THE ROLE OF CHROMOSOME 11 IN DETERMINING CELL SENSITIVITY TO DNA-DAMAGING AGENTS RELEASED BY ACTIVATED INFLAMMATORY CELLS

by

Amanda Jane Ward

BSc (Honours), The University of Wales, Aberystwyth, 1987

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the Department
of
Biological Sciences

© Amanda Jane Ward 1994

SIMON FRASER UNIVERSITY

July 1994

All rights reserved. This work may not be reproduced in whole or in part, by photocopy or other means, without permission of the author.

APPROVAL

Name: Amanda Jane Ward

Degree: Doctor of Philosophy

Title of Thesis: The role of chromosome 11 in determining cell sensitivity to DNA-damaging agents released by activated inflammatory cells

Examining Committee:

Chairperson: Dr. N.H. Haunerland

Dr. Miriam P. Rosin
Co-Senior Supervisor
Professor
Kinesiology

Dr. Arthur H. Burr
Co-Senior Supervisor
Associate Professor
Biological Sciences

Dr. Margo M. Moore
Assistant Professor
Biological Sciences

Dr. Brian A. McKeown
Professor
Biological Sciences

Dr. K.A. Skov
External Examiner

Honorary Professor, UBC
Pathology
Research Scientist
B.C. Cancer Research Centre
Medical Biophysics

Dr. S.S. Tsang
Internal Examiner
Adjunct Professor, Kinesiology
Honorary Assistant Professor, UBC
Medical Genetics
Research Scientist
B.C. Cancer Research Centre
Epidemiology, Biometry and
Occupational Oncology

Date approved: July 28, 1994

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

THE ROLE OF CHROMOSOME 11 IN DETERMINING CELL SENSITIVITY TO DNA-DAMAGING AGENTS RELEASED BY ACTIVATED INFLAMMATORY CELLS

Author: _____
(signature)

AMANDA JANE MARI
(name)

21 JULY 1994
(date)

ABSTRACT

An association between chronic inflammation and cancer is supported by both clinical observations and laboratory studies. Using laboratory models, there is evidence that suggests tumour promoters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) can activate inflammatory cells to release DNA-damaging agents. These agents include reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide as well as products of lipid peroxidation and arachidonic acid metabolism. A current hypothesis is that these agents damage DNA in nearby cells. DNA damage that is not repaired in a tissue can elevate chromosomal instability and increase the probability of acquiring the genetic alterations that are required for carcinogenesis. The hypothesis tested in this thesis was that the genotype of a nearby cell may influence its sensitivity to the DNA-damaging agents. Altered sensitivity may be inborn or may be acquired during the process of carcinogenesis. For example, a number of tumour cell lines and cells from the cancer-prone syndrome Ataxia-telangiectasia (AT) show increased sensitivity to radiation-induced DNA damage, an effect that has been attributed to a DNA repair defect located on chromosome 11.

A role of loci on chromosome 11 in determining a cell's sensitivity to genetic damage was assayed during co-culture of epithelial and fibroblast cells with TPA-activated neutrophils or with exposure to xanthine/xanthine oxidase (X/XO). The micronucleus (MN) assay was used to quantify chromosomal breakage and the comet assay the amount of initial strand breakage and the rate of rejoining of these breaks during DNA repair. Two model systems were studied: a bladder carcinoma model and an AT model.

In the first model, the results showed that bladder carcinoma cells exposed to TPA-activated neutrophils and X/XO were partially protected from MN induction by the microcell-mediated insertion of normal chromosome 11

($P<0.05$). Normal epithelial cells had no MN induction with X/XO treatment. The insertion of chromosome 11 did not alter the initial level of strand breakage or the efficiency of its rejoining in the bladder carcinoma cells when exposed to X/XO. In the second model, AT cells were shown to be more sensitive than normal cells to MN induction during the aforementioned treatments ($P<0.05$). This elevated sensitivity was also not due to an alteration in the level of initial strand breakage or its rejoining.

The capacity of TPA to cause genetic damage in epithelial and fibroblast cells in the absence of neutrophils was also studied. MN induction was observed only in the bladder carcinoma cells ($P<0.05$). To achieve the same level of MN induction observed in co-culture the treatment time with TPA alone had to be extended to a 24 hour period. The insertion of the normal chromosome 11 did not protect the bladder carcinoma cells against this DNA damage.

These studies support a role for TPA-activated neutrophils in inducing DNA damage in co-cultured cells and suggest that genetic alteration on chromosome 11 can increase the sensitivity of cells to this type of damage. The data also suggest that tumours may have an altered sensitivity to the clastogenic action of TPA.

To Darren and Leana

ACKNOWLEDGEMENTS

I would like to thank Dr. Jay Burr for giving me the opportunity to become a graduate student at Simon Fraser University and for allowing me to follow my interests. I would like to express my gratitude to Dr. Miriam Rosin for her time and advice throughout this research project. I appreciate the participation of Dr. Margo Moore and Dr. Brian McKeown as members of my supervisory committee, and I value the opinions and time taken by all members of my examining committee including Dr. K. Skov and Dr. S. Tsang.

I would like to thank Dr. Peggy Olive and Dr. Judit Banath for their assistance in teaching me the comet assay. In addition, I would like to thank Dr. O. Pereira-Smith for providing us with the bladder-carcinoma cell line A1698 and its microcell hybrid, and Dr. C. Arlett for the AT cell line AT2BR and the normal cell line 48BR.

I would like to acknowledge the continual support and patience of my husband, Darren Lemon, throughout my graduate work. I would like to express appreciation to our daughter, Leana, for taking long morning naps which allowed me the time to complete my thesis, and to Darren and Gerry Lemon in the last months for the many waking hours of childcare.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

D-MEM	Dulbecco's modified Eagle media
FCS	Fetal calf serum
K-SFM	Keratinocyte serum free media
MN	Micronucleus
NDI	Nuclear division index
PMN	Polymorphonuclear leucocyte (neutrophil)
ROS	Reactive oxygen species
TPA	12-O-tetradecanoylphorbol-13-actetate
X/XO	Xanthine/xanthine oxidase
SS	Single-strand
DS	Double-strand

THESIS FORMAT

This thesis is comprised of six chapters including a general introduction, research proposal, three papers written for publication, and a general discussion and conclusion.

The introduction (Chapter I) provides background information for the three papers which follow. It covers the concept that DNA-damaging agents generated during the respiratory burst of TPA-activated inflammatory cells may play an important role in the promotion of carcinogenesis, and in particular bladder cancer. In addition, AT is used as an example of a cancer-prone syndrome with an inherited predisposition for cancer and a sensitivity to agents that generate ROS. The possibility is explored that mutation on chromosome 11 which is inherited in the AT syndrome or acquired during the stages of carcinogenesis may result in a DNA processing error.

Chapter II covers the main hypotheses for the research and provides details, not given in the papers, on the MN and comet assays.

Chapter III has been published in *Mutation Research* (294: 299-308, 1993). This was a study of the sensitivity to oxidative stress of a bladder carcinoma culture and its microcell-mediated hybrid culture which contained a normal chromosome 11. We demonstrated that the hybrid culture had a reduced sensitivity to the DNA-damaging activity of TPA-activated neutrophils and X/XO. There was no induction of MN when normal epithelial cells were exposed to X/XO. We explored the possibility that reduction in sensitivity to oxidative stress of the hybrid culture was due to a correction of a DNA rejoining defect. It was found that this was not the case at the level of single-strand DNA damage and its rejoining.

Chapter IV has been accepted for publication in *Environmental and Molecular Mutagenesis*, Vol. 24, 2 or 3, 1994. In this paper, we demonstrated that fibroblast cultures from AT patients were more sensitive than cultures from normal individuals to the DNA-damaging activity of TPA-activated neutrophils and X/XO. We could not attribute increased sensitivity to oxygen-derived free radical DNA damage in the AT cells to a defect in the rejoining of single- or double-strand DNA damage.

Chapter V has been submitted for publication in *Carcinogenesis*. In this final study, we assessed the DNA-damaging potential of TPA treatment alone on both the epithelial and fibroblast cultures described above. From this study it was concluded that only the bladder carcinoma cells were sensitive to DNA damage by treatment with TPA alone. Insertion of a normal chromosome 11 in the hybrid culture did not protect against the DNA damage.

Chapter VI discusses the relevance of inflammation in tissues to carcinogenesis, the significance of this research project and the unresolved issues, as well as providing a final conclusion.

CHAPTER I

INTRODUCTION

1.1 The respiratory burst of inflammatory cells

Reactive oxygen species (ROS) are oxygen-derived free radicals or molecules generated by all living cells as byproducts during normal biological metabolism of oxygen (Harris et al., 1992). The production of ROS in some cells can be up-regulated by exposure to certain agents. Classical examples are neutrophils and macrophages, inflammatory cells that are the main phagocytes within the body (Babior, 1984). When stimulated these cells are capable of releasing large quantities of ROS during a phenomenon known as the respiratory burst. Important features of the respiratory burst include the release of ROS such as superoxide anion and hydrogen peroxide in an oxidative burst as well as products of lipid peroxidation and arachidonic acid metabolism (Gordon and Weitzman, 1988; Frenkel, 1992).

1.1.1 Activation of inflammatory cells

The major function of neutrophils and macrophages is to recognize, phagocytize and destroy invading microorganisms. At a site of invasion or injury specific inflammatory mediators are produced. Vasoactive molecules act to increase the vascular permeability, while chemotactic factors recruit white blood cells into the tissue. In addition, these white blood cells secrete further inflammatory mediators. Chemotactic factors are generated in high concentrations at the site of tissue injury, with a chemotactic factor gradient decreasing away from the tissue. Leucocytes migrate along the chemical gradient to the source of the chemotactic stimulus (Synderman and Pike, 1984; Cronstein and Weissmann, 1993). The most important chemotactic factors are C5a derived from the complement system, formylated peptides from bacteria, and products of lipid metabolism such as leukotriene B_4. Lymphokines released

from lymphocytes and monokines from monocytes and tissue macrophages also have chemotactic ability (Abramson et al., 1991; Haines et al., 1993).

Activation of inflammatory cells can be induced by binding of chemotactic mediators, by antigen-antibody binding complexation or by phagocytic stimuli. In addition, they can be activated by other agents such as opsonized particles and tumour promoters (Phillips et al., 1986). One mechanism by which tumour promoters are believed to promote carcinogenesis is through the activation of the inflammatory response (Troll et al., 1984; Gordon and Weitzman, 1988; Frenkel, 1992). In the inflammatory cell, activation is associated with a number of responses generally classed as the respiratory burst. These include enhanced lipid peroxidation and arachidonic acid metabolism, and degranulation with NADPH-dependent oxidase activation and the release of such ROS as superoxide anion and hydrogen peroxide (Babior, 1984; Gordon and Weitzman 1988).

The neutrophil is classically associated with initial acute inflammation. If the inflammation becomes chronic (long-term), macrophages become the predominant phagocyte.

1.1.2 The oxidative burst

Figure 1 is a diagram of the major pathways of ROS generation during a neutrophil's oxidative burst.

Inflammatory cell engulfment of a foreign agent such as a bacterium results in the formation of a phagolysosome into which granular enzymes are released. In addition, phagocytosis activates the oxidative burst. The oxidative burst begins with a rapid consumption of molecular oxygen followed by an almost simultaneous production of superoxide anion. This formation of superoxide anion is catalyzed by the NADPH-dependent oxidase system which

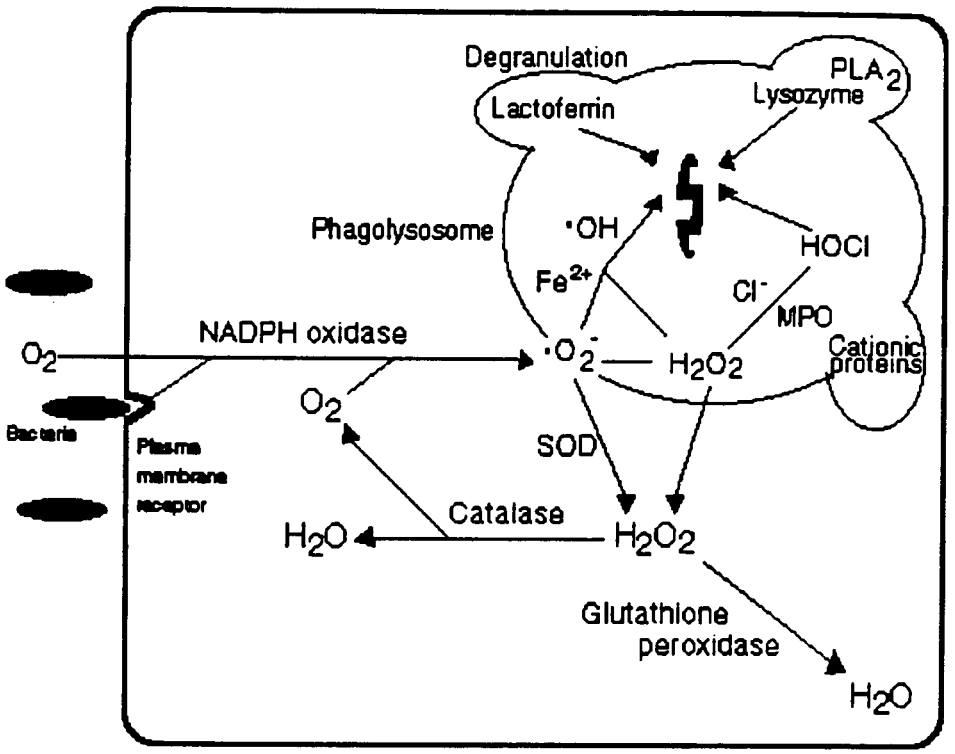

Figure 1. Major pathways of ROS generation during the oxidative burst of a neutrophil. MPO, myeloperoxidase; SOD, superoxide dismutase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate; PLA_2, phospholipase A_2. (References Root and Chen, 1981; Babior, 1984; Gordon and Weitzman, 1988)

is located on the cell membrane of inflammatory cells (Turrens and Boveris, 1980; Rossi, 1986):

$$O_2 + NADPH \text{ ----> } \cdot O_2^- + H^+ + NADP^+$$

The hexose monophosphate shunt enzymes use glucose as a substrate to regenerate NADPH. Spontaneous or enzymatic (superoxide dismutase) dismutation of superoxide results in hydrogen peroxide formation (Fridovich, 1978; Green and Hill, 1984):

$$2 \cdot O_2^- + 2H^+ \text{ ---> } H_2O_2 + O_2 \text{ (or } [^1O_2])$$

This hydrogen peroxide is the precursor of several bacteriocidal species: hydroxyl radical (·OH), hypochlorite/hypochlorous acid (OCl^-/HOCl) and singlet oxygen ($^1O_2$). Hydroxyl radical is generated in a series of iron-catalyzed reactions known as the Haber-Weiss reaction (Haber and Weiss, 1934; Aust et al., 1985):

$$Fe \text{ (III)} + \cdot O_2^- \text{ ---> } Fe \text{ (II)} + O_2 \qquad (1)$$

$$Fe \text{ (II)} + H_2O_2 \text{ ---> } \cdot OH + OH^- + Fe \text{ (III)} \qquad (2)$$

$$\cdot O_2^- + H_2O_2 \text{ --Fe--> } \cdot OH + OH^- + O_2 \qquad (3)$$

Hypochlorite and hypochlorous acid are formed by oxidation of chloride ions by hydrogen peroxide in a myeloperoxidase (MPO) catalyzed reaction (Stelmaszynska and Zgliczynski, 1974):

$$Cl^- + H_2O_2 \text{ ----> } OCl^- + H_2O$$

$$Cl^- + H_2O_2 + H^+ \text{ ----> } HOCl + H_2O$$

A long-lived and very powerful group of the oxidants called mono- and dichloramines are formed when hypochlorite reacts with primary amines (Weiss et al., 1983). Hydrogen peroxide, when in excess, acts as a reductant and reduces OCl^- back to Cl^- forming singlet oxygen in the process (Fantone and Ward, 1982):

$$OCl^- + H_2O_2 \text{ ---> } Cl^- + H_2O + {}^1O_2$$

(Reviewed by Babior, 1984; Warren, 1987; Gordon and Weitzman, 1988; Frenkel, 1992)

1.1.3 Lipid peroxidation and arachidonic acid metabolism

In the presence of oxygen, the hydroxyl radical can remove a hydrogen atom from the polyunsaturated fatty acids of membrane phospholipids and initiate the process of lipid peroxidation (Figure 2). This results in the formation of free lipid peroxide radicals called lipid hydroperoxy and endoperoxy radicals. Like the hydroxyl radical, the lipid peroxide radicals can then act as initiators of further lipid peroxidation. As lipid peroxides are unstable, they can break down into non-radical alkanes, alkenes and carbonylic compounds, which terminate the peroxidation (Horton and Fairhurst, 1987; Vaca et al., 1988; Harris et al., 1992).

Membrane receptor-mediated stimulation of either phospholipase A_2 or phospholipase C results in the release of arachidonic acid from membrane phospholipids (Figure 3). Phosopholipase A_2 catalyses the hydrolysis of arachidonic acid from phosphatidylcholine while phospholipase C enhances the metabolism of phosphatidylinositol to diacylglycerol and inositol phosphate. Diacylglycerol lipase then cleaves arachidonic acid from diacylglycerol. The first step in the production of active metabolites of arachidonic acid is lipid peroxidation. This is catalyzed by cyclooxygenase or lipoxygenase, or as described above by radicals such as the hydroxyl radical and lipid peroxide radical. The cyclooxygenase pathway results in the formation of prostaglandins and thromboxanes. Lipoxygenation generates mono- and dihydroxyeicosatetraenoic (HETEs and diHETEs) fatty acids and leukotrienes (Porter, 1980; Foegh et al., 1990; Harris, 1992).

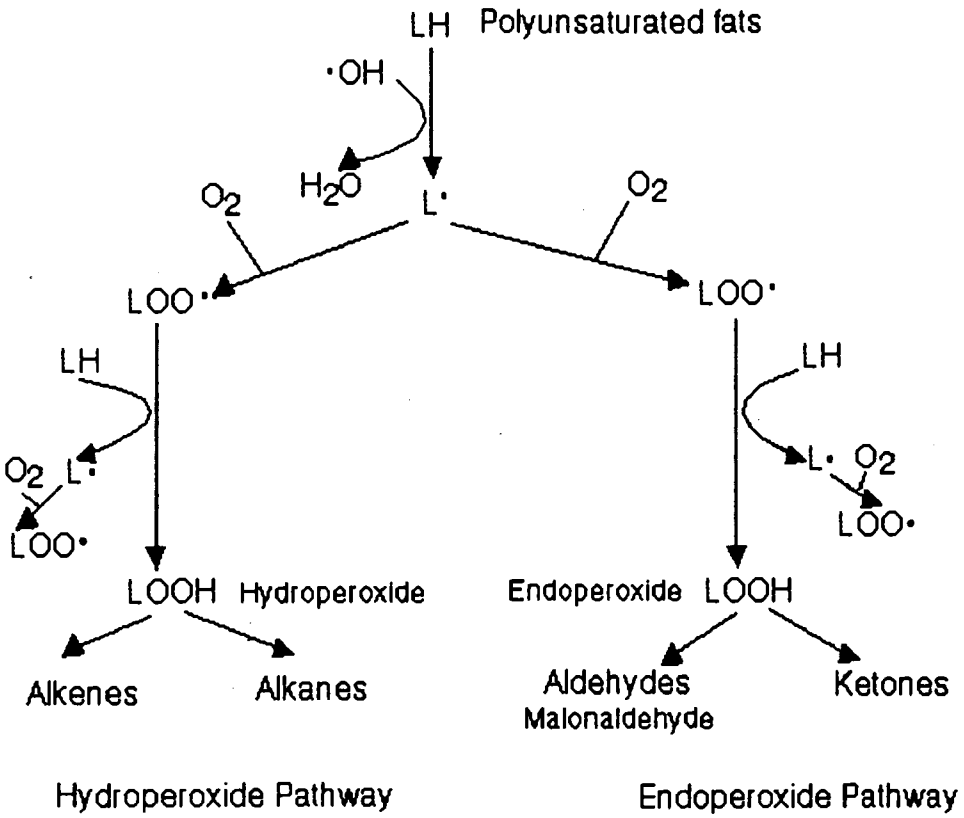

Figure 2. Lipid peroxidation.

LH, polyunsaturated fatty acid; L·, free lipid radical; LOO·, lipid peroxide radical; LOOH, hydro- and endoperoxides.

(References Horton and Fairhurst, 1987; Vaca et al., 1988; Harris et al., 1992)

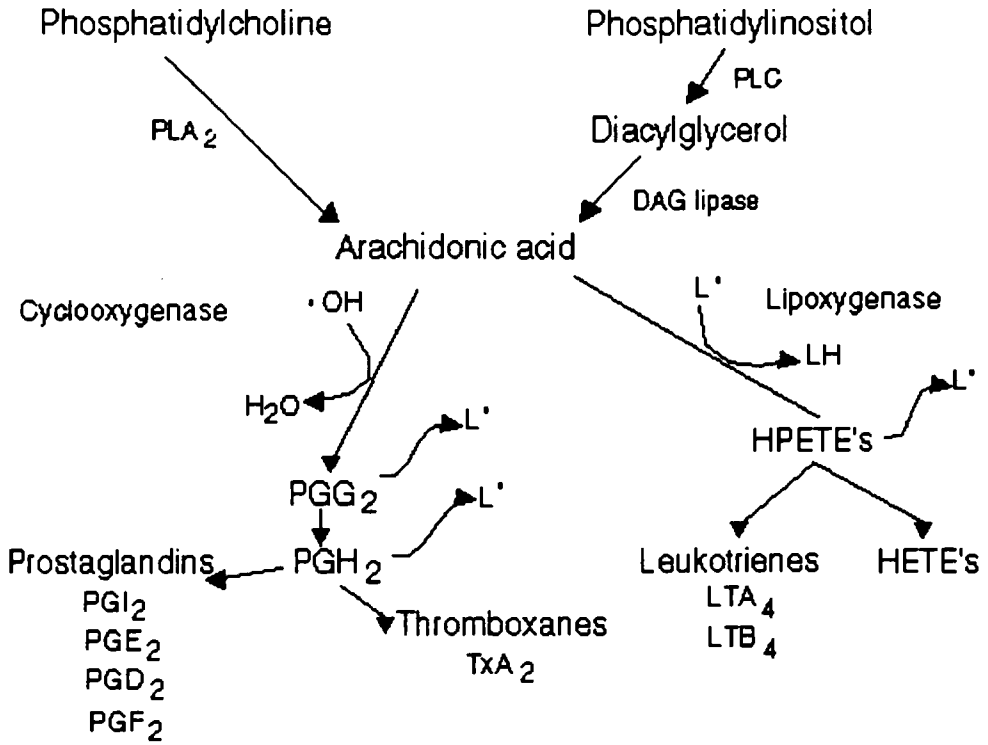

Figure 3. Arachidonic acid metabolism.

DAG, diacylglycerol; PLC, phospholipase C; PLA_2, phospholipase A_2; L·, free lipid radical; HPETEs and HETEs, hydroperoxy- and hydroxyeicosatetraenoic acids.

(References Porter, 1980; Harris et al., 1992)

1.2 DNA damaging agents generated by inflammatory cells

DNA damage has been observed in cells exposed to TPA-activated inflammatory cells (Kozumbo, et al., 1987; Shacter et al., 1988). Repeated exposure to TPA over prolonged periods is likely to result in accumulation of DNA damage in the cells of a tissue (Cerutti, 1985; Weitzman and Gordon, 1990; Frenkel, 1992). It has not yet been resolved which respiratory burst products released from stimulated inflammatory cells are important or what mechanism(s) of action are involved in damaging DNA. It may be a complex interaction between the different processes that ultimately generates the DNA damage (Cerutti, 1985; Weitzman and Gordon, 1990).

Birnboim (1982, 1983) and Dutton and Bowden (1985) first reported that TPA-activated inflammatory cells mediate formation of DNA strand breaks in their own DNA as well as in the cells with which they were co-cultured. Other forms of damage that have been generated in co-cultured cells include mutation, oxidized

DNA base derivatives, chromosomal aberrations and sister chromatid exchanges (Weitzman and Stossel, 1982, 1984; Cerutti, 1985; Lewis and Adams, 1985; Frenkel and Chrzan, 1987a; Frenkel and Chrzan, 1987b; Gordon and Weitzman, 1988; Schraufstatter, 1988; Shacter, 1988; Frenkel, 1992).

1.2.1 The hydroxyl radical

A number of hypotheses have been proposed for the mechanism(s) by which ROS induce DNA damage in cells. It is unlikely that oxy-radicals generated during the oxidative burst of the inflammatory cell can reach the DNA of co-cultured cells. For example, they can be either too short lived ($\cdot OH$ and OCl^-), or charged and require anion channels ($\cdot O_2^-$ and OCl^-). Hydrogen peroxide, however, is relatively stable, neutral and quite unreactive in the

absence of reduced transition metal ions and can penetrate plasma and nuclear membranes (Imlay and Linn, 1988; Chong et al., 1989). It is therefore hydrogen peroxide that is most likely to reach the nucleus where it can cause site-specific damage. Iron ions bound to the phosphate groups of nucleic acids may catalyze a Haber-Weiss reaction of hydrogen peroxide to form the hydroxyl radical. The hydroxyl radical has been widely supported as an agent responsible for DNA damage (Schraufstatter et al., 1988; Imlay and Linn, 1988; Frenkel, 1992). However, for it to be an agent of damage it must be formed in close proximity to the DNA as it has an extremely short life-time, approximately 4-8 x 10^{-9} seconds, and consequently a diffusion radius of 2.3 nm (Roots and Okada, 1975).

1.2.2 Products of lipid peroxidation and arachidonic acid metabolism

Due to general lack of stability and longevity of ROS released during the oxidative burst, a second hypothesis focuses on lipid peroxidation and, in particular, arachidonic acid metabolism. Lipid peroxides are good candidates for DNA-damaging agents. The lipid peroxides are not only highly reactive themselves but can initiate further lipid peroxidations in a chain reaction which can eventually result in loss of membrane integrity (Harris et al., 1992). Non-radical aldehydic products have the potential to traverse the distance to the DNA and interact directly. For example, malonaldehyde generated during the endoperoxide pathway has been shown to cause DNA damage (Ueda et al., 1985; Emerit and Lahoud-Maghani, 1989; Dargel, 1992).

Intermediates formed in the metabolism of arachidonic acid also have the potential to generate free radicals, induce DNA damage, and bioactivate carcinogens (Harris et al., 1992; Marnet, 1981; Battista and Marnet, 1985). It is the metabolites of the lipoxgenase pathway in particular that appear to have

important DNA-damaging activity. The hydroperoxy- and hydroxyeicosatetranoic acids (HPETEs and HETEs) have been shown to induce mutation and DNA strand breaks and cause cell transformation (Lewis et al., 1986; Ochi and Cerutti, 1987; Weitzman and Gordon, 1990).

1.2.3 Nitric oxide and *N*-nitrosamines

Another hypothesis which has gained recognition recently is that inflammatory cells may induce DNA damage by increasing the concentration of *N*-nitrosamine in co-cultured cells. *N*-nitrosamines are a group of compounds of known mutagenic potential (Marletta, 1988; Roediger, 1990). In stimulated inflammatory cells nitric oxide synthase catalyzes the conversion of arginine to citrulline and in so doing also generates nitric oxide (NO·) (Harris et al., 1992). When nitric oxide reacts with the superoxide anion it forms peroxynitrite, which in turn 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 (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 has been linked to increased exposure to *N*-nitrosamines (El-Aaser et al., 1984; Tricker et al., 1989).

A final point of interest is that non-phagocytic cells also have the potential to generated DNA-damaging agents when exposed to TPA. Therefore, not only are the products released by a neighbouring activated inflammatory cell potentially DNA-damaging, but also products generated within the cell by the interaction of TPA with protein kinase C receptors (Nakadate, 1989; Das, 1991;

Frenkel, 1992). As well as initiating arachidonic acid metabolism, TPA may also enhance the output of the pre-existing hydrogen peroxide-generation process(es) within the cell. In HeLa cells, TPA has been shown to induce hydrogen peroxide formation and dose-dependent formation of oxidized DNA base derivatives (Frenkel and Chrzan, 1987b; Bhimani and Frenkel, 1991; Frenkel and Gleichauf, 1991). ROS production has also been detected in keratinocytes and fibroblasts when treated with TPA (Fischer and Adama, 1985; Meier et al., 1990; Robertson et al., 1990). Treatment of mouse skin with TPA elevates the specific activity of xanthine oxidase, an enzyme that mediates production of superoxide and hydrogen peroxide (Reiners et al., 1987). TPA has been observed to induce chromosomal changes in keratinocyte (Harley et al., 1985; Petrusevska et al., 1988), fibroblast (Snyder, 1985) and lymphocyte cultures (Emerit et al., 1983).

1.3 Defence mechanisms against DNA-damaging agents of the respiratory burst

The many highly reactive radicals and molecules released in the respiratory burst of activated inflammatory cells can cause damage to cellular membranes and DNA. The cell is structurally organized to decrease the probability of indiscriminant free radical reactions. Nuclear DNA is protected by its distance from major sources of free radicals as well as by association with histones and DNA binding proteins (Frenkel, 1992; Harris et al., 1992). Various inhibitory agents exist to protect against membrane and DNA damage. Table 1 lists examples of enzymatic and nonenzymatic antioxidants, inhibitors of arachidonic acid metabolism, and DNA repair enzymes.

Table 1. Defence mechanisms and inhibitors which could be used to reduce DNA damage caused by TPA-activated inflammatory cells

Enzymatic antioxidants[a,b,c]	Nonenzymatic antioxidants[a,b,c]
superoxide dismutase	α-tocopherol
catalase	β-carotene
glutathione redox cycle	ascorbic acid
mitochondrial cytochrome oxidase system	albumin
	glutathione

Arachidonic acid metabolism inhibitors[a,d,e]	
Cyclooxygenase	Lipoxygenase
indomethacin	quercetin
aspirin	morin
ibuprofen	
flufenamic acid	

DNA repair enzymes[f,g,h]
endonucleases
exonucleases
glycosylase
polymerases
ligases

References: [a] Emerit et al., 1983; [b] Dutton and Bowden, 1985; [c] Birnboim and Kanabus-Kaminska, 1985 [d] Nakadate, 1989; [e] Paul et al., 1989; [f] Epe et al., 1993; [g] Thompson, 1991; [h] Reid and Loeb, 1993; Frenkel, 1992; Stewart, 1992; Harris et al., 1992.

Catalase is an example of an enzymatic antioxidant of particular interest to the research presented in this thesis. It is a peroxisomal enzyme that catalyzes the reduction and detoxification of hydrogen peroxide to water (Forman and Fisher, 1981):

$$2H_2O_2 \text{ ---> } 2H_2O + O_2$$

Although this reaction results in the formation of nontoxic products, superoxide dismutase is an enzymatic scavenger that generates DNA-damaging hydrogen peroxide (Bannister et al., 1987):

$$2 \cdot O_2^- + 2H^+ \text{ ---> } H_2O_2 + O_2$$

The impact of exogenous application of superoxide dismutase can therefore be potentially injurious to the cell. The removal of the hydrogen peroxide is then dependent on the availability of additional scavenging by catalase and peroxidase enzymes. Glutathione peroxidase is an example of a peroxidase enzyme. It oxidizes glutathione to glutathione disulfide and in so doing reduces hydroperoxides, including hydrogen peroxide, to an alcohol and water (Flohe, 1980).

1.4 DNA repair mechanisms

If the cell is unable to deal with elevated ROS levels then the potential arises for DNA damage to occur. Multiple pathways are involved in the repair of DNA damage. Much of our knowledge of these pathways comes from mutant cells which are deficient in the repair process (Paterson et al., 1984). The mutants most commonly studied are bacterial. However, successful use has been made of cells from humans with syndromes that exhibit high spontaneous chromosomal breakage and increased sensitivity to radiation-induced DNA damage. For example, cells from patients with the disease xeroderma

pigmentosum, after exposure to ultraviolet light, are defective in their ability to excise pyrimidine dimers from their DNA. These patients have a high frequency of skin cancers (Hoeijmakers et al., 1990; Krokan et al., 1990). Ataxia telangiectasia is another syndrome in which the cells may have a repair defect; however, the evidence for this is not as conclusive (Parshad et al., 1985).

There are a number of models proposed for the repair of single- and double-strand breakage. Error-free repair is most likely to occur when a lesion in one strand of the DNA is excised and replaced by replicating the compatible information from the undamaged strand (Paterson et al., 1984). This is referred to as excision repair and involves an endonuclease to cut the DNA near to the damage and an exonuclease for the removal of adjacent nucleotides. A polymerase then synthesizes new DNA using the intact DNA strand as a template. Finally, a ligase restores the continuity of the DNA strand (Krokan et al., 1990; Hoeijmaker et al., 1990; Perrino and Loeb, 1990). When the damage is to a single base the repair patch is small and a specific glycosylase is used before the action of an apurinic/apyrimidine endonuclease and exonuclease to remove the damaged base. The time to repair is rapid compared with more bulky lesions (Paterson et al., 1984; Stewart, 1992).

When DNA damage is initially bypassed or tolerated during replication there is an increased likelihood that the repair will be error-prone and give rise to mutations (Rauth, 1986). When the damage is tolerated, the post-replication gap left by the lesion may undergo recombinational repair which involves strand exchange with a separate, homologous DNA sequence, or it may simply be resealed by new DNA without a template (Cox, 1991). Double-strand breakage repair occurs at a lower rate than single-strand breakage and is more likely to be error-prone since the corresponding template is not available (Rauth, 1986; Stewart, 1992; Arrand and Michael, 1992).

1.5 Carcinogenesis

Chromosomal changes are a characteristic of cancer and may be critical driving forces in the alteration of a cell from normal to neoplastic. DNA damage to cells may increase the chances of acquiring some of these genetic changes required for carcinogenesis.

The process of carcinogenesis can generally be divided into three stages: (1) initiation, (2) promotion, and (3) progression.

1.5.1 Initiation, promotion and progression

The initiation, promotion and progression stages were first observed by Berenblum in the early 1940s using tumour induction in mouse skin (Berenblum, 1941). Since then the three-stage model of carcinogenesis has been validated in both laboratory animals and humans. Model systems include liver, bladder, colon and mammary gland, as well as cells in culture (Gordon and Weitzman, 1988). In humans, the stages are not as clearly defined as in animal models; however, events characteristic of these stages are observed. Continuous genetic changes are seen throughout the process of human carcinogenesis and are often followed by waves of proliferation and clonal expansion (Ames and Gold, 1990; Preston-Martin et al., 1990).

Initiation is normally a rapid process resulting in genetic changes that are irreversible. Initiators include carcinogenic agents that can interact with DNA directly or after metabolism (Ames et al., 1975; Miller and Miller, 1976).

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, rather than gene alteration, and proliferation followed by clonal expansion (Guyton and Kensler, 1993). Promoters may act through both epigenetic and genetic changes to

produce a benign tumour from initiated cells (Snyder, 1985). Examples include phorbol esters in mouse skin, saccharin in the bladder, and phenobarbital in the liver (Weitzman and Gordon, 1990).

Progression is the conversion of a benign tumour into a malignant tumour, which is usually accompanied by more rapid growth, invasiveness, metastasis, and increased genetic instability and irreversable genetic changes such as loss of tumour suppressor genes (Nigro et al., 1989; Weitzman and Gordon, 1990). For example, a benign papilloma in mouse skin can progress to a squamous cell carcinoma (O'Connell, 1986).

1.5.2 Involvement of DNA damage and chromosomal changes in carcinogenesis

The search to date for a single alteration common to all cancer cell types has been unsuccessful. It is most likely that a multitude of genetic and biochemical events results in the driving force behind carcinogenic potential.

The elevation of DNA damage observed in tissues exposed to chronic inflammation or any other agents with DNA-damaging potential increases the chance of producing the specific genetic alterations associated with the process of carcinogenesis (Rosin et al., 1989, 1994). Many premalignant conditions and dysplasias are characterized by chromosomal changes, and specific chromosomal aberrations appear to be associated with certain cancers (Mitelman and Heim, 1988; Berne et al., 1989; Parshad et al., 1992).

Strand breaks created by DNA damage can facilitate chromosomal translocations. Certain translocations can result in over-expression of oncogenes when they place a promoter region next to an oncogene site (Brown, 1986; Holladay, 1991). Oncogene expression is believed to be of critical importance in the process of carcinogenesis (Guyton and Kensler, 1993). The

activation of oncogenes as a result of translocation is believed to be important in chronic myelogenous leukemia (CML) and Burkitt's lymphoma. These two cancers are characterized by non-random reciprocal chromosome translocations. In Burkitt's lymphoma chromosomes 8 and 14 are involved in the reciprocal translocation. The *c-myc* gene is found at the breakage point on chromosome 8 and immunoglobulin genes at that of chromosome 14. The translocation results in *c-myc* oncogene activation as the gene expression becomes deregulated and enhanced by its proximity to the actively transcribed immunoglobulin genes. The normal function of the *c-myc* gene is in the growth regulation of lymphocytes and other cells. These cells will receive continuous growth stimulation as a result of the over-expression of the *c-myc* oncogene (Brown, 1986; Mitelman and Heim, 1988; Berne et al., 1989).

Another non-random event commonly noted in association with carcinogenesis is chromosome loss and gain. For example, an additional chromosome 7 is associated with some melanomas and may relate to the over-expression of epidermal growth factor in such cells (Sidransky and Messing, 1992). Gene dosage can also be increased by gene amplification. In neuroblastoma, amplification of the *c-myc* gene has been observed in the late, aggressive, stage of the tumour (Holladay, 1991). Leukemias are often characterized by chromosome loss (Mitelman and Heim, 1988; Berne et al., 1989). Sometimes specific gene deletions rather than whole chromosome loss occurs. Retinoblastoma and Wilms' tumour exhibit loss of tumour suppressor genes or 'antioncogenes' as a result of chromosome changes. Retinoblastoma typically shows deletion on the long arm of chromosome 13 while deletion in Wilms' tumour is associated with the short arm of chromosome 11 (Brown, 1986; Knudson, 1985; Flier et al., 1988; Holladay, 1991).

Further evidence for the involvement of DNA damage and genetic change in carcinogenesis comes from investigations of various cancer-prone heritable syndromes which exhibit elevated spontaneous chromosomal damage and a sensitivity to agents that act via free radical mechanisms to damage DNA. Examples include ataxia-telangiectasia, Bloom's syndrome, Fanconi anemia and xeroderma pigmentosum (German, 1983; Timme and Moses, 1988).

1.5.3 Tumour cells and chromosome 11

Microcell-mediated transfer of normal chromosomes into tumour cells has been found to suppress tumourigenesis. Tumourigenic expression has therefore been linked to allelic loss on specific chromosomes or whole chromosome loss. The specific chromosomal loss appears to be dependent on the type of malignant cell (Stanbridge, 1988). However, loss of some chromosomes harbouring general tumour suppressor genes may be a common feature of human cancers (Tsai et al., 1990).

Microcell-mediated transfer of chromosome 11 has been shown to supress tumourigenic properties in a number of malignant cell types. The tumourigenic phenotype is re-expressed with loss of the normal chromosome 11 (Saxon et al., 1986; Kaebling and Klinger, 1986; Stivatsan et al., 1986; Weissman et al., 1987). In Wilms' tumour cells, insertion of normal chromosome 11 corrected transformation ability but not tumourigenic phenotype thus suggesting that these two endpoints were under separate genetic control (Weissman et al., 1987). This dissociation has also been shown to occur in other tumour cells (Stanbridge and Wilkinson, 1978). Ning and co-workers in 1991 using bladder carcinoma cells with a chromosome 11 insert found that tumour suppression was not due to cellular senescence: the insert did not limit lifespan in culture.

In BK virus-transformed mouse cells, insertion of normal chromosome 11 suppresses tumourigenicity and anchorage-independent growth (Negrini et al., 1992), while in cells from human breast and rat prostrate cancer, it has been shown to be of importance in suppression of metastatic ability (Takita et al., 1992; Ichikawa et al., 1992). In the prostrate cancer cells the *in vivo* growth rate and tumourigenicity were unaffected by the chromosome 11 insertion (Ichikawa et al., 1992).

1.5.4 A DNA repair defect in tumour cells

Cancer-prone genetic conditions that have been extensively studied for their defective repair of DNA damage include: xeroderma pigmentosum, ataxia-telangiectasia, Fanconi's anemia and Bloom's syndrome (Timme and Moses, 1988; Knight et al., 1993). Researchers are now becoming interested in finding out if cancer patients with no known familial pre-disposition also exhibit a defective DNA repair mechanism when exposed to DNA-damaging agents. These patients may also be unknown gene carriers for cancer-prone genetic diseases (Knight et al., 1993).

Recent research supports a deficency in DNA repair mechanisms as an early step in neoplastic transformation of cells. A defect would increase the potential for accumulation of the genetic changes required for clonal development of cancer (Sanford, 1992). Genes on chromosome 11 have been associated with deficient repair of radiation-induced DNA damage in tumour cells. Parshad and co-workers (1992) reported an abnormally high frequency of chromatid breaks and gaps when various human tumours were x-irradiated. Insertion of normal chromosome 11 into cells from six tumour cell lines reduced radiation-induced DNA damage to that observed in normal cells. They attributed this to the restoration of a DNA repair defect. In four of the cell lines this

reduction in DNA damage was also associated with suppression of tumourigenicity. The fact that they were not linked in two of the cell lines would indicate that these two phenomena are not the responsibility of one gene. Embryonal rhabdomyosarcoma was one of the cell lines that did not exhibit this linkage; its hybrid cell line only received the long arm of chromosome 11. It may therefore be assumed that the repair gene(s) are localize on this arm. In Wilms' tumour the repair gene(s) were further localized to the segment between the centromere and q23 of chromosome 11.

Powell and co-workers (1992) found that it may be misrepair rather than lack of double-strand repair that influences radiosensitivity in tumour cells. They observed that DNA double-strand repair in a radiation-sensitive clone of a bladder carcinoma cells line was not related to the rate or extent of double-strand break rejoining but to the repair fidelity.

1.6 Chronic inflammation and cancer

The possibility that chronic inflammation exerts co-carcinogenic effects in tissue has been supported by many clinical observation. Table 2 shows that this association has been observed in many different tissues.

In the pathogenesis of bladder cancer several risk factors have been implicated : the use of dietary sweetners (eg. saccharin), tobacco (Armstrong and Doll, 1974), the intake of coffee (Cole, 1971), industrial exposure (The BAUS Subcommittee on Industrial Bladder Cancer, 1988), excessive exposure to motor vehicle exhaust fumes (Silverman et al., 1986), and infection with *Schistosoma haematobium* (Dimette et al., 1956). In some of these studies the actual risk is controversial because the risk evaluation has been influenced by

Table 2. Examples of chronic inflammation associated with increased risk for cancer

Cause of inflammation	Tissue site of cancer	Reference
Schistosoma haematobium infection	Bladder	Chen and Mott, 1989
Chronic and recurrent cystitis	Bladder	Locke et al., 1985
Schistosoma mansoni infection	Spleen	Andrade and Abrea, 1971
Schistosoma japonicum infection	Colon	Chen and Mott, 1988
Ulcerative colitis	Colon	Collins et al., 1987
Crohn's disease	Colon	Korelitz et al., 1983
Helicobacter infection	Stomach	Correa, 1992
Atrophic gastritis	Stomach	Correa, 1988
Chronic cholecystitis	Gall bladder	Diehl, 1983

variables such as multiple exposure, population drift, and the latency of carcinogenic effect. However, it has become clear that the use of tobacco, exposure to aniline dyes, and schistosomiasis can increase the risk of bladder cancer (Raghavan et al., 1990).

1.6.1 Bladder cancer

Bladder cancer appears to be increasing in incidence. It accounts for approximately 6% of male and 2% of all female cancers in Western society (Husband, 1992). Approximately 16 to 20 cases per 100,000 men and 5 cases per 100,000 women are diagnosed annually in the United States (Raghavan et al., 1990). In 1991, there were an estimated 50,200 new cases of bladder cancer in the United States with 9,500 deaths from the tumour (Boring et al., 1992). It usually presents late in life (60-70 years) (Husband, 1992).

1.6.2 Clinical characteristics of bladder cancer

Most bladder cancers are transitional cell tumours, the remainder being squamous cell tumours, mixed transitional cell and squamous cell tumours, adenocarcinomas, or undifferentiated lesions (Husband, 1992). In some cases the type of tumour has been linked to a specific causal agent; for example, squamous cell tumours of the bladder are more frequently seen in schistosomiasis-infected bladders (Dimette et al., 1956). Malignancies will vary in grade, from well differentiated 'low-grade' tumours (Grade 1) to poorly differentiated 'high-grade' tumours (Grade 3). Pattern of growth is also variable and classified as papillary, infiltrative, papillary and infiltrative, or non-papillary and non-infiltrative (carcinoma *in situ*)(Husband, 1992). Papillary tumours are the most common and most new cases diagnosed are papillary (Koss, 1975). Usually a correlation can be found between tumour grade and growth pattern.

Most well-differentiated tumours (Grade 1) are papillary. Most undifferentiated tumours (Grade 3) are solid and infiltrating and have a much poorer diagnosis (Husband, 1992).

There are 2 main staging systems for bladder cancer: the TMN system (International Union Against Cancer, 1982) and the Jewitt Strong Marshall System (Jewett et al., 1964). A number of new laboratory techniques are available to aid clinical diagnosis: chromosome complement analysis, total DNA content, and evaluation of the expression of blood-group antigens, epidermal growth factor receptor and certain oncogenes (Raghavan et al., 1990; Koss 1992; Sheinfeld, 1992).

1.6.3. Chromosome changes in bladder cancer

Allelic loss of specific chromosomes is a common feature in bladder cancer and appears to be a nonrandom event. Allelic loss occurs from chromosomes 1, 7, 9, 11 and 17 (Tsai et al., 1990, Hopman et al., 1991). The deletion of a putative tumour-suppressor gene on chromosome 9 (Sandberg, 1992; Cairns et al., 1993) and abnormal epidermal growth factor receptor expression are associated with the early stages of bladder carcinogenesis (Sidransky and Messing, 1992), while other changes such as mutation of the p53 gene and the functional loss of the retinoblastoma (RB) gene may be key events in the progression of the tumour (Sidransky et al., 1992). The allelic loss on chromosome 9 appears to be a primary genetic event, with further loss of genes on chromosomes 11 and 17 at a later stage. This loss of alleles on 11 and 17 has also been noted in breast cancer and in squamous cell carcinoma of the lung. It may be that epithelial tumours are characterized by the loss of specific alleles on these chromosomes (Tsai et al., 1990, Hopman et al., 1991).

Tyrkus and co-workers (1992) have studied the cytogenetics of 17 patients diagnosed with carcinoma *in situ* of the bladder. They found a correlation between the karyotype complexity (numerical and structural changes) and the disease course. It appeared that particular chromosomal changes were related to the expression of invasive potential. Patients that expressed a normal karyotype had superficial tumours, whereas an abnormal karotype was associated with a more aggressive tumour that progressed to invasive disease. Nonrandom chromosome changes were observed involving chromosomes 1, 5, 8, and 11. Structural rearrangements of chromosome 1 were a common event. The most common translocations involved chromosome 1 and 11.

1.6.4 A link between *Schistosoma haematobium* infection, inflammation and bladder cancer

Schistosoma haematobium is a parasitic infection of the bladder that results in chronic inflammation and irritation and is strongly associated with urinary bladder cancer. One possible reason for this association is the increased level of genetic change in the urothelium generated during chronic inflammation (Tawfik, 1988; Chen and Mott, 1989; Badawi, 1992).

Infection with the parasite occurs when an individual is exposed to the free-swimming laval stage of the parasite 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. It is the presence of the eggs in the mucosa that initiates a chronic inflammatory reaction (Rosin et al., 1994).

Rosin and Anwar in 1992 conducted *in vivo* population studies in Egypt using the micronucleus test as a measure of chromosomal damage in exfoliated urothelial cells of infected and uninfected individuals. They found chromosomal damage to be significantly higher in infected individuals. The use of an anti-schistosomal drug was associated with a decrease in the chromosomal damage in urothelial cells and a reduction in the number of eosinophils and neutrophils found in urine samples. The presence of infection and the associated inflammatory cells appeared to have a role in increasing the chromosomal damage. Biopsies from schistosomiasis patients were also examined by the investigators and a dysregulation of cell proliferation was shown to be associated with the presence of interstitial cystitis in these patients.

1.7 Ataxia-telangiectasia (AT), a syndrome with a predisposition to cancer and a sensitivity to ROS

1.7.1 Clinical characteristics of AT

AT is a genetic syndrome which shows autosomal recessive inheritance. Death usually occurs in the second decade of life and is primarily due to progressive pulmonary infection and/or to cancer. The distinctive clinical features are progressive development of cerebellar ataxia from early childhood and oculocutaneous telangiectasia appearing somewhat later. The ataxia is a failure of muscular coordination due to a disorder of the cerebellum. The telangiectasia is a permanent dilation of blood vessels over the eyes and ears. Table 3 shows additional features of AT that can aid in the diagnosis (reviewed by Boder, 1985; McKinnon, 1987; Gatti et al., 1991; Gatti, 1993; Swift et al.,

Table 3. Clinical and laboratory diagnostic hallmarks of the AT syndrome

Neurological markers
eg. cerebellar ataxia, oculomotor signs, dysarthric speech and postural attitude
Oculocutaneous telangiectasia
Progeric changes of hair and skin
Growth retardation
Cancer and pulmonary disease
Radiosensitivity
Spontaneous chromosome breaks and rearrangements in short term cultures of T-lymphocytes and fibroblasts
Immunodeficiency
eg. low levels of IgA, IgG2, IgE and diminished response to skin antigens
Endocrine abnormalities

Reviewed by Boder, 1985; McKinnon, 1987; Gatti et al., 1991; Gatti, 1993; Swift et al, 1993.

1993). Of interest in relation to the research presented in this thesis is the clinical and cellular sensitivity to agents that generate ROS and the spontaneous chromosome instability.

1.7.2 Cancer in AT homozygotes and AT heterozygotes

In a case study of 57 autopsy reports from AT homozygotes the causes of death were as follows: pulmonary disease 47%, malignancy 21%, a combination of pulmonary disease and malignancy 27%, and accidental 3%. Of the malignancies, lymphocytic leukemia and non-Hodkin's lymphoma in particular markedly predominated. The solid tumours included oral cavity, breast, stomach, pancreas, ovary, and bladder (Boder, 1985). Several patients had multiple neoplasms. About one fourth of the AT patients with a solid tumour subsequently developed leukemia or non-Hodgkin's lymphoma (Hecht and Hecht, 1990). Acute lymphocytic leukemias or lymphomas predominate in AT homozygotes under the age of 20, epithelial rather than lymphocytic malignancies increase steadily with age. (Taylor and Butterworth, 1986; Becher and Duhrsen, 1987). An estimated 1 in every 100 AT children from the age of 10 onward will develop a new cancer each year (Morrell et al., 1986; Taylor, 1992). The risk of developing cancer is 61 to 184 times higher in AT homozygotes than in the general population. The risk in black AT patients in the United States appears to be higher. This suggests that the AT allele is different or that other genetic or environmental factors are increasing the cancer rate in this population group (Morrell et al., 1986).

The AT gene also predisposes AT heterozygotes to cancer. In the United States AT heterozygotes make up 1.4% of the white population (Swift et al., 1986). They are susceptible to the same array of malignancies as the general

population. The estimated risk of cancer development compared with noncarriers is 3.8 times higher in men and 3.5 in women. Up to 5% of individuals dying of cancer under the age of 45 are believed to be AT heterozygotes (Swift et al., 1993). In heterozygote women who have been exposed to diagnostic ionizing radiation, the risk for breast cancer is 5.1 times higher than noncarriers. These women may constitute 9 to 18% of all persons with breast cancer in the United States (Swift et al., 1987; Borresen et al., 1990).

Immunodeficiency as well as chromosome instability may relate to the formation of malignancies. AT patients account for about one third of all cases in the Immunodeficiency Cancer Registry. Similarities can be drawn between AT patients and aquired immunodeficiency syndrome (AIDS) patients in which Kaposi's sarcoma occurs with mild immunodeficiency and *pneumocystis carinii* pneumonia occurs with more profound immunodeficiency (Hecht and Hecht, 1990).

1.7.3 Chromosomal instability in AT cells

Chromosome instability in the AT syndrome was first described by Hecht and co-workers in 1966. In lymphocytes and fibroblast cultures obtained from AT patients the frequency of chromosome fragments, breaks and gaps, non-stable chromosome rearrangements (dicentrics and rings), and stable chromosome rearrangements (translocations) are often increased (Gropp and Flatz, 1967; Hecht et al., 1973; Cohen et al., 1975; Oxford et al., 1975; Al Saadi et al., 1980; Taylor, 1982; Hecht and Kaiser-McCaw, 1982). Site-specific stable chromosome rearrangements occur primarily in T lymphocytes. They involve 7p14, 7q35, 14q12 and 14q32 (McCaw et al., 1975; Oxford et al., 1975; Aurias and Dutrillaux, 1986; Kojis et al., 1989), which correspond to T-cell receptors and immunoglobulin genes (Kennaugh et al., 1986). The development of

lymphoreticular neoplasia in the peripheral blood lymphocytes of AT patients appears to be initiated by increased chromosome breakage and rearrangements of chromosome 7 and 14, and especially 14:14 translocation. It can be speculated that if the 14:14 translocation is selected for within the cell population it may become a dominant malignant clone (Kaiser-McCaw and Hecht, 1982).

Increased instability is not always seen in all patients. Chromosome instability can vary with cell type. Therefore, there does not appear to be consistancy within or between patients in the chromosome instability found. If increased instability is found in a patient it may not be consistently higher at other sample times (Cohen et al., 1975; 1979; Hecht and Kaiser-McCaw, 1982; Gatti et al., 1991). In general chromosome instability appears to decline with time (Hecht et al., 1973). Rosin and co-workers in 1989 estimated the frequency distribution of spontaneous chromosomal breakage occuring *in vivo* in oral epithelia of AT patients. The levels of breakage were elevated in some but not all of the patients studied. There are also several *in vitro* reports in which the level of chromosomal breakage in cells from AT cells patients are no higher than that in normal individuals (Pfeiffer, 1970; Harnden, 1974; Bochkov et al., 1974; Taylor et al., 1981).

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 repair of DNA damaged by these radicals (Shaham et al., 1980; Emerit, 1982; Shiloh et al., 1983; Yi et al., 1990; Pandita and Hittelman, 1992a,b). This will be discussed in sections 1.7.4 and 1.7.6.

1.7.4 Sensitivity of AT cells to agents that generate ROS

The defective protein/enzyme that the AT gene expresses must be important from early development since the AT phenotype is expressed so early

in life and so uniformly. One possibility could be a defect in the ability of AT cells to monitor and repair oxidative damage to DNA resulting from normal aerobic metabolism (Gatti, 1993).

AT cells show a clear sensitivity to a large number of compounds which induce DNA strand breaks via ROS generation. AT fibroblast cells 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). When Shiloh and co-workers (1983) exposed AT and normal fibroblast cultures to hydrogen peroxide they found that the AT cells had lower colony-forming efficiency and significantly reduced transient inhibition of DNA synthesis.

Cultured AT cells are also more sensitive than cells from normal individuals to ionizing radiation and to radiomimetic cytotoxic drugs (Taylor et al., 1975; Morris et al., 1983; Shiloh et al., 1989). AT patients are extremely sensitive to the ionizing radiation used to treat malignancies (Gotoff et al., 1967; Cunliffe et al., 1975). Unlike normal cells which block DNA synthesis when the DNA is damaged, AT cells show no reduction in DNA synthesis after x-ray and bleomycin treatment (review in Shiloh et al., 1983). The altered response to ionizing radiation, found so consistently in all AT cells, is a phenotypic marker that is believed to relate closely to the primary genetic defect (Swift et al., 1993).

1.7.5 The AT gene(s) on chromosome 11

The present estimate is that there could be approximately 1 AT patient in every 20,000 births with a gene frequency of 0.007. If AT is genetically heterogeneous, as some evidence suggests, then the frequency of the AT alleles should be greater than 0.007 (Swift et al.,1993; Gatti, 1993). The discovery of 5 complementation groups suggests genetic heterogeneity and may also explain the complex multisystem nature of this disorder (Boder, 1985).

Complementation groups are assigned on the basis of reversal of the sensitivity to x-irradiation by cells from AT patients. Cells belonging to different complementation groups when fused together show a normal inhibition of DNA synthesis in response to x-irradiation (Jaspers et al., 1985). The 5 complementation groups are named: A,C,D, E and V1 (Jaspers et al., 1988). This complementation implies that different mutations are responsible for the AT phenotype. Approximately 97% of tested AT families exhibit genetic linkage to chromosome 11. This linkage is within a 4-megabase region of the human genome at 11q23 (McConville et al., 1990; Sanal et al., 1990; Ziv et al.,1991; Foroud et al., 1991; Kapp et al., 1992; Gatti, 1993).

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 in AT cells is responsible for the x-ray hypersensitivity to cell killing and chromosome breakage (Komatsu et al., 1990; Kodama et al., 1992). Ejima and co-workers (1990; 1991) have further localized this hypersensitivity to the q23 region in chromosome 11 by using transfer of various regions of chromosome 11.

1.7.6 A DNA repair defect in AT cells

Recent research has focused on the possibility that the defect in AT cells that gives rise to chromosome instability and sensitivity to DNA damaging agents is a defect in the repair of DNA damage or in the fidelity of the repair.

Both single-strand and double-strand DNA damage and their repair have been extensively studied. In most cases, the favoured agent for inducing DNA

damage is radiation. The use of alkaline filter elution to study single-strand DNA breakage and its repair has revealed no differences between AT and normal cells (Cantoni et al., 1989; Fornace et al., 1986; Hariharan et al., 1981; Forance and Little, 1980). The use of neutral filter elution to assay double-strand DNA breakage and its repair has revealed no defect in the initial level of double-strand breakage (Coquerelle and Weibezahn, 1981; Coquerelle et al., 1987; Radford and Hodgson, 1990; Pandita and Hittleman, 1992 a,b). However, there are some reports of a defect in the repair of the DNA damage (Coquerelle and Weibezahn, 1981; George and Cramp, 1987; Coquerelle et al., 1987; Debenham et al., 1987; Mozdarani and Bryant, 1989; Radford and Hodgson, 1990; Blocher et al., 1991; Pandita and Hittelman, 1992 a,b). The most recent work by Hittelman and co-workers (1992 a,b), using γ-irradiation to treat transformed lymphoblastoid cell lines derived from AT and normal individuals, suggests that AT cells have a defect in double-strand DNA fast repair.

Misrepair, rather than lack of DNA double-strand repair, may in fact be the basis of sensitivity in AT cells. It is possible that although AT cells are capable of repairing DNA breaks as well as normal cells, there is a significant increase in the misrepair of those breaks. The use of recombination vectors to study the recombination process in AT cells suggest that error-prone recombination events may result in misrepair (Thacker, 1989; Powell, et al., 1993; Ganesh et al., 1993).

1.9 References

Abramson, S.B., J. Leszczynska-Piziak and G. Weissmann (1991) Arachidonic acid as a second messenger: Interactions with a GTP-binding protein of human neutrophils, J. Immuno., 147, 231-236.

Al Saadi, A., M. Palutke and G. K. Kumar (1980) Evolution of chromosomal abnormalities in sequential cytogenetic studies of ataxia telangiectasia, Hum. Genet., 55, 23-29.

Ames, B.N. and L.S. Gold (1990) Too many rodent carcinogens: mitogenesis increases mutagenesis, Science, 249, 970-971.

Ames, B.N., J. McCann and E. Yamaski (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test, Mutat. Res., 31, 347-364.

Armstrong, B. and R. Doll (1974) Bladder cancer mortality in England and Wales in relation to cigarette smoking and saccharin consumption, Br. J. Prev. Soc. Med., 28, 233-240.

Andrade, Z.A. and W.N. Abreu (1971) Follicular lymphoma of the spleen in patients with hapatosplenic *Schistosomiasis mansoni*, Am. J. Trop. Med. Hyg., 21, 237-243.

Arrand, J.E. and B.D. Michael (1992) Workshop report: Recent advances in the study of ionizing radiation damage and repair, Int. J. Radiat. Biol., 61, 717-720.

Aurias, A. and B. Dutrillaux (1986) Probable involvement of immunoglobulin superfamily genes in most recurrent chromosomal rearrangements form ataxia telangiectasia, Hum. Genet., 72, 210-214.

Aust, S.D., L.A. Morehouse and C.E. Thomas (1985) Role of metals in oxygen radical reactions, J. Free Rad. Biol. Med., 1, 3-25.

Babior, B.M. (1984) The respiratory burst of phagocytes, J. Clin. Invest., 73, 599-601.

Badawi, A.F., M.H. Mostafa and P.J. O'Conner (1992) Involvement of alkylating agents in schistosome-associated bladder cancer: the possible basic mechanisms of induction, Cancer Lett., 63, 171-188.

Bannister, J.V., W.H. Bannister and G. Rotilio (1987) Aspects of the structure, function and application of superoxide dismutase, CRC Crit. Rev. Biochem., 22, 111-180.

BAUS Subcommittee on Industrial Bladder Cancer (1988) Occupational bladder cancer: a guide for clinicians, Br. J. Urol., 61, 183-191.

Becher, R. and C. Duhrsen (1987) Distinct chromosome abnormalities in ataxia telangiectasia with chronic T-cell lymphocytic leukemia, Cancer Genet. Cytogenet., 26, 217-225.

Berenblum, I. (1941) The carcinogenic action of croton resin, Cancer Res., 11, 44-48.

Berne, J., I. McColl and M. Adinolfi (1989) Recessive mutations and chromosome deletions leading to cancer, Br. J. Surg., 76, 327-330.

Bhimani, R., and K. Frenkel (1991) Supression of H_2O_2 production and oxidative DNA damage in HeLa cells by (-) epigallocatechin gallate (EGCG), Proc. Am. Assoc. Cancer Res., 32, 126.

Birnboim, H.C. (1982) Factors which affect DNA strand breakage in human leukocytes exposed to a tumor promoter, phorbol myristate acetate, Can. J. Physiol. Pharmac., 60, 1359-1366.

Birnboim, H.C. (1983) Importance of DNA strand-break damage in tumor promotion, In: O.F. Nygaard and M.G. Simic (Eds.), Radioprotectors and Anticarcinogens, Academic Press, New York, pp. 539-556.

Birnboim, H.C. and M. Kanabus-Kaminska (1985) The production of DNA strand breaks in human leukocytes by superoxide anion may involve a metabolic process, Proc. Natl. Acad. Sci. USA, 82, 6820-6825.

Blocher, D., D. Sigut and M. A. Hannan (1991) Fibroblasts for ataxia telangiectasia (AT) and AT heterozygotes show an enhanced level of residual DNA double-strand breaks after low dose-rate γ-irradiation as assayed by pulse field gel electrophoresis, Int. J. Radiat. Biol., 60 (5), 791-802.

Bochkov, N. P., Y. M. Lopukhin, N. P. Kuleshov and L. V. Kovalchuk (1974) Cytogenetic studies of patients with ataxia-telangiectasia, Humangenetik, 24, 115-128.

Boder, E. (1985) Ataxia-Telangiectasia: An Overview, In: R.A. Gatti, and M. Swift, (Eds.), Ataxia-Telangiectasia: Genetics, Neuropathology and Immunology of a Degenerative Disease of Childhood, Kroc Foundation Series, Vol. 19, Alan R. Liss, Inc., New York, pp.1-63.

Boring, C.C., T.S. Squires and T. Tong (1992) Cancer Statistics 1991, 41, 19-36.

Brown, K., M. Quintanella, M. Ramsden and A. Balmain (1986) The role of oncogenes in multistage carcinogenesis, In: J.F. Burns, A.C. Upton and G. Silini (Eds.), Radiation Carcinogenesis and DNA Alteration, Plenum Press, N.Y., pp.339-352.

Borresen, A-L, T.I. Anderson, S. Tretli and A. Heiberg (1990) Breast cancer and other cancers in Norwegian families with ataxia telangiectasia, Genes Chromosom. Cancer, 2, 339-340.

Cairns, P., M.E. Shaw and M.A. Knowles (1993) Initiation of bladder cancer may involve deletion of a tumour-suppressor gene on chromosome 9, Oncogene, 8, 1083-1085.

Cantoni, O., P. Sestili, M. Fiorilli, M. P. Santoro, M. C. Tannoia, G. Novelli, F. Cattabeni and B. Dallapiccola (1989) Identification of 4 ataxia

telangiectasia cell lines hypersensitive to γ-irradiation but not to hydrogen peroxide, Mutat. Res., 218, 143-148.

Cerutti, P. (1985) Prooxidant states and tumor promotion, Science, 227, 375-381.

Chen, M. and K. Mott (1988) Progress in assessment of morbidity due to *Schistosoma japonicum* infection, Trop. Dis. Bull., 85, 2056.

Chen, M. and K. Mott (1989) Progress in assessment of morbidity due to *Schistosoma haematobium* infection: a review of the recent literature, Trop. Dis. Bull., 86, 2-36.

Chiricolo, M., P. L. Tazzari, A. Abbondanza, A. Dinota and M. G. Battelli (1991) Cytotoxicity of, and DNA damage by, active oxygen species produced by xanthine oxidase, FEBS Letters, 291 (2), 173-176.

Chong, Y.C., G.H. Heppner, L.A. Paul and A.M. Fulton (1989) Macrophage-mediated induction of DNA strand breaks in target tumor cells, Cancer Res., 49, 6652-6657.

Cohen, M. M., M. Shaham, J. Dagan, E. Shnuell and G. Kohn (1975) Cytogenetic investigations in families with ataxia-telangiectasia, Cytogenet. Cell Genet., 15, 338-356.

Cohen, M. M., M. Sagi, Z. Ben-Hur, P. Schaap, R. Boos, G. Kohn and H. Bassat (1979) Ataxia telangiectasia: chromosomal stability in continuous lymphoblastoid cells, Cytogenet. Cell Genet., 15, 338-356.

Cole, P. (1971) Coffee-drinking and cancer of the lower urinary tract, Lancet, 1, 1335-1337.

Collins, R.H., M. Feldman, J.S. Fordtran (1987) Colon cancer, dysplasia and surveillance in patients with ulcerative colitis: A critical review, N. Engl. J. Med., 316, 1654.

Coquerelle, T. M., K. F. Weibezahn and C. Lucke-Huhle (1987) Rejoining of double strand breaks in normal human and ataxia-telangiectasia fibroblasts after exposure to ^{60}Co γ-rays, ^{241}Am α-particles or bleomycin, Int. J. Radiat. Biol., 51 (2), 209-218.

Coquerelle, T. M. and K. F. Weibezahn (1981) Rejoining of DNA double-strand breaks in human fibroblasts and its impairment in one ataxia telangiectasia and two Fanconi strains, J. Supramol. Struct. Cell. Biochem., 17, 369-376.

Correa, P (1988) A human model of gastric carcinogenesis, Cancer Res., 48, 3554-3559.

Correa, P. (1992) Human gastric carcinogenesis a multistep and multifactorial process - First American Cancer Society award lecture on cancer epidemiology and prevention, Cancer Res., 52, 6735-7740.

Cox, M.M. (1991) The RecA protein as a recombinational repair system, Mol. Microbiol., 5(6), 1295-1299.

Cunliffe, P. N., J. R. Mann, A. H. Cameron, K. D. Roberts and H. W. C. Ward (1975) Radiosensitivity in ataxia-telangiectasia, Br. J. Radiol., 48, 374-376.

Cronstein, B.N. and G. Weissmann (1993) The adhesion molecules of inflammation, Arthritis and Rheumatism, 36 (2), 147-157.

Dargel, R (1992) Lipid peroxidation - a common pathogenetic mechanism?, Exp. Toxicol. Pathol., 44, 169-181.

Das, U.N. (1991) Arachidonic acid as a mediator of some of the actions of phorbol myristate acetate, a tumor promoter and inducer of differentiation, Prostaglandins, Leukotrienes and Essential Fatty Acids, 42, 241-244.

Debenham, P. G., M. B. T. Webb, N. J. Jones and R. Cox (1987) Molecular studies of the nature of the repair defect in ataxia-telangiectasia and their implications for cellular radiobiology, J. Cell Sci. Suppl., 6, 177-189.

Deeley, J.O.T. and J.L. Moore (1992) Single-cell microgel electrophoresis: an in vitro assay of radiosensitivity, Brit. J. Radiol., 24 (Suppl.), 79-83.

Diehl, A.K. (1983) Gallstone size and the risk of gallbladder cancer, JAMA, 250, 2323.

Dimette, R.M., H.F. Sproat and E.S. Sayegh (1956) The classification of carcinoma of the urinary bladder associated with schistosomiasis and metaplasia, J. Urol., 75, 680-686.

Dutton, D.R. and G.T. Bowden (1985) Indirect induction of a clastogenic effect in epidermal cells by a tumor promoter, Carcinogenesis, 6, 1279-1284.

Eastmond, D.A. and J.D. Tucker (1989) Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocyes and an antikinetochore antibody, Environ. Mol. Mutagen., 13, 34-43.

Ejima, Y., M. Oshimura and M. S. Sasaki (1990) Establishment of a novel immortalized cell line from ataxia telangiectasia fibroblasts and its use for the chromosomal assignment of radiosensitivity genes, Int. J. Radiat. Biol., 58 (6), 989-997.

Ejima, Y., M. Oshima and M.S. Sasaki (1991) Determination of the chromosomal site for the human radiosensitive ataxia telangiectasia gene by chromosome transfer, Mutat. Res., 250, 337-343.

El-Aaser, A.A. and M.M. El-Mezrahani (1984) Etiology of bladder cancer, In: I. Elsebai (Ed.) Bladder Cancer, CRC Press, Boca Raton, FL, pp.39-58.

Eling, T.E., D.C. Thompson, G.L. Foureman, J.F.Curtis and M.F. Hughes (1990) Prostaglandin H synthesis and xenobiotic oxidation, Ann. Rev. Pharmacol. Toxicol., 30, 1-45.

Emerit, I. (1982) Chromosome breakage factors: origin and possible significance, Prog. Mutat. Res., 4, 61-74.

Emerit, I. and P.A. Cerutti (1981) Tumour promoter phorbol-12-myristate-13-acetate induced chromosome damage via indirect action, Nature, 293, 144-146.

Emerit, I. and M. Lahoud-Maghani (1989) Mutagenic effects of TPA induced clastogenic factor in Chinese hamster cells, Mutat. Res., 214, 97-104.

Emerit, I., A. Levy and P. Cerutti (1983) Suppression of tumor promoter phorbol myristate acetate-induced chromosome breakage by antioxidants and inhibitors of arachidonic acid metabolism, Mutat. Res., 110, 327-335.

Epe, B. M. Pflaum, M. Haring, J. Hegler and H. Rudiger (1993) Use of repair endonucleases to characterize DNA damage induced by reactive oxygen species in cellular and cell-free systems, Toxicol. Letters, 67,57-72.

Erexson, G.L., A. D. Klingerman and J.W. Allen (1987) Diaziquone-induced micronuclei in cytochalasin B-blocked mouse peripheral blood lymphocytes, Mutat. Res., 178, 117-122.

Farber, E. (1980) Chemical carcinogens, N. Engl. J. Med., 305, 1379.

Fantone, J.C. and P.A. Ward (1982) Role of oxygen-derived free radicals and metabolites in leukocyte-dependent imflammatory reactions, Am. J. Pathol., 107, 397-418.

Fenech, M. and A.A. Morley (1985) Measurement of micronuclei in lymphocytes, Mutat. Res., 147, 29-36.

Fischer, S.M. and L.M. Adams (1985) Suppression of tumor promoter-induced chemiluminescence in mouse epithelial cells by several inhibitors of arachidonic acid metabolism, Cancer Res., 45, 3130-3136.

Flier, J.S., L.H. Underhill, S.H. Friend, T.P. Dryja and R.H. Weinberg (1988) Oncogenes and tumor-suppressor genes, New. Engl. J. Med., 318 (10), 618-622.

Flohe, L. (1980) Glutathione peroxidase brought into focus, In: W.A. Pryor (Ed.), Free Radicals in Biology, New York, Academic Press, IV, pp. 223-254.

Foegh, M.L., G. Thomas and P.W. Ramwell (1990) Free radicals, arachidonic acid metabolites, and nutrition, J. Parent. Enter. Nutrit., 14 (5), 218S-222S.

Forman, H.J. and A.B. Fisher (1981) Antioxidant defenses, In: D.L. Gilbert (Ed.) Oxygen and Living Processes: An Interdisciplinary Approach, New York, Springer-Verlag New York, pp. 235-249.

Fornace, A. J., T. J. Kinsella, P. P. Dobson and J. B. Mitchell (1986) Repair of ionizing radiation DNA base damage in ataxia-telangiectasia cells, Cancer Res., 46, 1703-1706.

Fornace, A. J. and J. B. Little (1980) Normal repair of DNA single-strand breaks on patients with ataxia telangiectasia, Biochim. Biophys. Acta, 607, 432-437.

Foroud, T., S. Wei, Y. Ziv, E. Sobel, E. Lange, A. Chao, T. Goradia, Y. Huo, A. Tolun, L. Chesse, P. Charmley, O. Sanal, N. Salman, C. Julier, P. Concannon, C. McConville, A. M. R. Taylor, Y. Shiloh, K. Lange and R. A. Gatti (1991) Localization of an ataxia-telangiectasia locus to a 3-cM interval on chromosome 11q23: Linkage analysis of 111 families by an international consortium, Am. J. Hum. Genet., 49, 1263-1279.

Frenkel, K. (1992) Carcinogen-mediated oxidant formation and oxidative DNA damage, Pharmac. Ther., 53, 127-166.

Frenkel, K. and K. Chrzan (1987a) Hydrogen peroxide formation and DNA base modification by tumour promoter-activated polymorphonuclear leukocytes, Carcinogenesis, 8, 455-460.

Frenkel, K. and K. Chrzan (1987b) Radiation-like modification of DNA and H_2O_2 formation by activated human polymorphonuclear leukocytes (PMNs), In: P.A. Cerutti, O.F. Nygaard and M.G. Simic (Eds.), Anticarcinogenesis and Radiation Protection, Plenum Publishing Corp., New York, pp.97-102,

Frenkel, K. and C. Gleichauf (1991) Hydrogen peroxide formation by cells treated with a tumor promoter, Free Rad. Res. Commun., 12-13, 783-794.

Fridovich, I. (1978) The biology of oxygen radicals, Science, 201, 875-880.

Ganesh, A., P. North and J. Thacker (1993) Repair and misrepair of site-specific DNA double-strand breaks by human cell extracts, Mutat. Res., 299, 251-259.

Gatti, R. A. (1993) Candidates for the molecular defect in ataxia telangiectasia, Adv. Neurol., 61, 127-132.

Gatti, R. A., E. Boder, H. V. Vinters, R. S. Sparkes, A. Norman and K. Lange (1991) Ataxia-telangiectasia: An interdisciplinary approach to pathogenesis, Medicine, 70 (2), 99-117.

George, A.M. and W.A. Cramp (1987) The effect of ionizing radiation on the structure and function of DNA, Prog. Biophys. Molec. Biol., 50, 121-169.

German, J. (1983) Patterns of neoplasia associated with the chromosome-breakage syndromes, In: J. German (Ed.), Chromosome Mutation and Neoplasia, Liss, New York, pp.97-134.

Gordon, L.I., and S.A. Weitzman (1988) The respiratory burst and carcinogenesis, In: A.J. Sbarra and R.R. Strauss (Eds.), The Respiratory Burst and Its Physiological Significance, Plenum Press, New York, pp. 277-298.

Green, M.J. and Hill H.A.D. (1984) Chemistry of dioxygen, In: L. Packer (Ed.), Methods in Enzymology, New York, Academic Press, 105, pp.3-22.

Gropp, A. and G. Flatz (1967) Chromosome breakage and blastic transformation of lymphocytes in atiaxia-telangiectasia, Humangenetik, 5, 77-79.

Guyton, K.Z. and T.W. Kensler (1993) Oxidative mechanisms in carcinogenesis, Brit. Med. Bull., 49 (3), 523-544.

Haber, F. and J. Weiss (1934) The catalytic decomposition of hydrogen preoxide by iron salts, Proc. R. Soc. Lond., 147, 332.

Haines, K.A., S.L. Kolasinski, B.N. Cronstein, J. Reibman, L.I. Gold and G. Weissmann (1993) Chemoattraction of neutrophils by substance P and transforming growth factor β1 is inadequately explained by current models of lipid remodeling, J. Immunol., 151 (3), 1491-1499.

Hariharan, P. V., S. Eleczko, B. P. Smith and M. C. Paterson (1981) Normal rejoining of DNA strand breaks in ataxia telangiectasia fibroblast lines after low x-ray exposure, Radiat. Res., 86, 589-597.

Harnden, D. G. (1974) Ataxia-telangiectasia syndrome: cytogenetic and cancer aspects, In: J. German (Ed.) Chromosomes and Cancer, J. Wiley, New York, pp.619-636.

Harris, M.L., H.J. Schiller, P.M. Reilly, M. Donowitz, M.B. Grisham and G.B. Bulkley (1992) Free radicals and other reactive oxygen metabolites in inflammatory bowel disease: cause, consequence or epiphenomenon?, Pharmac. Ther., 53, 375-408.

Hartley, J.A., N.W. Gibson, L.A. Zwelling and S.H. Yuspa (1985) Association of DNA strand breaks with accelerated terminal differentiation in mouse epidermal cells exposed to tumor promoter, Cancer Res., 45, 4864-4870.

Hecht, F. and B. K. Hecht (1990) Cancer in ataxia-telangiectaia patients, Cancer Genet. Cytogenet., 46, 9-19.

Hecht, F. and B. Kaiser-McCaw (1982) Ataxia-telangiectasia; chromosomes before cancer, In: B. A. Bridges and D. G. Harnden (Eds.), Ataxia-telangiectasia - A cellular and Molecular Link between Cancer, Neuropathology and Immune Defeciency, J. Wiley and Sons Ltd., London, England, pp. 235-241.

Hecht, F., B. K. McCaw and R. D. Koler (1973) Ataxia-telangiectasia - clonal growth of translocation lymphocytes, New Engl. J. Med., 289, 286-291.

Hecht, F., R. D. Koler, D. A. Rigas, G. S. Dahnke, M. P. Case, V. Tisdale and R. W. Miller (1966) Leukemia and lymphocytes in ataxia-telangiectasia, Lancet, 2, 1193.

Heddle, J.A., M. Hite, B. Kirkhart, K. Mavournin, J.T. MacGregor, G.W. Newell and M.F. Salamone (1983) The induction of micronuclei as a measure of genotoxicity, Mutat. Res., 123, 61-118.

Heddle, J.A., M.C. Cimino, M. Hayashi, F. Romagna, M.D. Shelby, J.D. Tucker, Ph. Vanparys and J.T. MacGregor (1991) Micronuclei as an index of cytogenetic damage: past, present and future, Environ. Mol. Mutagen., 18, 277-291.

Hoeijmakers, J.H.J., A.P.M. Eker, R.D. Wood and P. Robins (1990) Use of *in vivo* and *in vitro* assays for the characterization of mammalian excision repair and isolation of repair proteins, Mutat. Res., 236, 223-238.

Holladay, K., H. Fujiki and T. Bowden (1991) Okadaic acid, a non phorbol ester tumour promoter and phosphatase inhibitor, induces the expression of both early and late response genes in mouse keratinocytes, Proc. Am. Assoc. Cancer Res., 32, 156.

Hopman, A.H.N., O. Moesker, A. W.G.B. Wmeets, R. P.E. Pauwels, G.P. Vooijs and F.C.S. Ramaekers (1991) Numerical chromosome 1,7,9, and 11 aberrations in bladder cancer detected by *in situ* hybridization, Cancer Res., 51, 644-651.

Horton, A.A and S. Fairhurst (1987) Lipid peroxidation and mechanisms of toxicity, CRC Crit. Rev. Toxicol., 18, 27-79.

Husband, E. (1992) Staging bladder cancer, Clinical Radiology, 46, 153-159.

Iarotski, L.S. and S. Davis (1981) The schistosomiasis problem in the world: results of a WHO questionnaire survey, Bull. WHO, 59, 115-127.

Ichikawa, T., Y. Ichikawa, J. Dong, A.L. Hawkins, C. A. Griffin, W. B. Isaacs, M. Oshimura, J.C. Barrett and J.T. Isaacs (1992) Localization of metastasis suppressor gene(s) for prostatic cancer to the short arm of human chromosome 11, Cancer Res., 52, 3486-3490.

Imlay, J.A. and S. Linn (1988) DNA damage and oxygen radical toxicity, Science, 240, 1302-1309.

International Union Against Cancer (1982) TNM Classification of Malignant Tumours, 3rd ed., Springer-Verlag, Berlin.

Jaspers, N. G., R. B. Painter, M. C. Paterson, C. Kidson and T. Inoue (1985) Complementation analysis of ataxia-telangiectasia, Found. Ser., 19, 147-162.

Jaspers, N. G. J., R. A. Gatti, C. Baan, P. C. M. L. Linssen and D. Bootsma (1988) Genetic complementation analysis of ataxia-telangiectasia and Nijmegen breakage syndrome: a survey of 50 patients, Cytogenet. Cell Genet., 49, 259-263.

Jewett, H.J., L.R. King, W.M. Shelley (1964) A study of 365 cases of infiltrating bladder cancer: relation of certain pathological characteristics to prognosis after extirpation, J. Urol., 92, 668-678.

Kaelbling, M. and H. P. Klinger (1986) Suppression of tumourigenicity in somatic cell hybrids. III. Consegregation of human chromosome 11 of a normal cell and suppression of tumourigenicity in intraspecies hybrids of normal diploid x malignant cells, Cytogenet. Cell Genet., 41, 65-70.

Kaiser-McCaw and F. Hecht (1982), Ataxia-telangiectasia: chromosomes and cancer, In: B. A. Bridges and D. G. Harnden (Eds.) Ataxia-Telangictasia: A Cellular and Molecular Link Between Cancer, Neuropathology and Immune Deficiency, J. Wiley and Sons Ltd., Chichester, England, pp.243-257.

Kaplan, R.P. (1987) Cancer complicating chronic ulcerative and scarifying mucocutaneous disorders, Adv. Dermatol., 2, 19-46.

Kapp, L. N., R. B. Painter, L-C. Yu, N. Van Loon, C.W. Richard III, M. R. James, D. R. Cox and J. P. Murnamne (1992) Cloning of a candidate gene for ataxia-telangiectasia group D, Am. J. Hum. Genet., 51, 45-54.

Kastan, M. B., Q. Zhan, W. S. El-Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett. B. Vogelstein and A. J. Fornace (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia, Cell, 71, 587-597.

Kennaugh, A. A., S. V. Butterworth, R. Hollis, R. Baer, T. H. Rabbitts and A. M. R. Taylor (1986) The chromosome breakpoint at 14q32 in an ataxia telangiectasia t(14:14) T cell clone is different from the 14q23 breakpoint in Burkitts and an inv(14) T cell lymphoma, Hum. Genet., 73, 346-349.

Khan, S.H. and I. Emerit (1985) Lipid peroxidation products and clastogenic material in culture media of human leukocytes exposed to the tumor promoter phorbol-myristate-acetate, J. Free Rad. Biol. Med., 1, 443-449.

Kinzel V., G. Furstenberger, H. Loehoke and F. Marks (1986) Three stage tumourigenesis in mouse skin. DNA synthesis as a prerequisite for the conversion stage induced by TPA prior to initiation, Carcinogenesis, 7, 779-782.

Knight, R.D., R. Parshad, F.M. Price, R.E. Tarone and K.K. Sanford (1993) X-ray-induced chromatid damage in relation to DNA repair and cancer incidence in family members, Int. J. Cancer, 54, 589-593.

Knudson, A.G. (1985) Hereditary cancer, oncogenes, and antioncogenes, Cancer Res., 45, 1437-1443.

Kodama, S., K. Komatsu, Y. Okumura and M. Oshimura (1992) Suppression of x-ray-induced chromosome aberrations in ataxia telangiectasia cells by introduction of a normal human chromosome 11, Mutat. Res., 293, 31-37.

Korelitz, B.I. (1983) Carcinoma of the intestinal tract in Crohn's disease: Results of a survey conducted by the National Foundation for Ileitis and Colitis, Am. J. Gastroenterol, 78, 44.

Kojis, T. L., R. R. Schreck, R. A. Gatti and R. S. Sparkes (1989) Tissue specificity of chromosomal rearrangements in ataxia-telangiectasia, Hum. Genet., 83, 337-353.

Komatsu, K., S. Kodam, Y. Okumura, M. Koi and M. Oshimura (1990) Restoration of radiation resistance in ataxia telangiectasia cells by the introduction of normal human chromosome 11, Mutat. Res., 235, 59-63.

Koss, L.G. (1992) Bladder cancer from a perspective of 40 years, J. Cell. Biochem., Supp. 161, 23-29.

Koss, L.G. (1975) Tumors of the urinary bladder, Fasicle II in Atlas of Tumor Pathology, Armed Forces Institute of Pathology, Washington, DC.

Kozumbo, W.J., D. Muehlematter, A. Jorg, I. Emerit and P. Cerutti (1987) Phorbol ester-induced formation of clastogenic factors from human monocytes, Carcinogenesis, 8 (4), 521-526.

Krishna, G., M.L. Kropko and J.C. Theiss (1989) Use of the cytokinesis-block method for the analysis of micronuclei in V79 chinese hamster lung cells: results with mitomycin C and cyclophosphamide, Mutat. Res., 222, 63-69.

Krokan, H.E., L.C. Olsen, R. Aasland, G. volden, G. Eggset, B. Myrnes, B. Johansen, A. Haugen and D.E. Helland (1990) DNA repair in mammalian

tissues and cells, In: B.M. Sutherland and A.D. Woodhead (Eds.), DNA Damage and Repair in Human Tissues, Plenum Press, New York, pp. 175-189.

Leaf, C.D., J.S. Wishnok and S.R. Tannenbaum (1991) Endogenous incorporation of nitric oxide from L-arginine into N-nitrosomorpholine stimulated Escherichia coli lipopolysaccharide in the rat, Carcinogenesis, 12 (3), 537-539.

Lewis, J.G. and D.O. Adams (1985) Induction of 5,6-ring saturated thymine bases in NIH-3T3 cells by phorbol ester-stimulated macrophages: role of reactive oxygen intermediates, Cancer Res., 45, 1270-1275.

Lewis, J.G., T. Hamilton and D.O. Adams (1986) The effect of macrophage development on the release of reactive oxygen intermediates and lipid oxidation products, and their ability to induce oxidative DNA damage in mammalian cells, Carcinogenesis, 7, 813-818.

Locke, J.R., D.E. Hill, and Y. Walzer (1985) Incidence of squamous cell carcinoma in patients with long-term catheter drainage, J. Urol., 133, 1034.

Marletta, M.A. (1988) Mammalian synthesis of nitrite, nitrate, nitric oxide and N-nitrosating agents, Chem. Res. Toxicol., 1, 249-257.

Masters, J.R., S.G. Vesey, C.F. Munn, G.I. Evan, J.V. Watson (1988) *C-myc* oncoprotein levels in bladder cancer, Urol. Res., 16, 341-344.

McCaw, B. K., F. Hecht, D. G. Harnden and R. L. Teplitz (1975) Somatic rearrangements of chromosome 14 in human lymphocytes, Proc. Nat. Acad. Sci. USA, 72, 2071-2075.

McConville, C. M. C. J. Formstone, D. Hernandez, J. Thick and A. M. R. Taylor (1990) Fine mapping of the chromosome 11q22-23 region using PFGE, linkage and haplotype analysis; localization of the gene for ataxia-

telangiectasia to a 5cm region flanked by NCAM/DRD2 and STMY/S385, phage 2.22, Nucleic Acids Res., 18, 4335-4343.

McCord, J.M. (1987) Radical explanations for old observations, Gastroenterology, 92, 2026-2037.

McKelvey-Martin, V.J., M.H.L. Green, P. Schmezer, B.L. Pool-Zobel, M.P. De Meo and A. Collins (1993) The single cell gel electrophoresis assay (comet assay): A European review, Mutat. Res., 288, 47-63.

McKinnon, P. J. (1987) Ataxia-Telangiectasia: an inherited disorder of ionizing-radiation sensitivity in man, Hum. Genet., 75, 197-208.

Meier, B., H.H. Radeke, S. Selle, G.G. Habermehl, K. Resch and H. Sies (1990) Human fibroblasts release low amounts of reactive oxygen species in response to the potent phagocyte stimulants, serum-treated zymosan, N-formyl-methionyl-leucyl-phenylalanine, leukotriene B4 or 12-O-tetradecanoylphorbol-13-acetate, Biol. Chem. Hoppe-Seyler, 371, 1021-1025.

Meredith, and Dodson (1987) Impaired glutathione biosynthesis in cultured human ataxia telangiectasia cells, Cancer Res., 47, 4576-4581.

Miller, E.C. and J.A. Miller (1976) The metabolism of chemical carcinogens to reactive electrophiles and their possible mechanisms of action in carcinogenesis, In: C.E. Searle (Ed.) Chemical Carcinogens, American Chemical Society, Washington, DC, pp. 737-762.

Mitelman, F. and S. Heim (1988) Consistent involvement of only 71 of the 329 chromosomal bands of the human genome in primary neoplasia-associated rearrangements, Cancer Res., 48, 7115-7119.

Morrell, D., E. Cromartie and M. Swift (1986) Mortality and cancer incidence in 263 patients with ataxia-telangiectasia, J. Natl. Cancer Inst., 77, 89-92.

Morris, C., R. Mohamed and M. F. Lavin (1983) DNA replication and repair in ataxia telangiectasia cells exposed to bleomycin, Mutat. Res., 112, 67-74.

Mozdarani, H. and P. E. bryant (1989) Induction and rejoining of chromatid breaks in x-irradiated A-T and normal human G_2 fibroblasts, Int. J. Radiat. Biol., 56 (5), 645-650.

Mulligan, M.S., J.M. Hevel, M.A. Marletta and P.A. Ward (1991) Tissue injury caused by deposition of immune complexes is L-arginine dependent, Proc. Natl. Acad. Sci. USA, 88, 6338-6342.

Nakadate, T. (1989) The mechanism of skin tumor promotion caused by phorbol esters: possible involvement of arachidonic acid cascade/lipoxygenase, protein kinase C and calcium/calmodulin systems, J. Pharmacol., 49, 1-9.

Negrini, M., A. Castagnoli, J.V. Pavan, S.Sabbioi, D. Araujo, A. Corallini, F. Gualandi, P. Rimessi, A. Bonfatti, C. Giunta, A. Sensi, E.J. Stanbridge and G. Barbanti-Brodano (1992) Suppression of tumorigenicity and anchorage-independent growth of BK virus-transformed mouse cells by human chromosome 11, Cancer Res., 52, 1297-1303.

Nigro, J. S. Baker, A. Preisinger, J. Jessup, R. Hostetter, K. Cleary, S. Bigner, N. Davidson, D. Baylin, P. Devilee, T. Glover, F. Collins, A. Weston, R. Modali, C. Harris and B. Vogelstein (1989) Mutations in the p53 gene occur in diverse human tumour types, Nature, 342, 705-708.

Ning, Y., J. W. Shay, M. Lovell, L. Taylor, D. H. Ledbetter and O. M. Pereira-Smith (1991) Tumor suppression by chromosome 11 is not due to cellular senescence, Exp. Cell Res., 192, 220-226.

O'Connell, J.F., A.J.P. Klein-Szanto, D.M. DiGiovanni, J.W. Fries and T.J. Slaga (1986) Enhanced malignant progression of mouse skin tumours by the free-radical generator benzoyl peroxide, Cancer Res., 46, 2863-2865.

Ochi, T and P.A. Cerutti (1987) Clastogenic action of hydroperoxy-5,8,11,13-icosatetraenoic acids on the mouse embryo fibroblasts C3H 10T1/2, Proc. Natl. Acad. Sci., USA, 84, 990-994.

Olive, P.L., J.P. Banath and R.E. Durand (1990a) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay, Radiat. Res., 122, 86-94.

Olive, P.L., J.P. Banath and R.E. Durand (1990b) Detection of etoposide resistance by measuring DNA damage in individual Chinese hamster cells, J. Nat. Cancer Inst., 82, 779-783.

Olive, P.L., D. Wlodek and J.P. Banath (1991) DNA double-strand breaks measured in individual cells subjected to gel electrophoresis, Cancer Res., 51, 4671-4676.

Olive, P.L., D. Wlodek, R.E. Durand and J.P. Banath (1992) Factors influencing DNA migration from individual cells subjected to gel electrophoresis, Exp. Cell Res., 198, 259-267.

Oxford, J. M., D. G. Harnden, J. M. Parrington and J. D. A. Delhanty (1975) Specific chromosome aberations in ataxia telangiectasia, J. Med. Genet., 12, 251-262.

Pandita, T. K. and W. N. Hittelman (1992a) Initial chromosome damage but not DNA damage is greater in ataxia telangiectasia cells, Radiat. Res., 130, 94-103.

Pandita, T. K. and W. N. Hittelman (1992b) The contribution of DNA and chromosome repair deficiencies to the radiosensitivity of ataxia-telangiectasia, Radiat. Res., 131, 214-223.

Parada, L.F., C.J. Tabin, C. Shih, R.A. Weinberg (1982) Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus *ras* gene, Nature, 297, 474-478.

Parshad, R., K.K. Sanford and G.M. Jones (1985) Chromosomal radiosensitivity during the G_2 cell cycle period of skin fibroblasts from individuals with familial cancer, Proc. Natl. Acad. Sci. USA, 82, 5400-5403.

Parshad, R., F.M. Price, M. Oshimura, J.C. Barrett, H. Satoh, B.E. Weissman, E.J. Stanbridge and K.K. Sanford (1992) Complementation of a DNA repair deficiency in six human tumor cell lines by chromosome 11, Hum. Genet., 88, 524-528.

Paterson, M.C., N.E. Gentner, M.V. Middlestadt and M. Weinfeld (1984) Cancer predisposition, carcinogen hypersensitivity and aberrant DNA metabolism, J. Cell Physiol. Suppl., 3, 45-62.

Paul, L.A., A.M. Fulton and G.H. Heppner (1989) Reactive oxygen-mediated damage to murine mammary tumor cells, Mutat. Res., 215, 223-234.

Perrino, F.W. and L.A. Loeb (1990) Animal cell DNA polymerases in DNA repair, Mutat. Res., 236, 289-300.

Petrusevska, R.T., G. Furstenberger, F. Marks and N.E. Fusenig (1988) Cytogenetic effects caused by phorbol ester tumor promoter in primary mouse keratinocyte cultures: correlation with the convertogenic activity of TPA in multistage skin carcinogenesis, Carcinogenesis, 9, 1207-1215.

Pfeiffer, R. A. (1970) Chromosomal abnormalities in ataxia-telangiectasia (Louis-Bar syndrome), Humangenetik, 8, 302-306.

Phillips, B.J., D. Anderson and S.D. Gangolli (1986) Influence of phagocyte-derived active oxygen species in tissue responses to tumour promoters and irritants, Fd. Chem. Toxic., 24 (6/7), 681-683.

Ponder B. A .J. (1991) Genetic predisposition to cancer, Brit. J. Cancer, 64, 203-204.

Porter, N.A. (1980) Prostaglandin endoperoxides, In: W.A. Pryor (Ed.) Free Radicals in Biology, New York, Academic Press, IV, pp.261-294.

Powell, S.N., S.J. Whitaker, S.M. Edwards and T.J. McMillan (1992) A DNA repair defect in a radiation-sensitive clone of a human bladder carcinoma cell line, Brit. J. Cancer, 65, 798-802.

Powell, S., S. Whitaker, J. Peacock and T. McMillan (1993) Ataxia telangiectasia: An investigation of the repair defect in the cell line AT5BIVA by plasmid reconstruction, Mutat. Res., 294, 9-20.

Preston-Martin, S., M.C. Pike, R.K. Ross, P.A. Jones, and B.E. Henderson (1990) Increased cell division as a cause of human cancer, Cancer Res., 50, 7415-7421.

Proctor, A. J., L. M. Coombs, J. P. Cairns and M. A. Knowles (1991) Amplification of chromosome 11q13 in transitional cell tumours of the bladder, Oncogene, 6, 789-795.

Radford, I. R. (1986) Effect of radiomodifying agents on the ratios of X-ray-induced lesions in cellular DNA: use in lethal lesion determination, Int. J. Radiat. Biol., 49, 621-637.

Radford, I. R. and G. S. Hodgson (1990) A comparison of the induction of DNA double-strand breakage and letal lesions by X irradiation in ataxia telangiectasia and normal fibroblasts, Radiat. Res., 124, 334-335.

Raghavan, D., W.U. Shipley, M.B. Garnick, P.J.Russell, J.P. Richie (1990) Biology and management of bladder cancer, New Engl. J. Med., 322 (16), 1129-1138.

Rauth, A.M. (1986) The induction and repair of ultraviolet light damage in mammalian cells, In: F.J. Burns, A.C. Upton and G. Silini (Eds.), Radiat. Carcinogenesis and DNA Alterations, Plenum Press, New York, pp.212-226.

Reid, T.M. and L.A. Loeb (1993) Effect of DNA-repair enzymes on mutagenesis by oxygen free radicals, Mutat. Res., 289, 181-186.

Reiners, J.J.Jr., B.C. Pence, M.C.S. Barcus and A.R. Cantu (1987) 12-O-tetradecanoylphorbol-13-acetate-dependent induction of xanthine dehydrogenase and conversion to xanthine oxidase in murine epidermis, Cancer Res., 47, 1775-1779.

Robertson, F.M., A.J. Beavis, T.M. Oberyszyn, S.M. O'Connell, A. Dokidos, D.L. Laskin, J.D. Laskin and J.J.Jr. Reiners (1990) Production of hydrogen peroxide by murine epidermal keratinocytes following treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, Cancer Res., 50, 6062-6067.

Roediger, W.E.W., M.J. Lawson and B.C. Radcliffe (1990) Nitrite from inflammatory cells - a cancer risk factor in ulcerative colitis? Dis. Colon Rectum, 33, 1034-1036.

Roots, R. and S. Okada (1975) Estimation of lifetime and diffusion distance of radicals involved in X-ray induced DNA strand breaks or killing of mammalian cells, Radiat. Res., 64, 306-320.

Roots, R.K. and M.S. Chen (1981) The microbicidal mechanisms of human neutrophils and eosinophils, Rev. Infect. Dis., 3, 565-598.

Rosin, M.P. (1991) Genetic and proliferative markers in clinical studies of the premalignant process, Cancer Bull., 43, 507-514.

Rosin, M.P. (1992) The use of the micronucleus test on exfoliated cells to identify anti-clastogenic action in humans: a biological marker for the efficacy of chemopreventive agents, Mutat. Res., 267, 265-276.

Rosin, M.P. and W. Anwar (1992) Chromosomal damage in urothelial cells from Egyptians with chronic *Schistosoma haematobium* infections, Int. J. Cancer, 51, 1-5.

Rosin, M. P., H. D. Ochs, R. A. Gatti and E. Boder (1989) Heterogeneity of chromosomal breakage levels in epithelial tissue of ataxia-telangiectasia homozygotes and heterozygotes, Hum. Genet., 83, 133-138.

Rosin, M.P., S.S. El Din Zaki, A.J. Ward and W.A. Anwar (1994) Involvement of inflammatory reactions and elevated cell proliferation in the development of bladder cancer in schistosomiasis patients, Mutat. Res., 305, 283-292.

Rossi, F. (1986) The superoxide anion-forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function, Biochim. Biophys. Acta, 853, 65-89.

Sanal, O. S. Wei, T. Foroud U. Malhortra P. Concannon, P. Charmley, W. Salser, K. Lange and R. A. Gatti (1990) Further mapping of an ataxia-telangiectasia locus to the chromosome 11q23 region, Am. J. Hum. Genet., 47, 860-866.

Sandberg, A.A. (1992) Chromosome changes in early bladder neoplasms, J. Cell Biochem. Supp. 161, 76-79.

Sanford, K., R. Parshad, F.M. Price, G.M. Jones, R.E. Tarone, L. Eierman, P. Hale and T.A. Waldmann (1990) Enhanced chromatid damage in blood lymphocytes after G_2 phase X irradiation, a marker of the ataxia-telangiectasia gene, J. Nat. Cancer Inst., 82 (12), 1050-1054.

Sanford, K.K., F.M. Price, J.S. Rhim, M.R. Stampfer and R. Parshad (1992) Role of DNA repair in malignant neoplastic transformation of human mammary epithelial cells in culture, Carcinogenesis, 13 (7), 1137-1141.

Santos, E., S.R. Tronick, S.A. Aaronson, S. Pulciani and M. Barbacid (1982) T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALA- and Harvey-MSV transforming genes, Nature, 298, 343-347.

Saxon, P. J., E. S. Srivatsan and E. J. Stanbridge (1986) Introduction of normal human chromosome 11 via microcell transfer controls tumourigenic expression of HeLa cells, EMBO Journal, 5, 3461-3466.

Schraufstatter, I., P.A. Hyslop, J.H. Jackson, and C.G. Cochrane (1988) Oxidant-induced DNA damage of target cells, J. Clin. Invest., 82, 1040-1050.

Shacter, E., E.J. Beecham, J.M. Covey, K.W. Kohn, and M. Potter (1988) Activated neutrophils induce prolonged DNA damage in neighboring cells, Carcinogenesis, 9, 2297-2304.

Shaham, M. Y. Becker and M. M. Cohen (1980) A diffusable clastogenic factor in ataxia-telangiectasia, Cytogenet. Cell Genet., 27, 155-161.

Sheinfeld, J. (1992) Markers of early lesions, J. Cell. Biochem, Supp. 161, 48-49.

Shiloh, Y., E. Tabor and Y. Becker (1983) Abnormal response of ataxia telangiectasia cells to agents that break the deoxyribose moiety of DNA via a targeted free radical mechanism, Carcinogenesis, 4, 1317-1322.

Shiloh, Y, R. Parshad, M. Frydman, K.K. Sanford, S. Portnoi, Y. Ziv and G.M. Jones (1989) G2 chromosomal radiosensitivity in families with ataxia telangiectasia, Hum. Genet., 84, 15-18.

Sidransky, D. and E. Messing (1992) Molecular genetics and biochemical mechanisms in bladder cancer, Urolog. Clinica of North Amer., 19 (4), 629-639.

Sidransky, D., T. Mikkelsen, K. Schwecheimer, M.L. Rosenblum, W. Cavanee and B. Vogelstein (1992) Clonal expansion of p53 mutant cells is associated with brain tumour progression, Nature, 355, 846-847.

Silverman, D.T., R.N. Hoover, T.J. Mason and G.M. Swanson (1986) Motor exhaust-related occupations and bladder cancer, Cancer Res., 46, 2113-2116.

Snyder, R.D. (1985) An examination of the DNA damaging and repair inhibitory capacity of phorbol myristate acetate in human diploid fibroblasts, Carcinogenesis, 6, 1667-1670.

Srivatsan, E.S., W. F. Benedict and E. J. Stanbridge (1986) Molecular analysis of the chromosomal control of neoplastic expression in human cell hybrids: chromosome 11 is implicated in the suppression of tumourigenicity, Cancer Res., 46, 6174-6179.

Stanbridge, E.J. (1988) Genetic analysis of human malignancy using somatic cell hybrids and monochromosome transfer, Cancer Surveys, 7, 317-324.

Stanbridge, E. J. and J. Wilkinson (1978) Analysis of malignancy in human cells: malignant and transformed phenotypes are under separate genetic control, Proc. Nat. Acad. Sci. USA, 75, 1466-1469.

Stewart, B.W. (1992) Role of DNA repair in carcinogenesis, In: H. Vainio, P.N. Magee, D.B. McGregor and A.J. McMichael (Eds.), International Agency for Research on Cancer, Lyon, France, pp. 307-320.

Stelmaszynska,T. and J.M. Zgliczynski (1974) Myeloperoxidase of human neutrophilic granulocytes as chlorinating enzymes, Eur. J. Biochem., 45, 305-312.

Stich, H.F. and M.P. Rosin (1983) Micronuclei in exfoliated human cells as an internal dosimeter for exposure to carcinogens, In: H.F. Stich (Ed.) Carcinogens and Mutagens in the Environment, Vol. II: Naturally Occurring Compounds: Endogenous Formation and Modulation, CRC Press, Boca Raton, Florida, pp. 17-25.

Swift, M., R. A. Heim and N. J. Lench (1993) Genetic aspects of ataxia telangiectasia, Adv. Neurol., 61, 115-125.

Swift, M., D. Morrell, E. Cromartie, A. R. Chamberlin, M. H. Skolnick and D. T. Bishop (1986) The incidence and gene frequency of ataxia-telangiectasia in the United States, Am. J. Hum. Genet., 39, 573-583.

Swift, M., P.J. Reitnauer and D. Morrell (1987) Breast and other cancers in families with ataxia telangiectasia, N. Engl. J. Med., 316, 1289-1294.

Synderman, R. and M.C. Pike (1984) Chemoattractant receptors on hpagocytic cells, Ann. Rev. Immun., 2, 257-281.

Takita, K., T. Sato, M. Miyagi, M. Watatani, F. Akiyama, G. Sakamoto, F. Kasumi, R. Abe and Y. Nakamua (1992) Correlation of loss of alleles on the short arms of chromosomes 11 and 17 with metastasis of primary breast cancer to lymph nodes, Cancer Res., 52, 3914-3917.

Tanigami, A., T. Tokino, K-I. Takita, M. Ueda, F. Kasumi and Y. Nakamura (1992) Detailed analysis of an amplified region at chromosome 11q13 in malignant tumors, Genomics, 13, 21-24.

Tawfik, H.N. (1988) Carcinoma of the urinary bladder associated with schistosomiasis in Egypt: the possible causal relationship, In: R.W. Miller (ed.), Unusual Occurrences as Clues to Cancer Ethology, Japan Sci. Soc. Press, Tokyo, pp.197-209.

Taylor, A. M. R. (1982) Cytogenetics of ataxia-telangiectasia, In: B. A. Bridges, D. G. Harnden (Eds.), Ataxia-Telangiectasia - A Cellular and Molecular Link between Cancer, Neuropathology and Immune Deficiency, J. Wiley and Sons Ltd., London, England, pp. 53-81.

Taylor, A. M. R. (1992) Ataxia telangiectasia genes and predisposition to leukaemia, lymphoma and brest cancer, Brit. J. Cancer, 66 (1), 5-9.

Taylor, A.M. and S.V. Butterworth (1986) Clonal evolution of T-cell chronic lymphocytic leukaemia in a patient with ataxia telangiectasia, Int. J. Cancer., 37, 511-516.

Taylor, A. M. R., J. M. Oxford and J. A. Metcalfe (1981) Spontaneous cytogenetic abnormalities in lymphocytes from thirteen patients with ataxia-telangiectasia, Int. J. Cancer, 27, 311-319.

Taylor, A. M. R., D. G. Harnden, C. F. Arlett, S. A. Harcourt, A. R. Lehmann, S. Stevens and B. A. bridges (1975) Ataxia telangiectasia: a human mutation with abnormal radiation sensitivity, Nature, 258, 427-429.

Thacker, J. (1989) The use of integrated DNA vectors to analyse the molecular defect in ionizing radiation-sensitive mutants of mammalian cells including ataxia telangiectasia, Mutat. Res., 220, 187-204.

Thompson, L.H (1991) Properties and applications of human DNA repair genes, Mutat. Res., 247, 213-219.

Timme, T.L. and R.E. Moses (1988) Review: Diseases with DNA damage-processing defects, Amer. J. Med. Sci., 295 (1), 40-48.

Tricker, A.R., M.H. Mostafa, B. Spiegelhalder and R. Preussmann (1989) Urinary excretion of nitrate, nitrite and N-nitroso compounds in schistosomiasis and bilharzia bladder cancer patients, Carcinogenesis, 10, 547-552.

Trush, M.A., and T.W. Kensler (1991) An overview of the relationship between oxidative stress and chemical carcinogenesis, Free Rad. Biol. Med., 10, 201-209.

Tsai, Y.C., P.W. Nichols, A.L. Hiti, Z. Williams, D.G. Skinner and P.A. Jones (1990) Allelic losses of chromosomes 9, 11, and 17 in human bladder cancer, Cancer Res., 50, 44-47.

Turrens, J.F. and A. Boveris (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria, Biochem. J., 191, 421-427.

Tyrkus, M., I. Powell and W. Fakr (1992) Cytogenetic studies of carcinoma in situ of the bladder: prognostic implications, J. Urol., 148, 44-46.

Ueda, K., S. Kobayashi, J. Morita and T. Komano (1985) Site-specific DNA damage caused by lipid peroxidation products, Biochim. Biophy. Acta, 824, 341-348.

Vaca, C.E., J. Wilhelm and M. Harms-Ringdahl (1988) Interaction of lipid peroxidation products with DNA. A review, Mutat. Res., 195, 137-149.

Vuillaume, M. (1987) Reduced oxygen species, mutation, induction and cancer initiation, Mutat. Res., 186, 43-72.

Wakata, A. and M.S. Sasaki (1987) Measurement of micronuclei by cytokinesis-block method in cultured chinese hamster cells: comparison with types and rates of chromosome aberrations, Mutat. Res., 190, 51-57.

Warren, J.S., K.J. Johnson and P. Ward (1987) Oxygen radicals in cell injury and cell death, Pathol. Immunopathol. Res., 6, 301-315.

Weiss, S.J., M.B. Lampert and S.T. Test (1983) Long-lived oxidants generated by human neutrophils: characterization and bioactivity, Science, 222, 625-628.

Weissman, B. E., P. J. Saxon, S. R. Pasquale, G. R. Jones, A. G. Geiser and E. J. Stanbridge (1987) Introduction of a normal human chromosome 11 into a Wilms' tumor cell line controls its tumourigenic expression, Science, 236, 175-180.

Weitberg, A.B. and D. Corvese (1990a) translocation of chromosomes 16 and 18 in oxygen radical transformed human fibroblasts, Biochem. Biophys. Res. Commun., 169, 70-74.

Weitberg, A.B. and D. Corvese (1990b) Oxygen radical-induced malignant transformation of cultured human lung cells: an in vitro model, Proc. Am. Ass. Cancer Res., 31, 147.

Weitzman, S.A. and L.I. Gordon (1990) Inflammation and cancer: Role of phagocyte-generated oxidants in carcinogenesis, Blood, 76 (4), 655-662.

Weitzman, S.A. and T.P. Stossel (1982) Effect of oxygen radical scavangers and anti-oxidants on phagocyte-induced mutagenesis, J. Immunol., 128, 2770-2772.

Weitzman, S.A. and T.P. Stossel (1984) Phagocyte-induced mutation in Chinese hamster ovary cells, Cancer Lett., 22, 337-340.

Weitzman, S.A., A.B. Weitberg, E.P. Clark and T.P. Stossel (1985) Phagocytes and carcinogenesis: Malignant Transformation produced by human neutrophils, Science, 227, 1231-1233.

Weitzman, S.A., C. Schmeichel, C. Turk, P. Stevens, S. Tolsma and N. Bouck (1988) Phagocyte-mediated carcinogenesis. DNA from phagocyte-transformed C3H 10T 1/2 cells can transform NIH/3T3 cells, Ann. NY Acad. Sci., 551, 103-109.

Yi, M., M. P. Rosin and C.K. Anderson (1990) Response of fibroblast cultures from ataxia-telangiectasia patients to oxidative stress, Cancer Lett., 54, 43-50.

Ziv, Y., G. Rotman, M. Frydman, J. Dagan, T. Cohen, T. Foroud, R. A. Gatti and Y. Shiloh (1991) The ATC (ataxia-telangiectasia complementation group C) locus localized to 11q22-q23, Genomics, 9, 373-379.

CHAPTER II

EXPERIMENTAL APPROACH

2.1 Central Hypotheses

a) The tumour promoter TPA can induce DNA damage in cells of a tissue through direct interaction and/or through the activation of nearby inflammatory cells to release DNA-damaging agents. Possible agents of DNA damage include hydrogen peroxide and superoxide anion.

b) Genetic defects which are inborn or acquired during the process of carcinogenesis increase a cell's sensitivity to TPA-generated DNA-damaging agents.

c) Loci on chromosome 11 are associated with the protection of cells from oxidative DNA damage.

d) The mechanism underlying this protection is an increase in the efficiency of rejoining of DNA strand breaks.

2.2 Techniques

The micronucleus (MN) test was used to quantitate DNA breakage and the comet assay was used to measure initial single- and double-strand breakage and its rejoining.

2.2.1 The micronucleus (MN) assay

The MN assay is a sensitive method of assaying chromosomal breakage in cell culture and has been widely used to identify genotoxic agents (Heddle et al., 1983; 1991; Stich and Rosin, 1983). Micronuclei are formed when a cell divides and chromosome fragments or entire chromosomes which lack attachment to the spindle apparatus lag behind and are excluded from the main nuclei in the daughter cells. The Feulgen reaction stains the DNA of the nucleus and MN pink. To delineate the cell cytoplasm, a counterstain with Fast Green is used (Yi et al., 1990). MN are identified using a high-power objective (1000X) in both bright-field and phase-contrast microscopy. The MN are clearly separate from the main nucleus, lie in the same optical plane, and resemble the nucleus in both chromatin texture and staining pattern. The upper size range of the MN is usually a fifth the size of the nucleus. The lower size limits vary between laboratories. We score all Feulgen-positive bodies, including small irregular shaped structures (Rosin, 1992).

The number of micronuclei observed in a given number of cells is dependent on the proportion of cells that have responded to the treatment and of that proportion the number that have divided. To establish the number of dividing cells, Fenech and Morley (1985) introduced the cytokinesis-block MN assay. Cytochalasin B when added to a cell culture will act on actin polymerization to prevent cytokinesis. A cell that has divided and is thus at risk for MN formation will appear as binucleated and easily identifiable from those cells which have not divided. Therefore, only the population of cells at risk for micronuclei production are analyzed and the number of micronuclei is not diluted by unknown cell turnover. In recent years, there has been extensive use of the cytokinesis-block method for analyzing MN frequencies (Wakata and Sasaki, 1987; Erexson et al,. 1987; Krishna et al., 1989; Eastmond and Tucker, 1989; Yi et al., 1990).

2.2.2 Developing the co-culture assay for MN formation

Co-culture of activated inflammatory cells with tissue cells is often avoided for a number of technical reasons and model systems such as xanthine/xanthine oxidase (X/XO) are favoured. The X/XO mixture generates superoxide anion and hydrogen peroxide. Since it is known that an array of products are released from activated inflammatory cells and the formation of the DNA damage may be dependent on the interaction of such products, a co-culture assay for MN formation was developed during this research. This is the first time that MN have been observed in a co-culture system.

Details of the procedure can be found on page 80. In developing the co-culture assay for MN formation, the following technical problems were encountered:

Macrophages were unsuitable for co-culture in the MN assay

Macrophages were not suitable for the MN assay since they adhered strongly to the glass coverslips on which the tissue cell cultures were grown. As a result of this adhesiveness the macrophages often obstructed the view of the cultured cells and prohibit scoring. The macrophages could not be removed with repeated washing after the treatment was complete. Neutrophils were therefore selected as the inflammatory cells since they did not show the same adhesive quality.

Fresh blood was needed for maximum neutrophil activation capacity

As neutrophil activation capacity was found to decrease with time after removal from the body, fresh blood was used for all studies. This blood was drawn from 10 volunteers in the laboratory. Once the neutrophils had been

separated from a blood sample they were used within the hour for the co-culture treatment.

Variations existed in activation capacity of neutrophil preparations

Variations in neutrophil activation existed between people and also within a person over time (weeks). To monitor these variations, photon emission generated by the oxidative burst of activated neutrophils was measured using chemiluminescence.

ROS released during the oxidative burst are in an unstable, excited form. They release energy in the form of photons of light and so obtain a more stable ground state. Luminol is used to enhance the photon emission so that it is easily detectable by a photomultiplyer tube in the luminometer. The chemiluminescence of luminol is a result of its oxidization to a high energy state intermediate in the presence of oxidizing species (Easmon et al., 1980).

A neutrophil preparation was used when the counts/sec were within an optimum range (8×10^4 - 2×10^5). The counts would remain within this range for an average time interval of 15 - 20 minutes. The oxidative burst was complete within 1 hour.

Media type affected DNA damage

The media used during co-culture affected the level of DNA-damaging agents able to interact with the tissue cells. Co-cultures performed in RPMI media sustained the most DNA damage. In contrast, D-MEM with and without FCS appeared to quench the DNA-damaging effect of the activated neutrophils.

Neutrophils needed to be in close proximity to tissue cells to generate DNA damage

Cell-to-cell contact was found to be important for the induction of DNA damage by activated neutrophils in tissue cells. To generate optimum cell-to-cell contact, the total reaction volume as well as the length of the exposure was found to be critical. Rapid cell contact was facilitated by using a small volume during co-culture. An exposure time of one hour enabled the inflammatory cells to remain in close proximity to the tissue cells long enough to complete their respiratory burst and generate DNA damage.

A number of different techniques for ensuring cell contact were tested:

a.) "Sandwich" coverslips. Tissue cells were grown on a coverslip and sandwiched with macrophages on another coverslip. A separation distance of one coverslip width was created between the two coverslip cultures by placing small pieces of a broken coverslip between them.

b.) Mixed cell suspension. Tissue cells and neutrophils were suspended together in varying volumes (500 µl - 2 ml) of media for various time intervals (15, 30, 60 minutes) before being seeded into tissue culture dishes.

c.) Mixed cell suspension and spin down. Cells were suspended as described above, centrifuged (200 xg), and left in a pellet for varying time intervals before seeding into dishes.

d.) Transwell co-culture dishes. These culture dishes have a hanging insert with pores on it into which the inflammatory cells could be placed. The tissue cell cultures were seeded on to glass coverslips in the lower chamber of these specialized dishes.

None of the above procedures were particularly effective. However, further adaption of methods tested in (b) resulted in the development of a suitable co-culture that was optimum for cell contact and induction of MN in the tissue cells:

The activated neutrophils were suspended in a drop (250 µl) of medium over the coverslip culture for 1 hour. This procedure worked because of the moist coverslip on which the tissue cells were seeded was transferred to a dry petri dish. The 250 µl of solution containing the activated neutrophil was held in a crescent-shaped meniscus over the surface of the coverslip. The neutrophils settled quickly onto the surface of the cultured cells.

Neutrophil cell concentration reflected level of MN induction

The number of neutrophils used in the cell cultures was crucial. When too many neutrophils were used, cell toxicity became apparent as demonstrated by a reduction in the nuclear division index and the loss of the cytoplasmic membrane on the treated cells. The ratio of neutrophils:cultured cells used in these studies resulted in the induction of chromosomal breakage with a limited amount of toxicity.

2.2.3 The comet assay

The comet assay is a sensitive technique which can be used to quantify single- and double-strand DNA breakage and repair in cells that have been exposed to DNA-damaging agents. Single cells are embedded in agarose, lysed, subjected to electrophoresis, stained with a fluorescent DNA-binding dye, and then observed using a fluorescence image-processing system. Alkaline and neutral lysing solutions allow the detection of single- and double-strand DNA breakage respectively (Olive et al., 1990a,1991; 1992; Deeley and Moore, 1992; McKelvey-Martin et al., 1993).

Unlike other DNA breakage and repair assays that only generate an average response for cells, the comet assay can also look at the response of individual cells. The average response of cells is often not representative of the diversity within a sometimes heterogeneous cell culture or tissue. With this method it can be determine whether all cells within a population show equal amounts of damage and whether all the cells rejoin DNA damage at the same rate. For example, it can be used to identify subpopulations of drug-resistant and sensitive cells within a tumour (Olive et al., 1990a,b).

When a cell containing DNA breakage is subjected to an electric current, the negatively charged broken and relaxed DNA is pulled away from the nucleus towards the anode. When imaged the cell resembles a comet with a brightly fluorescent head and tail region which contains the fragments and relaxed supercoils of DNA. The tail has a length and intensity which is representative of the amount of DNA damage produced in the cell. The distance the DNA moves is related to the size of free or relaxed pieces. The smaller the pieces the farther they will move. The intensity of the tail is an indication of the number of pieces that have migrated (Olive et al., 1990a; McKelvey-Martin et al., 1993). Olive and co-workers use a single number called the "tail moment" to provide a

measurement of the intensity as well as the distance migrated by the damaged DNA. This number is calculated by multiplying the percentage of DNA in the tail by the displacement between the means of the head and tail distributions. All measurements are made from a digitized pixel matrix image of the comet. This image is created using a program written by Dr. R. Durand of the B.C. Cancer Research Centre (Olive et al., 1990a).

2.3 References

Deeley, J.O.T. and J.L. Moore (1992) Single-cell microgel electrophoresis: an *in vitro* assay of radiosensitivity, Brit. J. Radiol., 24 (Suppl.), 79-83.

Easmon, C.S.F., P.J. Cole, A.J. Williams and M. Hastings (1980) The measurement of opsonic and phagocytic function by Luminol-dependent chemiluminescence, Immunol., 41, 67-74.

Eastmond, D.A. and J.D. Tucker (1989) Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocyes and an antikinetochore antibody, Environ. Mol. Mutagen., 13, 34-43.

Erexson, G.L., A. D. Klingerman and J.W. Allen (1987) Diaziquone-induced micronuclei in cytochalasin B-blocked mouse peripheral blood lymphocytes, Mutat. Res., 178, 117-122.

Fenech, M. and A.A. Morley (1985) Measurement of micronuclei in lymphocytes, Mutat. Res., 147, 29-36.

Heddle, J.A., M. Hite, B. Kirkhart, K. Mavournin, J.T. MacGregor, G.W. Newell and M.F. Salamone (1983) The induction of micronuclei as a measure of genotoxicity, Mutat. Res., 123, 61-118.

Heddle, J.A., M.C. Cimino, M. Hayashi, F. Romagna, M.D. Shelby, J.D. Tucker, P.H. Vanparys and J.T. MacGregor (1991) Micronuclei as an index of cytogenetic damage: past, present and future, Environ. Mol. Mutagen., 18, 277-291.

Krishna, G., M.L. Kropko and J.C. Theiss (1989) Use of the cytokinesis-block method for the analysis of micronuclei in V79 chinese hamster lung cells: results with mitomycin C and cyclophosphamide, Mutat. Res., 222, 63-69.

McKelvey-Martin, V.J., M.H.L. Green, P. Schmezer, B.L. Pool-Zobel, M.P. De Meo and A. Collins (1993) The single cell gel electrophoresis assay (comet assay): A European review, Mutat. Res., 288, 47-63.

Olive, P.L., J.P. Banath and R.E. Durand (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay, Radiat. Res., 122, 86-94.

Olive, P.L., J.P. Banath and R.E. Durand (1990) Detection of etoposide resistance by measuring DNA damage in individual Chinese hamster cells, J. Nat. Cancer Inst., 82, 779-783.

Olive, P.L., D. Wlodek and J.P. Banath (1991) DNA double-strand breaks measured in individual cells subjected to gel electrophoresis, Cancer Res., 51, 4671-4676.

Olive, P.L., D. Wlodek, R.E. Durand and J.P. Banath (1992) Factors influencing DNA migration from individual cells subjected to gel electrophoresis, Exp. Cell Res., 198, 259-267.

Rosin, M.P. (1992) The use of the micronucleus test on exfoliated cells to identify anti-clastogenic action in humans: a biological marker for the efficacy of chemopreventive agents, Mutat. Res., 267, 265-276.

Stich, H.F. and M.P. Rosin (1983) Micronuclei in exfoliated human cells as an internal dosimeter for exposure to carcinogens, In: H.F. Stich (Ed.) Carcinogens and Mutagens in the Environment, Vol. II: Naturally Occurring Compounds: Endogenous Formation and Modulation, CRC Press, Boca Raton, Florida, pp. 17-25.

Wakata, A. and M.S. Sasaki (1987) Measurement of micronuclei by cytokinesis-block method in cultured chinese hamster cells: comparison with types and rates of chromosome aberrations, Mutat. Res., 190, 51-57.

Yi, M., M. P. Rosin and C.K. Anderson (1990) Response of fibroblast cultures from ataxia-telangiectaisia patients to oxidative stress, Cancer Lett., 54, 43-50.

CHAPTER III

A sensitivity to oxidative stress is linked to chromosome 11 but is not due to a difference in single-strand DNA breakage or its rejoining

Mutation Research, 294, 299-308, 1993.

3.1 Abstract

Defects in loci on chromosome 11 have been associated with tumourigenicity, anchorage-independent growth, metastasis and radiosensitive DNA repair in tumour cells. The introduction of normal chromosome 11 into these tumour cells suppresses these responses. In the present study we tested two hypotheses: 1) that microcell fusion of normal chromosome 11 into bladder carcinoma cells (A1698) can protect the cells against chromosomal damage by oxidative stress; and 2) that insertion of normal chromosome 11 corrects a single-strand (SS) DNA rejoining defect. Cultures of A1698 (termed parent) and its microcell-mediated hybrid (termed hybrid) were exposed for 1 hour to X/XO or co-incubated with human neutrophils activated with TPA. The frequencies of micronucleated cells (an indication of chromosome damage) were significantly higher in parent cultures after treatment than in hybrid ($P<0.0001$). The level of SS DNA breakage and its rejoining were assayed in X/XO-treated cultures with the alkaline comet assay. There was no significant difference between parent and hybrid in the amount of SS DNA breakage at treatment ($P>0.1$) or after 20 mins of rejoining ($P>0.1$). The data support the involvement of a defect in chromosome 11 leading to sensitivity to oxidative stress and suggest this defect is not in the initial amount or rate of rejoining of SS DNA breakage.

3.2 Introduction

An association between chronic inflammation and increased risk for cancer is supported by several clinical observations (reviewed in Kaplan, 1987; Gordon and Weitzman, 1988; Preston-Martin et al., 1990; Weitzman and Gordon, 1990; Trush and Kensler, 1991). For example, patients with ulcerative colitis are at elevated risk for colorectal cancer (Simmonds et al., 1992); patients with infections such as *Helicobacter pylori* are at elevated risk for stomach cancer (Correa, 1992); and infection with *Schistosoma haematobium* is strongly correlated with bladder tumourigenesis (Chen and Mott, 1989). In such conditions, tumours develop in the tissue undergoing chronic (long-term) inflammation. Animal studies also show an association between inflammation and tumour development. A variety of irritants, both mechanical and chemical (e.g. tumour promoters such as TPA), are known to increase tumourigenesis in carcinogen-exposed animals. Among the plethora of changes induced by these irritants are two which appear ubiquitously: the induction of inflammation in the tissue in which the tumour will develop and the stimulation of cell proliferation.

One mechanism by which inflammation may be acting to increase tumour development is by induction of genetic damage in the inflamed tissue through release of ROS by activated inflammatory cells. Support for this mechanism comes from *in vitro* studies in which TPA-activated inflammatory cells have been co-incubated with a variety of target cells. DNA strand breakage, chromosomal change and malignant transformation are reported in the target cells (Cerutti, 1985; Gordon and Weitzman, 1988; Schacter et al., 1988; Schraufstatter et al., 1988; Frenkel, 1992). Activation of inflammatory cells results in the release of many products, including superoxide anion, hydrogen peroxide, and numerous secondary oxidants, as well as products of the arachidonic acid cascade (Lewis et al., 1986; Ochi and Cerutti, 1987; Gordon and Weitzman, 1988; Frenkel,

1992). Although the path by which inflammatory cells induce DNA damage is unresolved, a favored mechanism involves the interaction of hydrogen peroxide with metals bound to the nucleic acid in a metal catalyzed Haber-Weiss reaction that produces hydroxyl radical. It is this radical which may be responsible for DNA damage (Frenkel, 1992).

One of the interests of this laboratory is the identification of host factors that increase sensitivity to oxidative damage and, more specifically, the products of activated inflammatory cells. Recent studies suggest that chromosome 11 may have loci that affect this sensitivity. Sanford and co-workers have reported the presence of elevated chromosomal damage in tumour cell lines compared to normals after x-irradiation (Parshad et al., 1992). They attribute this radiosensitivity to a defect in DNA repair. Insertion of normal chromosome 11 into these tumour cultures results in restoration of the level of radiation-induced chromosomal damage in these cells to that observed in normal cultures. Chromosome 11 alteration is a common event in tumourigenesis; for example, it is frequently present in bladder tumours (Tsai et al., 1990; Hopman et al., 1992). We propose that alterations in chromosome 11 in tissues undergoing chronic inflammation may play a critical role in tumourigenesis. Such alterations would elevate genetic instability in the tissue and increase the probability of acquiring the multiple alterations to specific oncogenes or suppressor genes required for tumour development (Rosin et al., 1994).

This hypothesis was tested by assaying two cell cultures for sensitivity to oxidative stress: a bladder carcinoma culture (termed parent) and its microcell hybrid, containing a normal chromosome 11 insert (termed hybrid). These cultures were exposed to X/XO, a treatment which produces a mixture of superoxide and hydrogen peroxide, or co-incubated with TPA-activated neutrophils. The MN test was used to quantitate chromosomal damage.

Micronuclei are produced from acentric chromosomal fragments or entire chromosomes excluded from the main nucleus when the cell divides. SS DNA breakage and its rejoining was quantitated with the alkaline comet assay. This assay analyzes individual cells within a population and provides data on the heterogeneity of DNA breakage and its rejoining. The results of these studies support the hypothesis that chromosome 11 protects against chromosomal damage in bladder carcinoma cells although this protection does not involve alteration in SS DNA breakage or its rejoining.

3.3 Materials and methods

3.3.1 Cell lines and culture

The primary culture of normal human epidermal breast keratinocytes, NHEK-267-1, was obtained from Clonetics (San Diego, CA). The bladder carcinoma cell line A1698 and its microcell hybrid clone, A1698 + der(11) clone 1, were obtained from Dr. O. Pereira-Smith, Baylor College of Medicine, Houston,TX. This hybrid contains an intact der(11), which consists of a derivative chromosome 11 from a normal donor which 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 et al., 1991). Ning and co-workers have shown that this insertion results in a loss of tumourigenicity in the hybrid. Karyotyping of the parent and its hybrid revealed a near-triploid chromosome complement with a der[11] insert in the hybrid.

Bladder cultures were grown in Dulbecco's modified eagle media (D-MEM, 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 µg/ml). The medium for the hybrid was also supplemented with HAT (hypoxanthine 10^{-4} M, aminopterin 5 x 10^{-7}M and thymidine 10^{-5} M) to maintain selection for the der(11). The keratinocyte culture was maintained in keratinocyte serum free medium supplemented with bovine pituitary extract (50 µg/ml) and epidermal growth factor (5 ng/ml) (K-SFM, Clonetics, San Diego, CA). All stock cultures were maintained in 75 cm^2 culture flasks at 37°C in incubators with a 5% CO_2/95% air atmosphere.

3.3.2 The MN assay

The MN test was performed on cell cultures growing on sterile 22 mm^2 glass coverslips placed in 10 x 30 mm^2 tissue culture dishes. These coverslip cultures were prepared by seeding bladder cell cultures in D-MEM medium with 10% FCS and the keratinocyte culture in K-SFM. The bladder and keratinocyte cultures were seeded at 1.5 x 10^5 cells/dish and treated on the following day. At this time, the cell density of all cultures was approximately 65% of a monolayer.

The X/XO treatment was performed as follows: The medium was removed by suction from the dishes and the cells were washed twice in 1 ml of fresh medium, D-MEM minus FCS for the bladder cultures and K-SFM for the keratinocyte cultures. Cultures were exposed for 1 h at 37°C to xanthine (Sigma, St. Louis, MO, sodium salt, 174 µg/ml) and xanthine oxidase (Sigma, St. Louis, MO, Grade III from buttermilk, 6.04 x 10^{-7} - 3.02 x 10^{-6} units/µl) prepared in phosphate-buffered saline (PBS, components/liter: 8 g sodium chloride, 0.2 g potassium chloride, 1.15 g dibasic sodium phosphate and 0.2 g monobasic potassium phosphate, pH 7.4). Following treatment, the X/XO solution was removed with suction, the cells were washed twice and fresh culture media was added. One hour later, cytochalasin B (Sigma, St. Louis, MO.) was added to the media in each dish (final concentration, 2 µg/ml) in order to identify cells that have passed through one division cycle after treatment. This drug prevents cytokinesis and thus cells undergoing karyokinesis after the treatment will appear binucleated (Fenech and Morley, 1985). For bladder cultures, the cells were incubated with cytochalasin B for 24 h prior to harvest. Pilot studies with the keratinocyte cultures showed that harvests at 24 h did not allow sufficient time to accumulate an adequate number of cells since the cell cycle time was longer than that of the bladder cultures. Subsequently, keratinocyte cultures were left for 48 h in cytochalasin-containing media prior to harvest.

In the co-incubation experiments with bladder cultures and activated neutrophils, the neutrophils were separated by placing 6 ml of heparanized human blood onto a Histopaque density gradient consisting of 3 ml of Histopaque #1077 overlaid on 3 ml of Histopaque #1119 (Sigma, St. Louis, MO). This gradient was centrifuged (700 x g) at room temperature for 30 min. The separated neutrophils were washed twice in PBS and resuspended at a cell concentration of 2×10^6 cells/ml in RPMI 1640 (Gibco Laboratories, Grand Island, NY). Using Wright Stain, these preparations were determined to consist of 95 $\pm$ 1.15% neutrophils. Prior to co-culture, the activation capacity of each neutrophil isolation was monitored by measuring the luminol-enhanced chemiluminescence produced when the neutrophils were exposed to TPA (Easmon et. al. 1980; Fischer et. al. 1985). 75 µl of the neutrophil suspension was added to a mixture consisting of 100 µl of TPA (Sigma, St. Louis, MO, 25 ng/ml) and 75 µl of luminol (Sigma, St. Louis, MO, 0.1 mg/ml) prepared in RPMI medium and the chemiluminescence generated in the sample was quantitated on a Packard Model 6100 (Pico-lite) Luminometer. Preparations were used if the counts/sec were within the range of 7.6×10^4 to 1.6×10^5. The counts remained within this range for 17 $\pm$ 2 min after initial activation of the neutrophils, the maximum intensity occurred at 14 $\pm$ 2 min and the photon emission was complete within 1 h.

To co-incubate, each coverslip was removed from its tissue culture dish, washed by dipping 5 times in RPMI (without FCS) and placed in a fresh dish. A treatment mixture consisting of 75 µl of neutrophils, 100 µl of TPA and 75 µl of RPMI was prepared and immediately pipetted onto the centre of the coverslip. As the coverslip was wet from washing and the surrounding dish dry, the drop of 250 µl was held in a crescent-shaped meniscus over the coverslip. As controls, coverslip cultures were exposed to either TPA or neutrophils in RPMI.

Treatment was for 1 h in the dark at room temperature. Each coverslip was then removed from its dish, washed by dipping 5 times in RPMI, and placed into a fresh dish with 2 ml of D-MEM with FCS. Cytochalasin B was added after 1 h and the cells were harvested 24 h later.

For both X/XO and co-incubation treatments, coverslip cultures were harvested for MN analysis by removing the coverslips from the dishes, dipping them in PBS, air-drying and fixing them for 20 min with carnoys (methanol: glacial acetic acid in a ratio of 3:1). The cellular DNA was stained with the Feulgen reaction as described in Yi et al. (1990). To delineate the cell cytoplasm, cells were counterstained for 15 sec with a 0.5% solution of fast green (Fisher Scientific, Fairlawn, NJ) prepared in 95% ethanol. The coverslip cultures were then dehydrated through an alcohol-xylene series and mounted in permount on glass slides.

The slides were blind-coded and a total of 500 binucleated cells per slide were analyzed for the presence of micronuclei using criteria described in Rosin, 1992. Each experiment was repeated a minimum of three times. In addition to calculating the percentage of binucleated cells with micronuclei, the nuclear division index (NDI) was determined for each slide. This index is used as a measurement of toxicity and/or inhibition of cell growth in different cultures. It is calculated by dividing the percentage binucleated cells present in the treated culture by the percentage of such cells in the untreated culture and multiplying this ratio by 100.

3.3.3 Alkaline comet assay for single-strand DNA damage

Bladder cells were seeded directly into 10 x 30 mm^2 tissue culture dishes. One day later, the culture medium was removed by suction, the cells were washed twice in 1 ml of D-MEM (minus FCS) and then exposed at room

temperature for 15 min to xanthine (174 μg/ml) and xanthine oxidase (2.42 x 10^{-6} units/μl) prepared in PBS. After this treatment, the X/XO solution was removed and the cultures were washed and trypsinized under ice-cold conditions. The cells were kept on ice until analysis. In dishes where DNA rejoining was to be studied, cells were washed twice in 1ml of medium after the treatment and incubated at 37°C in medium with FCS for the allocated time of rejoining. The same harvest procedure was followed for these cells.

The cell suspensions were processed by mixing 0.5 ml of the suspension with 1.5 ml of 1% agarose (low-temperature-gelling, Sigma type VII) kept at 40°C. This mixture was pipetted rapidly onto a glass slide and placed on a ice-cold metal block for 1 min to gel. In the rest of the procedure, the slides were always covered from direct fluorescent light exposure which can cause SS breakage. The slides were immersed in lysing solution (0.03 M NaOH, 1 M NaCl and 0.1% *N*-lauroylsarcosine) at room temperature for 1 h and then washed 3 times (20 min each) in electrophoresis solution (0.03 M NaOH, 2 mM EDTA in double distilled water) to remove the salt. Slides were placed in a horizontal gel electrophoresis chamber (Bethesda Research laboratory model H-4) and a potential of 0.5V/cm for 25 min was applied. The slides were washed in distilled water for 10 min and then stained with 2.5 μg/ml propidium iodide for 10 min. They were then stored in a covered, humidified, air-tight box for up to 24 h.

The individual cells or comets were observed using a Zeiss epifluorescence microscope with 25X Plan-neofluor objective and a 100 W mercury power source with a 580 nm reflector and a 590 nm barrier filter. The microscope was attached to an imaging system consisting of a F4577 intensified camera (ITT electro-Optical Production, Fort Wayne, IN) and an IBM AT computer with an ITEX 100 image processor and framegrabber board from Imaging Technology Inc., Woburn, MA. The gain setting on the camera was set

at 350 for all experiments. Overlapping comets and those at the edges of the gel were omitted from analysis. From 50-200 comets were analyzed per slide. Since there was minimal DNA breakage in samples from untreated controls and from treated cultures with 4 h of repair, only 50 comets were analyzed for these samples. For all other samples, a minimum of 100 comets were analyzed. Using a program written by Dr. R. Durand of the B.C. Cancer Research Centre, the images of comets were digitized into a pixel matrix. An algorithm was used which defined the limits of the comet by applying an edge filter, the background was subtracted (defined as the image intensity at the edge of the comet) and head and tail distributions subsequently formed were analyzed. Several calculations were made for each individual comet image, the most important being the "tail moment". Tail moment was defined as the product of the amount of DNA in the tail (fluorescence intensity of the tail above background) multiplied by the tail length (distance between edge of comet head and end of tail) (Olive et al., 1990).

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 Bonferroni multiple comparisons. Comparisons between different cell lines were performed using a split plot design ANOVA with Bonferroni multiple comparisons. Comparisons of spontaneous micronucleus frequencies in parent and hybrid cultures when n=12 were done with an unpaired t-test. The P-value chosen for significance in these studies was 0.05.

3.4 Results

3.4.1 The effect of chromosome 11 insertion on spontaneous MN frequencies

In order to determine whether the spontaneous MN frequency of the parent bladder culture was affected by insertion of der(11), a comparison was made of MN frequencies in untreated parent and hybrid cultures. Cells were seeded onto glass coverslips and cytochalasin B was added after 1 day of growth to collect cells as they passed through karyokinesis. The percentage of binucleated cells with micronuclei was ascertained in cultures harvested 24 h later. No significant difference was observed in the spontaneous MN frequencies of the parent and hybrid cultures (MN frequencies $\pm$ S.E.: parent, 3.26 $\pm$ 0.36; hybrid, 3.96 $\pm$ 0.28; $P = 0.14$, $n = 12$ experiments).

3.4.2 The effect of chromosome 11 insertion on induction of MN by X/XO treatment

Parent and hybrid coverslip cultures were exposed for 1 h to X/XO, cytochalasin B was added to the cultures, and MN frequencies were determined in binucleated cells after 24 h growth. The response of the 2 cultures to this treatment was significantly different ($P = 0.0001$). Both cultures responded with a significant increase in MN frequencies (parent culture, $P = 0.0001$; hybrid culture, $P < 0.01$); however, the response was greater in the parent. Figure 4A shows the MN frequencies which were induced in each cell culture by X/XO treatment. Spontaneous values for each culture have been subtracted to make it easier to see the effect of the treatment. As an example of the differential response of the 2 cell lines, induced MN frequencies were 3.8-fold higher for parent than for hybrid cultures when treated with 1.81×10^{-6} units/μl of xanthine

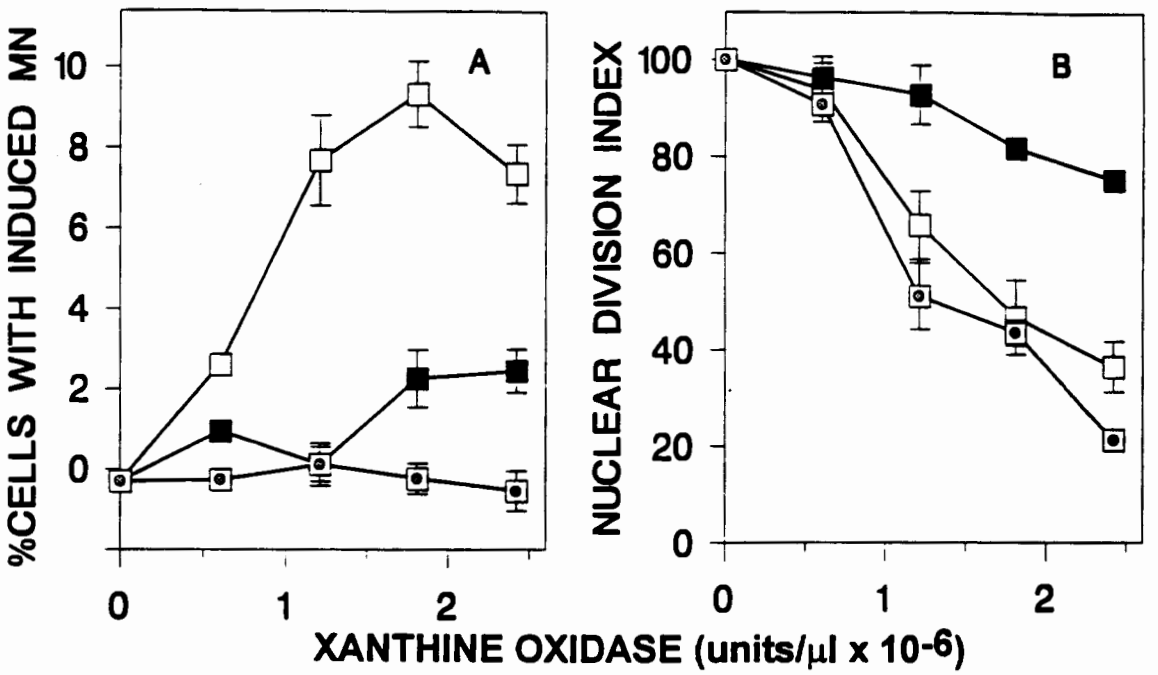

Figure 4. The effect of X/XO treatment on A) MN formation and B) NDI in a bladder carcinoma culture termed parent (□), its hybrid culture containing normal chromosome 11 (■), and a primary epithelial keratinocyte culture (⊡). Mean ± S.E. (n=3 experiments). Induced MN, is the frequency of MN at treatment minus the spontaneous MN frequency. Spontaneous values for untreated cultures: for parent, 2.28 ± 0.18; for hybrid, 3.37 ± 0.62; for keratinocyte, 2.01 ± 0.10.

oxidase and 174 µg/ml of xanthine (mean induced frequency for parent culture ± S.E., 9.64 ± 0.81; for hybrid cultures, 2.56 ± 0.54, $P < 0.001$).

Figure 4B shows the effect of the X/XO treatment on the NDI of parent and hybrid cultures. The two cultures responded differently to treatment ($P = 0.0001$) with a larger inhibition of nuclear division occurring in the parent. For example, at the treatment level mentioned above, the NDI of parent and hybrid cultures were 47% and 82% respectively ($P< 0.01$).

3.4.3 Response of keratinocyte cultures to X/XO treatment

Primary bladder epithelial cultures were not available for us to use in a comparative study of sensitivity to oxidative damage. In order to obtain some information on the sensitivity of normal epithelial cells to this treatment we made use of a commercially available breast keratinocyte cell culture, prepared from a clinically normal individual. The spontaneous MN frequency of this culture was 2.01 ± 0.10% (n=3), a value which did not differ significantly from the parent ($P = 0.62$; n=3) and which differed only marginally from the hybrid ($P = 0.04$, n=3). In contrast to the results observed with both the parent and hybrid cultures, this keratinocyte culture showed no significant induction of micronucleated cells at any of the X/XO doses used (Fig. 4A). However, the X/XO treatment did have an effect on the NDI of the keratinocyte culture (Fig. 4B). A similar drop in the NDI was observed for keratinocyte and parent cultures ($P = 0.12$). Control treatments with X alone or XO alone did not affect spontaneous MN or NDI frequencies in any culture.

3.4.4 The effect of chromosome 11 insertion on cellular response to TPA-activated neutrophils

Parent and hybrid cultures were co-incubated with TPA-activated neutrophils and the effect of this treatment on MN frequencies and NDI were determined. A significant induction of micronucleated cells occurred only in the parent culture (induced MN frequency for parent, 5.53 $\pm$ 0.71%, P = 0.0001; for hybrid, 0.50 $\pm$ 0.58%, P = 0.34, Fig. 5A). Both cultures showed a significant decrease in NDI; however, the change in parent cultures was larger (index for parents, 42%; for hybrids, 80%, Fig. 5B). Cultures co-incubated with unstimulated neutrophils had no significant change in NDI or MN frequencies. There was a slight increase in the frequency of micronucleated cells in cultures receiving the TPA treatment alone. This effect was significant for the parent cultures (P = 0.02) but not hybrid (P = 0.31). The TPA treatment produced a significant drop in the NDI of both cultures; however, a greater inhibition of nuclear division occurred in the parent (change in NDI, for parent, P = 0.0001; for hybrid, P = 0.02).

3.3.5 The effect of chromosome 11 on the formation of single-strand DNA breakage and its rejoining

Single-strand DNA breakage and rejoining was measured in single cells using the alkaline comet assay. Parent and hybrid cells were exposed to X/XO, embedded in agarose, lysed *in situ* in the presence of detergent and alkali and exposed to an electric field. Cells containing DNA breakage resembled comets with long tails. DNA breakage was quantified by measuring the tail moment, a product of the tail length and the amount of DNA in the tail.

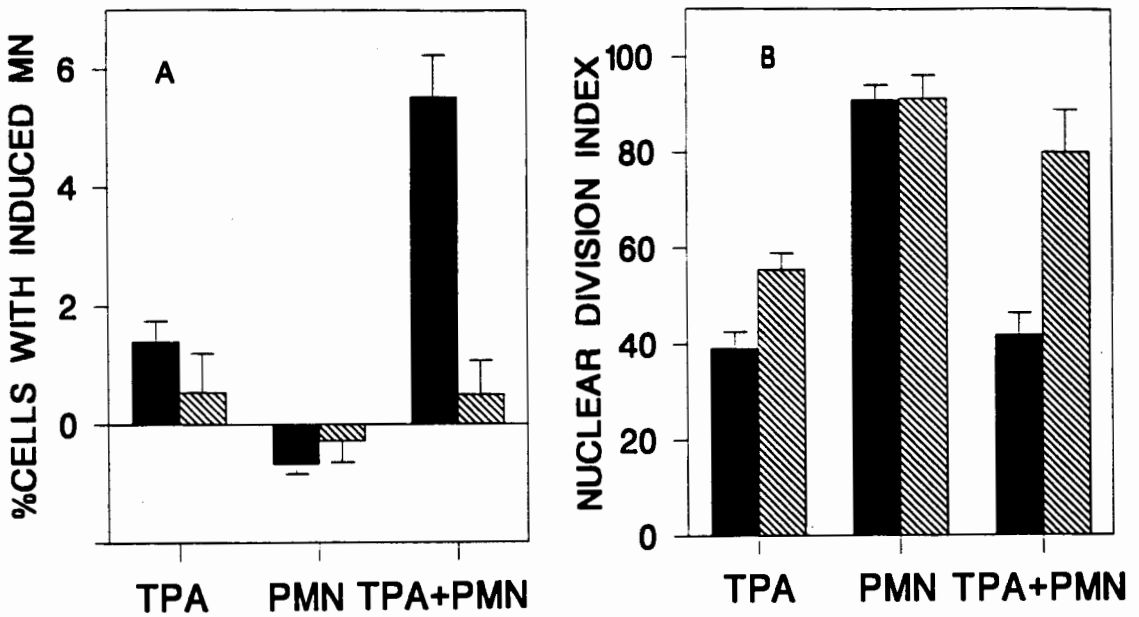

Figure 5. A) The induction of MN in parent (solid bars) and hybrid (striped bars) cultures co-incubated with TPA-activated neutrophils; B) NDI in these treatments. Mean ± S.E. (n=3 experiments). Spontaneous MN frequencies have been subtracted. Spontaneous values for untreated cultures: for parent, 2.36 ± 0.33; for hybrid, 4.10 ± 0.51.

PMN, polymorphonuclear leucocyte (neutrophil).

Figure 6 is a histogram showing the distribution of comet tails in untreated and treated cultures, and the rejoining of X/XO-induced DNA breakage with time. Compared to x-ray-induced breakage (Olive et al., 1992), both parent and hybrid cultures displayed a large variability in the amount of DNA breakage induced in different cells within the population by the treatment. This variability did not appear to be associated with any specific stage of the cell cycle since there was no relationship between DNA content (measured as the total intensity of fluorescence in each comet) and the tail moment (data not shown). Figure 7 shows the mean tail moments and standard error for the sample times shown in these histograms. X/XO treatment induced single-strand DNA breakage in the parent and hybrid; however, the amount of damage induced initially was not significantly different ($P = 0.68$). A close examination of the histograms in Figure 6 would suggest the possibility of a difference in the fast rejoining component of DNA breakage. A larger number of comets with tail moments similar to those in untreated cells was observed in the hybrid culture at 20, 40, and 60 min after treatment. However, in repetitions of the 20 min sample, this effect was shown not to be significant ($P = 0.09$).

It is also possible to use the comet assay to quantify double-strand DNA breakage by employing neutral rather than alkali conditions (Olive et al., 1991, 1992). Under neutral conditions DNA remains double-stranded and therefore DNA migrating in the electrophoretic field will be double-stranded. Using this approach we were unable to detect any increase in migration of DNA in X/XO treated cells compared to untreated cells, even when the enzyme concentration was increased 10-fold (data not shown).

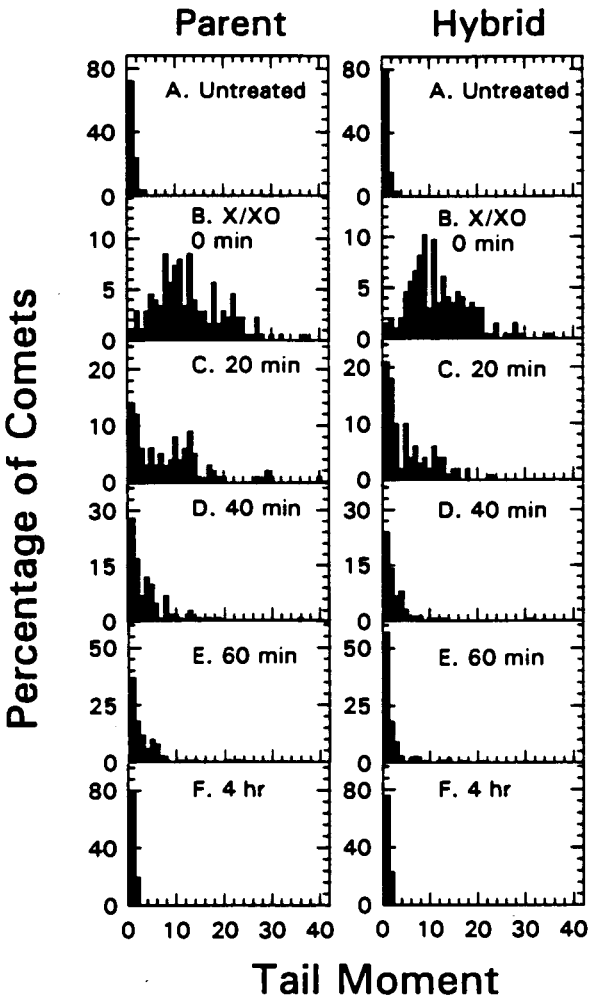

Figure 6. Heterogeneity in DNA damage and its rejoining in parent and hybrid cells treated with X/XO, as measured by the alkaline comet assay.

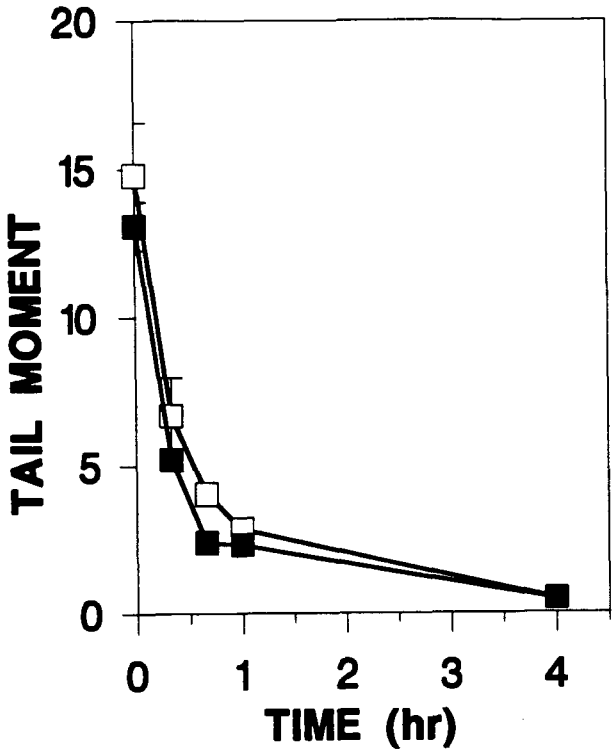

Figure 7. Rejoining of DNA damage induced by X/XO treatment with time in parent (open symbol) and hybrid (closed symbol) cultures. Mean tail moments ± S.E. (n=3 experiments) are shown for time 0 and for 20 minutes repair. Values for untreated cultures: for parent, 0.40 ± 0.12; for hybrid, 0.28 ± 0.13.

3.5 Discussion

When normal human chromosomes are introduced into tumour cell lines by microcell mediated transfer, alterations occur in phenotypic responses of some of these cultures. Insertion of chromosome 11 is associated with correction of radiosensitive DNA repair, restoration of anchorage dependence, suppression of tumourigenesis and inhibition of metastasis in tumour cell lines (Weissman et al., 1987; Stanbridge, 1988; Oshimura et al., 1990; Ichikawa et al., 1992; Negrini et al., 1992; Takita et al., 1992; Parshad et al., 1992). In this paper, we present the first evidence that insertion of chromosome 11 into a bladder carcinoma cell line is associated with a decrease in sensitivity to oxidative stress. Treatment of a bladder carcinoma culture with X/XO or with activated neutrophils results in the induction of micronucleated cells (an indication of chromosomal breakage and/or chromosome loss) and a decrease in the nuclear division index (an indication of toxicity and growth inhibition). Both of these phenomena are reduced by the introduction of chromosome 11 into the bladder carcinoma cell line.

At this point it is only possible to speculate on the mechanism underlying this reduction in sensitivity to oxidative stress in hybrid cultures. Previous reports have implicated genes on chromosome 11 in the repair of radiation-induced DNA damage. Parshad and co-workers (1992) report an abnormally high frequency of chromatid breaks and gaps when human tumours are x-irradiated during the G_2-phase of the cell cycle. This effect occurs in tumours of different tissue origin and/or histopathology (Parshad et al., 1992). Insertion of normal chromosome 11 into these tumour cell lines results in a reduction of radiation-induced damage to the level observed in normal cells. These workers attribute this effect to a restoration of a defective DNA repair process. It is possible that the same mechanism is affecting the sensitivity of the bladder

carcinoma cultures to oxidative stress induced by X/XO and TPA-activated neutrophils.

In our studies we explored the possibility that the insertion of chromosome 11 into the bladder parent cell line was decreasing its sensitivity to chromosomal damage by altering initial DNA breakage or its rejoining. The initial amount of SS DNA breakage and the rate of its rejoining were analyzed with the alkaline comet assay in parent and hybrid cells treated with X/XO. Chromosome 11 insertion did not affect either of these parameters. These results do not eliminate the possibility that a double-strand (DS) DNA rejoining defect is involved. We were unable to detect DS DNA breakage in X/XO-treated cells with the neutral comet assay. However, other investigators have measured double strand breakage with hydrogen peroxide treatment using neutral filter elution techniques (Price et al., 1989; Iliakis et al., 1992). Price and co-workers suggest that DNA DS breaks and not SS breaks are involved in hydrogen peroxide-induced cell killing. This possibility has yet to be resolved.

It is of interest to note that Sanford and co-workers report a G_2-phase chromosomal radiosensitivity, similar to that observed in tumour cell lines, in individuals belonging to the cancer prone syndrome ataxia-telangiectasia (AT) (Parshad et al., 1985; Sanford et al., 1989; Shiloh et al., 1989). Furthermore, Kodama and co-workers (1992) have shown that this G_2-phase sensitivity in AT patients is correctable by insertion of chromosome 11. Genetic linkage analyses have localized the AT gene(s) to 11q22-23 (Sanal et al., 1992). AT fibroblast cultures show an elevated sensitivity to ionizing radiation and to other agents which act via free radical mechanisms targeted at the DNA, including hydrogen peroxide (Shiloh et al., 1983). Yi and co-workers (1990) report that hydrogen peroxide treatment induces a greater increase in micronucleus frequencies in AT fibroblasts than that observed in normal cultures. The hybrid cell line used in

our study contains an intact der 11 chromosome insert, which includes most of chromosome 11, with a break occurring in q23 (Ning et al., 1991). Whether the hybrid retains the putative AT locus is not known. However, this locus does present a possible candidate for the genes on chromosome 11 involved in the response to oxidative damage reported in this paper.

Another mechanism by which chromosome 11 insertion could be affecting sensitivity to oxidative stress is by altering the level of catalase in the cell, since this gene is located at 11p13. This hypothesis does not appear to be correct, since one would expect that the level of SS DNA breakage as measured by the comet assay would be lower in the hybrid cell than in the parent. The catalase would act to reduce the hydrogen peroxide to oxygen and water, thereby decreasing the quantity available to cause DNA damage. Furthermore, we have studied the expression of the catalase gene in these cultures and have observed no significant differences (unpublished data).

A question left unresolved by this study is how sensitive normal bladder epithelial cells are to chromosomal damage by oxidative agents. The breast keratinocyte culture tested in this study showed a reduction in NDI similar to that seen in the parent bladder tumour cell line, but without the associated increase in MN frequency. The data suggest that normal epithelial cells are less sensitive to chromosomal damage by oxidative stress than the bladder carcinoma cultures. Whether normal bladder cultures will show a similar response is not known. In our laboratory, normal fibroblast cell lines show a significant induction of MN with X/XO treatment (Ward et al., in press). In fact, most researchers have used fibroblast or short-term lymphocyte cultures to study cellular damage due to oxidative stress. As most cancers develop in epithelial tissues, further work exploring the sensitivity of normal epithelial cells to oxidative stress and its alteration during tumourigenesis is highly relevant. As a final point, it should be

noted that the breast keratinocyte response in these studies suggests that inhibition of cell division does not result from chromosome damage, a possibility that requires further study.

In summary, these studies suggest an important role for chromosome 11 in controlling the level of chromosomal damage in bladder carcinogenesis during exposure to oxidative stress. The mechanism underlying this phenomenon is at this point unresolved. Future studies will be aimed at its clarification.

3.6 References

Cerutti, P. (1985) Prooxidant states and tumor promotion, Science, 227, 375-381.

Chen, M., and K. Mott (1989) Progress in assessment of morbidity due to *Schistosoma haematobium* infection: a review of the recent literature, Trop. Dis. Bull., 84, 1-30.

Correa, P. (1992) Human gastric carcinogenesis: a multistep and multifactorial process - first American Cancer Society award lecture on cancer epidemiology and prevention, Cancer Res., 52, 6735-6740.

Easmon, C. S. F., P.J. Cole, A.J. Williams, and M. Hastings (1980) The measurement of opsonic and phagocytic function by Luminol-dependent chemiluminescence, Immun., 41, 67-74.

Fenech, M., and Morley, A.A. (1985) Measurement of micronuclei in lymphocytes, Mutat. Res. 147, 29-36.

Fischer, S.M., and L.M. Adams (1985) Suppression of tumor promoter-induced chemiluminescence in mouse epithelial cells by several inhibitors of arachidonic acid metabolism, Cancer Res., 45, 3130-3136.

Frenkel, K. (1992) Carcinogen-mediated oxidant formation and oxidative DNA damage, Pharmac. Ther., 53, 127-166.

Gordon, L.I., and S.A. Weitzman (1988) The respiratory burst and carcinogenesis, In: A.J. Sbarra and R.R. Strauss (Eds.), The Respiratory Burst and Its Physiological Significance, Plenum Press, New York, pp. 277-298.

Hopman, A.H.N., O. Moesker, A. W.G.B. Smeets, R. P.E. Pauwels, G.P. Vooijs, and F.C.S. Ramaekers (1991) Numerical chromosome 1,7,9, and 11

aberrations in bladder cancer detected by *in situ* hybridization, Cancer Res., 51, 644-651.

Ichikawa, T., Y. Ichikawa, J. Dong, A.L. Hawkins, C. A. Griffin, W. B. Isaacs, M. Oshimura, J.C. Barrett, and J.T. Isaacs (1992) Localization of metastasis suppressor gene(s) for prostatic cancer to the short arm of human chromosome 11, Cancer Res., 52, 3486-3490.

Iliakis, G.E., G.E. Pantelias, R. Okayasu, and W.F. Blakely (1992) Induction by H2O2 of DNA and interphase chromosome damage in plateau-phase Chines Hamster Ovary cells, Radiat. Res., 131, 192-203.

Kaplan, R.P. (1987) Cancer complicating chronic ulcerative and scarifying mucocutaneous disorders, Advances in Dermatol., 2, 19-46.

Kodama, S., K. Komatsu, Y. Okumura, and M. Oshimura (1992) Suppression of x-ray-induced chromosome aberrations in ataxia telangiectasia cells by introduction of a normal human chromosome 11, Mutation Res., 293, 31-37.

Lewis, J.G., T. Hamilton, and D.O. Adams (1986) The effect of macrophage development on the release of reactive oxygen intermediates and lipid oxidation products, and their ability to induce oxidative DNA damage in mammalian cells, Carcinogenesis, 7, 813-818.

Negrini, M., A. Castagnoli, J.V. Pavan, S.Sabbioi, D. Araujo, A. Corallini, F. Gualandi, P. Rimessi, A. Bonfatti, C. Giunta, A. Sensi, E.J. Stanbridge, and G. Barbanti-Brodano (1992) Suppression of tumorigenicity and anchorage-independent growth of BK virus-transformed mouse cells by human chromosome 11, Cancer Res., 52, 1297-1303.

Ning, Y. (1991) Tumor suppression by chromosome 11 is not due to cellular senescence, Exp. Cell Res., 192, 220-226.

Ochi, T., and P.A. Cerutti (1987) Clastogenic action of hydroperoxy-5,8,11,13-icosatetraenoic acids on the mouse embryo fibroblasts C3H 10T1/2, Proc. Natl. Acad. Sci, USA, 84, 990-994.

Olive, P. L., J.P. Banath, and R.E. Durand (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay, Radiat. Res., 122, 86-94.

Olive, P.L., D. Wlodek, and J.P. Banath (1991) DNA double-strand breaks measured in individual cells subjected to gel electrophoresis, Cancer Res., 51, 4671-4676.

Olive, P.L., D. Wlodek, R.E. Durand, and J.P. Banath (1992) Factors influencing DNA migration from individual cells subjected to gel electrophoresis, Exp. Cell Res., 198, 259-267.

Oshimura, M., H. Kugoh, M.Kol, M. Shimizu, H. Yamada, H. Satoh, and J.C. Barrett (1990) Transfer of a normal human chromosome 11 suppresses tumorigenicity of some but not all tumor cell lines, J. Cellular Biochem., 42, 135-142.

Parshad, R., K.K. Sanford, and G.M. Jones (1985) Chromosomal radiosensitivity during the G2 cell-cycle period of skin fibroblasts from individuals with familial cancer, Proc. Natl. Acad. Sci. USA, 82, 5400-5403.

Parshad, R., F.M. Price, M. Oshimura, J.C. Barrett, H. Satoh, B.E. Weissman, E.J. Stanbridge, and K.K. Sanford (1992) Complementation of a DNA repair deficiency in six human tumor cell lines by chromosome 11, Hum. Genet., 88, 524-528.

Preston-Martin, S., M.C. Pike, R.K. Ross, P.A. Jones, and B.E. Henderson (1990) Increased cell division as a cause of human cancer, Cancer Res., 50, 7415-7421.

Price, K.M., S. Davies, and B.D. Michael (1989) Cell killing and DNA damage in Chinese hamster V79 cells treated with hydrogen peroxide, Int. J. Radiat. Biol., 55, 583-592.

Rosin, M.P. (1992) The use of the micronucleus test on exfoliated cells to identify anti-clastogenic action in humans: a biological marker for the efficacy of chemopreventive agents, Mutat. Res., 267, 265-276.

Rosin, M.P. , S.S. El Din Zaki, A.J. Ward, and W.A. Anwar (1994) Involvement of inflammatory reactions and elevated cell proliferation in the development of bladder cancer in schistosomiasis patients, Mutat. Res., 305, 283-292.

Sanal, O., E. Lange, M. Telatar, E. Sobel, J. Salazar-Novak, F. Ersoy, A. Morrison, P. Concannon, A. Tolun, and R.A. Gatti (1992) Ataxia-telangiectasia: linkage analysis of chromosome 11q22-23 markers in Turkish families, FASEB, 6, 2848-2852.

Sanford, K.K., R. Parshad, R. Gantt, R.E. Tarone, G.M. Jones, and F.M. Price (1989) Factors affecting and significance of G2 chromatin radiosensitivity in predisposition to cancer, Int. J. Radiat. Biol., 55, 963-981.

Schraufstatter, I., P.A. Hyslop, J.H. Jackson, and C.G. Cochrane (1988) Oxidant-induced DNA damage of target cells, J. Clin. Invest., 82, 1040-1050.

Scott, A.F., J.A. Phillips III, and B.R. Migeon (1979) DNA restriction endonuclease analysis for localization of human β- and δ-globin genes on chromosome 11, Proc. Natl. Acad. Sci. USA., 76, 4563-4565.

Shacter, E., E.J. Beecham, J.M. Covey, K.W. Kohn, and M. Potter (1988) Activated neutrophils induce prolonged DNA damage in neighboring cells, Carcinogenesis, 9, 2297-2304.

Shiloh, Y., E. Tabor, and Y. Becker (1983) Abnormal response of ataxia-telangiectasia cells to agents that break the deoxyribose moiety of DNA via a targeted free radical mechanism, Carcinogenesis, 4, 1317-1322.

Shiloh, Y., R. Parshad, M. Frydaman, K.K. Sanford, S. Portnoi, Y. Ziv, and G.M. Jones (1989) G2 chromosomal radiosensitivity in families with ataxia-telangiectasia, Hum. Genet., 84, 15-18.

Simmonds, N.J., R.E. Allen, T.R.J. Stevens, R.N.M. Van Someren, D.R. Blake, and D.S. Rampton (1992) Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease, Gastroenterology, 103, 186-196.

Stanbridge, E.J. (1988) Genetic analysis of human malignancy using somatic cell hybrids and monochromosome transfer, Cancer Surveys, 7, 317-324.

Takita, K., T. Sato, M. Miyagi, M. Watatani, F. Akiyama, G. Sakamoto, F. Kasumi, R. Abe, and Y. Nakamua (1992) Correlation of loss of alleles on the short arms of chromosomes 11 and 17 with metastasis of primary breast cancer to lymph nodes, Cancer Res., 52, 3914-3917.

Trush, M.A., and T.W. Kensler (1991) An overview of the relationship between oxidative stress and chemical carcinogenesis, Free Radical Biol. Med., 10, 201-209.

Tsai, Y.C., P.W. Nichols, A.L. Hiti, Z. Williams, D.G. Skinner, and P.A. Jones (1990) Allelic losses of chromosomes 9, 11, and 17 in human bladder cancer, Cancer Res., 50, 44-47.

Ward, A.J., M.P. Rosin, P.L. Olive, and A.H. Burr, Response of fibroblast cultures from ataxia-telangiectasia patients to reactive oxygen species generated during inflammatory reactions, Environ. Mol. Mutagen., manuscript in press.

Weissman, B.E., P.J. Saxon, S.R. Pasquale, G.R. Jones, A.G. Geiser, and E.J. Stanbridge (1987) Introduction of a normal human chromosome 11 into a Wilms' Tumor cell line controls its tumorigenic expression, Science, 236, 175-180.

Weitzman, S.A., and L.I. Gordon (1990) Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis, Blood, 76(4), 655-664.

Yi, M., M.P. Rosin, and C.K. Anderson (1990) Response of fibroblast cultures from ataxia-telangiectasia patients to oxidative stress, Cancer Lett., 54, 43-50.

CHAPTER IV

Response of fibroblast cultures from ataxia-telangiectasia patients to reactive oxygen species generated during inflammatory reactions

Environmental and Molecular Mutagenesis, 24, 2 or 3, 1994.

4.1 Abstract

Cells from patients with AT are more sensitive than cells from normal individuals to a number of compounds which induce DNA damage via oxygen-derived free radical attack. We tested the hypothesis that AT cells would show a sensitivity to ROS generated by activated inflammatory cells. AT cells were exposed to neutrophils activated with TPA or to X/XO, an enzyme system which generates superoxide and hydrogen peroxide. Induced MN frequencies (corrected for spontaneous MN frequencies) were significantly higher in AT cell cultures than in cultures from normal individuals (comparison of MN frequencies of AT vs normal cultures: for treatment with activated neutrophils, $P = 0.003$; for X/XO, $P = 0.05$). The comet assay was used to determine whether the elevated chromosomal damage in the treated AT cells was due to a difference in strand breakage or its rejoining. X/XO treatment was used in studies of SS DNA breakage and x-ray treatment for DS DNA damage. AT and normal cells showed no significant difference in the initial levels of SS ($P = 0.29$) or DS ($P = 0.91$) DNA damage. Likewise, they exhibited similar rejoining kinetics (rejoining half-time for SS = 10 min, for DS = 30 min). These data support the involvement of the AT loci in determining a cell's ability to deal with oxidative stress although the mechanism underlying this effect has yet to be resolved. The data also suggest that AT patients are at elevated risk of sustaining DNA damage in tissues undergoing inflammatory reactions.

4.2 Introduction

Clinical studies have shown an association between the development of cancer in a tissue and the presence of chronic (long-term) inflammation (reviewed by: Kaplan, 1987; Preston-Martin et al., 1990; Kensler et al., 1992; Rosin et al., 1994). Although the nature of this relationship is unresolved, a favored hypothesis is that DNA damage is induced in tissues during inflammatory reactions. Stimulated inflammatory cells undergo a respiratory burst and release ROS such as superoxide anion, hydrogen peroxide, and numerous secondary oxidants as well as products of the arachidonic acid cascade (Lewis et al., 1986; Ochi and Cerutti, 1987; Gordon and Weitzman, 1988; Frenkel, 1992). Many of these products have DNA-damaging potential. *In vitro* studies have shown that DNA damage is induced in cell cultures incubated with TPA-activated neutrophils or macrophages (Birnboim, 1983; Dutton and Bowden, 1985; Frenkel and Chrzan, 1987; Dizdaroglu et al., 1993).

Our laboratory is interested in identifying host factors that control sensitivity to the inflammatory response and in particular the oxidative DNA damage created by oxygen-derived free radicals or molecules (ROS). We have recently shown that insertion of a normal chromosome 11 into tumour cells protects against the DNA damaging effects of activated neutrophils (Ward et al., 1993). In this paper we have extended these studies to include cells from individuals with AT. AT cells were selected because of their known sensitivity to DNA damage by x-rays or by hydrogen peroxide treatment (Shiloh et al., 1983; Yi et al., 1990; Sanford et al., 1990). Furthermore, the AT gene defect is localized to chromosome 11 at the site 11q22-23 (Gatti, 1993).

This study involves an exposure of AT and normal fibroblast cultures to either X/XO treatment or to incubation with TPA-activated human neutrophils. Two endpoints were used to assess genetic damage. The MN test was used to

quantitate chromosome damage. The comet assay was used to quantitate initial DNA breakage and its rejoining. The results of these studies support the hypothesis that AT cells have an increased sensitivity to the products released by activated neutrophils and in particular ROS. Furthermore, the results from the comet assay suggest that the mechanism underlying the AT sensitivity is not due to an inability of these cells to rejoin DNA strand breaks effectively.

4.3 Materials and methods

4.3.1 Cell lines and culture

Fibroblast cultures used in this study included GM01588, GM00647A, GM02052, and AT2BR from AT patients and GM05757, GM05659A, GM043903, and 48BR from apparently normal individuals. All cultures were obtained from the National Institute of General Medical Sciences, Human Genetic Cell Repository (NIGMS, Camden, NJ) with the exception of AT2BR and 48BR, which were obtained from Dr. Collin Arlett, MRC Cell Mutation Unit, University of Sussex, Brighton, U.K. Cultures were grown in Dulbecco's modified eagle media (D-MEM, 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 μg/ml). All stock cultures were maintained in 75 cm^2 culture flasks at 37°C in incubators with a 5% CO_2/95% air atmosphere.

4.3.2 The MN assay

The micronucleus test was performed on cell cultures growing on sterile 22 mm^2 glass coverslips placed in 10 x 30 mm^2 tissue culture dishes. These coverslip cultures were prepared by seeding fibroblast cell cultures in D-MEM medium with 10% FCS at 6 x 10^4 cells/dish and treating them after 2 days growth. At this time, the cell density of all cultures was approximately 65% of a monolayer.

The xanthine/xanthine oxidase (X/XO) treatment was performed as follows: The medium was removed by suction from the dishes and the cells were washed twice in 1 ml of fresh D-MEM (without FCS). Cultures were exposed for 1 hr at 37°C to xanthine (Sigma, St. Louis, MO, sodium salt, 174 μg/ml) and xanthine oxidase (Sigma, St. Louis, MO, Grade III from buttermilk, 6.0-30.0 x 10^-

^{7} units/μl) prepared in phosphate-buffered saline (PBS, components/liter: 8 g sodium chloride, 0.2 g potassium chloride, 1.15 g dibasic sodium phosphate and 0.2 g monobasic potassium phosphate, pH 7.4). Following treatment, the X/XO solution was removed with suction, the cells were washed twice and fresh culture media was added. One hour later, cytochalasin B (Sigma, St. Louis, MO) was added to the media in each dish (final concentration, 2 μg/ml) in order to identify cells that have passed through one division cycle after treatment. This drug prevents cytokinesis and thus cells undergoing karyokinesis after the treatment will appear binucleated (Fenech and Morley, 1985). The cells were incubated with cytochalasin B for 48 hr prior to harvest.

In the co-incubation experiments with activated neutrophils, the neutrophils were separated by placing 6 ml of heparanized human blood onto a Histopaque density gradient consisting of 3 ml of Histopaque #1077 overlaid on 3 ml of Histopaque #1119 (Sigma, St. Louis, MO). This gradient was centrifuged (700 x g) at room temperature for 30 min. The separated neutrophils (95 $\pm$ 1.15 % pure) were washed twice in PBS and resuspended at a cell concentration of 2 x 10^{6} cells/ml in Roswell Park Memorial Institute medium (RPMI 1640, Gibco Laboratories, Grand Island, NY). Prior to co-culture, the activation capacity of each neutrophil isolation was monitored by measuring the luminol-enhanced chemiluminescence produced when the neutrophils were exposed to TPA (Easmon et al., 1980). 75 μl of the neutrophil suspension was added to a mixture consisting of 100 μl of TPA (25 ng/ml) and 75 μl of luminol (Sigma, St. Louis, MO, 0.1 mg/ml) prepared in RPMI medium and the chemiluminescence generated in the sample was quantitated on a Packard Model 6100 (Pico-lite) Luminometer. Preparations were used if the counts/sec were within the range of 7.6 x 10^{4} to 1.6 x 10^{5}. The counts remained within this range for 17 $\pm$ 2 min

after initial activation of the neutrophils, the maximum intensity occurred at 14 ± 2 min and the photon emission was complete within 1 hr.

To co-incubate, each coverslip was removed from its tissue culture dish, washed by dipping 5 times in RPMI (without FCS) and placed in a fresh dish. A treatment mixture consisting of 75 µl of neutrophils, 100 µl of TPA and 75 µl of RPMI was prepared and immediately pipetted onto the center of the coverslip. As the coverslip was wet from washing and the surrounding dish dry, the drop of 250 µl was held in a crescent-shaped meniscus over the coverslip. As controls, coverslip cultures were exposed to either TPA or neutrophils in RPMI. Treatment was for 1 hr in the dark at room temperature. Each coverslip was then removed from its dish, washed by dipping 5 times in RPMI, and placed into a fresh dish with 2 ml of D-MEM with FCS. Cytochalasin B was added after 1 hr and the cells were harvested 48 hr later.

For both X/XO and co-incubation treatments, coverslip cultures were harvested for MN analysis by removing the coverslips from the dishes, dipping them in PBS, air-drying and fixing them for 20 min with methanol and glacial acetic acid in a ratio of 3:1. The cellular DNA was stained with the Feulgen reaction as described in Yi et al., 1990. To delineate the cell cytoplasm, cells were then counterstained for 15 sec with a 0.5% solution of fast green (Fisher Scientific, Fairlawn, NJ) prepared in 95% ethanol. The coverslip cultures were then dehydrated through an alcohol-xylene series and mounted in permount on glass slides.

The slides were blind-coded and a total of 500 binucleated cells per slide were analyzed for the presence of micronuclei using criteria described by Rosin (1992). Each experiment was repeated a minimum of three times. In addition to calculating the percentage of binucleated cells with micronuclei, the nuclear division index was determined for each slide. This index is used as a

measurement of toxicity and/or inhibition of cell growth in different cultures. It is calculated by dividing the percentage binucleated cells present in the treated sample by the percentage of such cells in untreated cultures and multiplying this ratio by 100.

4.3.3 Alkaline comet assay for single-strand DNA damage

Cells (6×10^4) were seeded directly into 10 x 30 mm^2 tissue culture dishes. Two days later the culture medium was removed by suction, the cells were washed twice in 1 ml of D-MEM (without FCS), and then the cells were exposed for 15 min to xanthine (174 µg/ml) and xanthine oxidase (2.42×10^{-6} units/µl) prepared in PBS. Subsequent to this treatment, the X/XO solution was removed and the cultures were washed and trypsinized under ice-cold conditions. The cells were kept on ice until analysis. In dishes where DNA rejoining was to be studied, cells were washed twice in 1ml of medium after the treatment and incubated at 37°C in medium with FCS for the allocated time of rejoining. The same harvest procedure was followed for these cells.

The cell suspensions ($2 - 4 \times 10^4$ cells/ml) were processed by mixing 0.5 ml of the suspension with 1.5 ml of 1% agarose (low-temperature-gelling, Sigma type VII) kept at 40°C. This mixture was pipetted rapidly onto a glass slide and placed on a frozen metal block for 1 min to gel. In the rest of the procedure, the slides were always covered from direct fluorescent light exposure which can cause SS DNA damage. The slides were immersed in lysing solution (0.03 M NaOH, 1 M NaCl and 0.1% *N*-lauroylsarcosine) at room temperature for 1 hr and then washed 3 times (20 min each) in electrophoresis solution (0.03 M NaOH, 2 mM EDTA in double distilled water) to remove the salt. Slides were placed in a horizontal gel electrophoresis chamber (Bethesda Research laboratory model H-4) and a potential of 0.5 V/cm for 25 min was applied. The slides were washed

in distilled water for 10 min and then stained with 2.5 μg/ml propidium iodide for 10 minutes. They were then stored in a covered, humidified, air-tight box for up to 24 hr.

4.3.4 Neutral comet assay for double-strand DNA damage

For the neutral comet assay the treatment was either X/XO as above or x-ray exposure. For the x-ray exposure cells were trypsinized, washed in complete medium and resuspended at a density of 3-4 x 10^4 cells/ml in ice-cold phosphate-buffered saline. They were then irradiated on ice at a dose rate of 3.2 Gy/min.

The neutral procedure differed from the alkaline procedure as follows. Fully frosted glass microscope slides were used to prevent the gelled cell suspension detaching from the slide during the lysing (30mM EDTA, 0.5 % SDS, pH. 8.0) for 4 hr at 50 ^{o}C. The slides were washed free of detergent in Tris borate buffer (TBE; 90mM Tris, 2 mMEDTA, 90 mM boric acid in double distilled water, pH. 8.5) for 20 hr at room temperature. This was followed by a second wash for 2 hr. Electrophoresis was performed in TBE buffer at 0.6 V/cm for 25 min. The slides were stained for 1 hr and then viewed immediately.

4.3.5 Analysis of comets

The individual cells or comets were observed using a Zeiss epifluorescence microscope with 25X Plan-neofluor objective and a 100 W mercury power source with a 580 nm reflector and a 590 nm barrier filter. The microscope was attached to an imaging system consisting of a F4577 intensified camera (ITT Electro-Optical Production, Fort Wayne, IN) and an IBM AT computer with an ITEX 100 image processor and framegrabber board from Imaging Technology Inc., Woburn, MA. Overlapping comets and those at the

edges of the gel were omitted from analysis. Heavily damaged cells with no visable head region were excluded from the analysis. From 30-100 comets were analyzed per slide. A minimum of 30 comets were analyzed in the untreated controls, since there was minimal DNA damage. Using a program written by Dr. R. Durand of the B.C. Cancer Research Centre, the images of comets were digitized into a pixel matrix. An algorithm was used which defined the limits of the comet by applying an edge filter, the background was subtracted (defined as the image intensity at the edge of the comet), and head and tail distributions subsequently formed were analyzed. Several calculations were made for each individual comet image, the most important being the "tail moment". Tail moment was defined as the product of the amount of DNA in the tail (fluorescence intensity of the tail above background) multiplied by the tail length (distance between edge of comet head and end of tail) (Olive et al., 1990). The rejoining of DNA damage was expressed as the percentage of initial damage remaining. This was calculated by subtracting the untreated tail moment value from the treated tail moment value at each time point, this number was divided by the initial tail moment before rejoining (minus untreated value) and then multiplied by 100.

4.3.6 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 done a minimum of 3 times. Tests for the effect of treatment within each cell line were performed using a randomized block design ANOVA with *Bonferroni* multiple comparisons. Comparisons between different cell lines were performed using a split plot design ANOVA with *Bonferroni* multiple comparisons. The P-value chosen for significance in these studies was 0.05.

4.4 Results

4.4.1 Induction of micronuclei by X/XO treatment

AT and normal coverslip cultures were exposed to X/XO for 1 hr, cytochalasin B was added to the cultures, and MN frequencies were determined in binucleated cells 48 hr later. Table 4 shows MN frequencies in untreated cultures (spontaneous frequencies) and in cultures treated with X (174 mg/ml) and XO (1.8×10^{-6} units/μl). Induced MN frequencies are also shown to assist in interpretation of results. Spontaneous frequencies were 6-fold higher in the AT group as compared to the normal group ($P = 0.0006$).

Individual AT and normal cultures showed variation in the extent of micronuclei induction by the X/XO treatment. However, despite this variation, when the data were compared for the 2 groups the MN induction was significantly larger in the AT cultures (Table 4). All cultures showed a dose-dependent increase in MN frequencies, with differences in induced MN frequencies between AT and normal cultures becoming significant at the 2 upper doses of xanthine oxidase (1.8×10^{-6} units/μl XO, $P = 0.03$; 2.4×10^{-6} units/μl XO, $P = 0.05$). Figure 8A shows the average response of AT and normal cultures to a dose range of XO.

All cultures showed a dose-dependent decrease in the NDI after X/XO treatment. NDI values for individual cultures treated with one dose of XO (1.8 x 10-6 units/μl) are shown in Table 4 and average values for the 2 groups are shown in Figure 8B. In contrast to the results observed with MN frequencies, the reduction of NDI with treatment did not differ significantly when the AT and normal groups were compared (Fig. 8B, $P = 0.85$). However, the variability in response of individual AT and normal cultures was substantial. For example, among the normal cultures GM05659 was very sensitive to both induction of MN

TABLE 4: Variability in response of AT and normal cultures to X/XO treatment[a]

Cell Type	Micronuclei frequency (%)[b]			Nuclear division index (%)[b]
	Untreated	Treated	Induced[c]	
AT				
2052	14.7 ± 0.5	27.3 ± 1.0	12.7 ± 0.9	60.6 ± 1.0
647A	11.6 ± 0.6	33.2 ± 0.7	21.6 ± 0.1	28.7 ± 2.3
1588	12.4 ± 0.7	22.1 ± 1.1	9.7 ± 0.8	82.7 ± 3.7
x ± S.E.	12.9 ± 0.9[d]	27.5 ± 3.2	14.6 ± 3.6[e]	57.3 ±15.7[f]
Normal				
5757	2.5 ± 0.5	6.0 ± 0.8	3.6 ± 0.3	24.6 ± 1.4
4390	0.9 ± 0.3	2.0 ± 0.3	1.3 ± 0.1	75.1 ± 8.2
5659[g]	2.9 ± 0.2	*12.0*	*9.1*	*6.0*
x ± S.E.	2.1 ± 0.6[d]	6.7 ± 2.9	4.7 ± 2.3[e]	35.2 ±20.7[f]

[a] X (174 µg/ml) and XO (1.8 x 10^{-6} units/µl) for 1 hour.

[b] Mean ± S.E. (n = 3 experiments).

[c] Treated MN frequencies minus the spontaneous MN frequencies in untreated cultures.

[d] P = 0.0006, means are significantly different.

[e] P = 0.03, means are significantly different.

[f] P = 0.44, means are not significantly different.

[g] Only 100 cells analyzed for each experiment at this dose due to low NDI values.

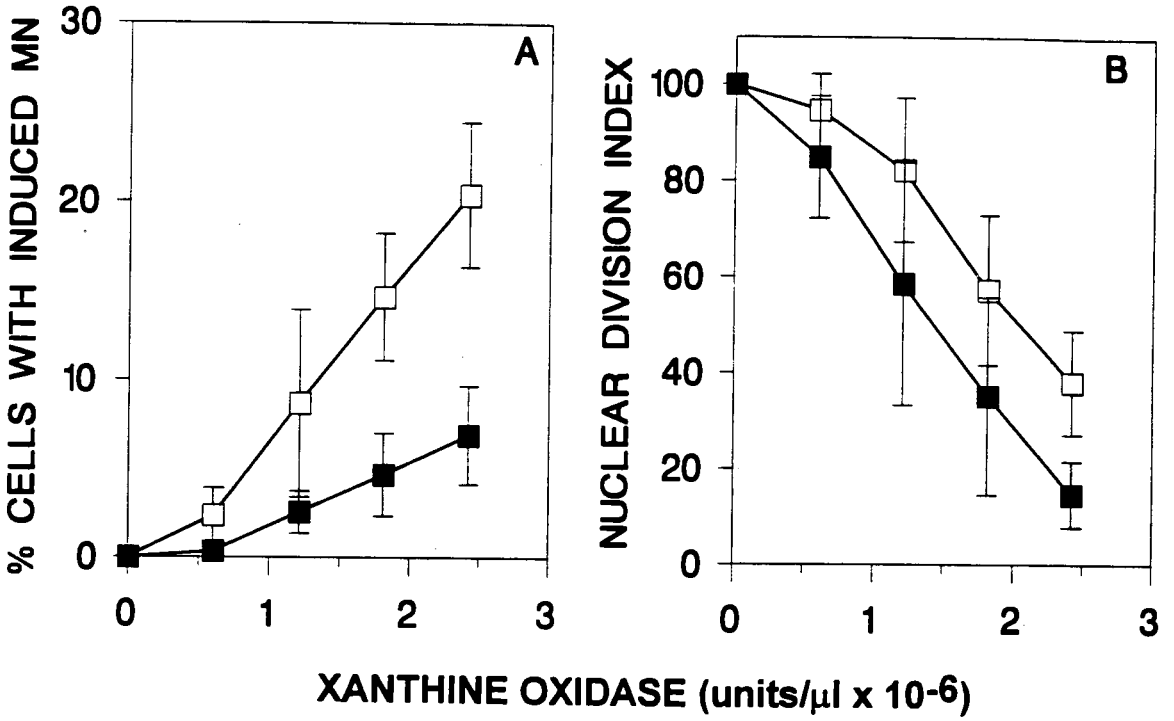

Figure 8. The effect of X/XO treatment on A) MN formation and B) NDI in AT (open symbols) and normal (closed symbols) cultures. Values shown are the mean values ± S.E. for 3 AT and 3 normal cultures (listed in Table 4). The spontaneous frequencies shown in Table 4 have been subtracted from the MN data.

and decrease in NDI with treatment. Control treatments with X alone or XO alone did not affect spontaneous MN or NDI frequencies in any culture.

4.4.2 Induction of micronuclei in cultures co-incubated with TPA-activated neutrophils

AT (GM01588) and normal (GM05757) cultures were each co-incubated with TPA-activated neutrophils and the effect of this treatment on MN frequencies and NDI was determined. As controls, cultures were also exposed to unstimulated neutrophils and to TPA alone. Only the AT culture showed a significant induction of micronucleated cells when co-incubated with TPA-activated neutrophils (induced MN frequencies: for AT, 4.5 $\pm$ 0.5%, P = 0.0006; for normal, 0.1 $\pm$ 0.5%, P = 0.85, Figure 9A). However, a significant decrease in the NDI was observed in both cultures with the drop being slightly larger in the AT culture (NDI for AT, 42.2 $\pm$ 3.9%; for normal, 58.0 $\pm$ 0.7%, P = 0.02, Figure 9B). Unstimulated neutrophils and TPA alone caused no significant induction of MN but did result in a drop in the NDI in the AT culture (for unstimulated neutrophils, 86.7 $\pm$ 2.4%, P = 0.03, for TPA alone, 83.8 $\pm$ 2.9%, P = 0.01).

4.4.3 The effect of X/XO treatment on single-strand DNA damage and rejoining

The alkaline comet assay was used to measure SS DNA damage and rejoining after X/XO treatment in individual cells from AT (GM01588) and normal (GM05757) cultures. The cultures were treated with X/XO, embedded in agarose, lysed *in situ* in the presence of detergent, exposed to alkaline conditions, and then subjected to electrophoresis. Cells in which DNA damage had occurred resembled comets with long tails (Figure 10).

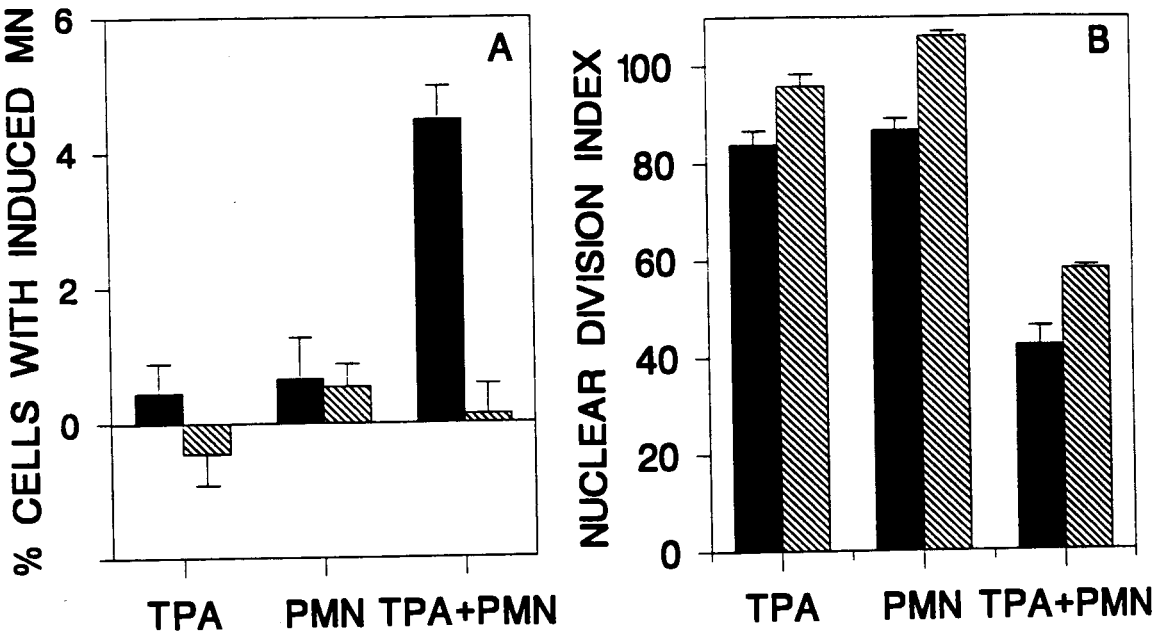

Figure 9. A) The induction of MN in AT (solid bars) and normal (striped bars) cultures co-incubated with TPA-activated neutrophils; B) NDI. Values shown are the mean values ± S.E. Spontaneous MN frequencies (Table 4) have been subtracted.

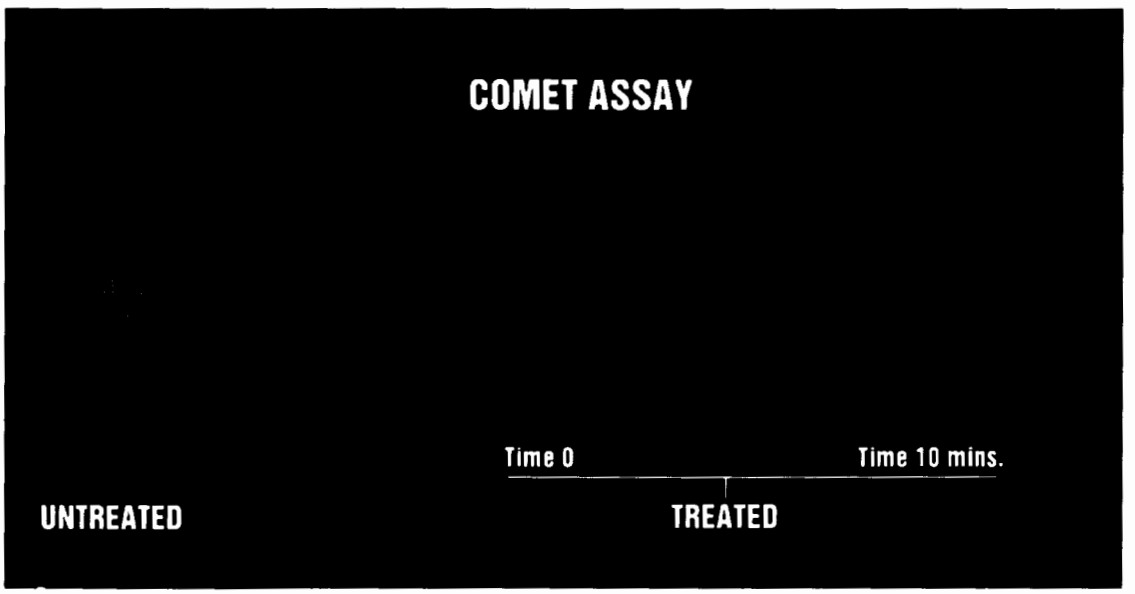

Figure 10. Fluorescence photomicrograph of AT cells exposed to X/XO and then examined for single-strand DNA damage and rejoining using the alkaline comet assay.

X/XO treatment induced SS DNA damage in both cultures (tail moment for AT culture: untreated cells, 0.7 $\pm$ 0.2; treated cells, 19.6 $\pm$ 1.6, P = 0.0002; for normal culture: untreated cells, 0.6 $\pm$ 0.07; treated cells, 16.3 $\pm$ 2.2, P = 0.0006); however, the initial level of damage did not differ between the AT and normal cultures (P = 0.29). Figure 11 is a representative histogram of the tail moment distributions in untreated cultures, treated cultures, and treated cultures with DNA undergoing rejoining. Both the AT and the normal culture displayed considerable heterogeneity in the amount of DNA damage induced in different cells when treated. As there was no relationship between the tail moment and the total DNA content (measured as the total intensity of fluorescence in each comet) the heterogeneity observed was probably not associated with any specific stage of the cell cycle (data not shown). The rejoining of the DNA damage was measured by allowing the cells to remain in media at 37^{o}C and harvesting the cells at regular time intervals up to 4 hr after treatment. The 2 cultures exhibited similar rejoining kinetics (Figure 12). Ten minutes after treatment, the level of damage had been reduced in both cultures by roughly half with no significant difference in mean tail moment (P = 0.46). After 4 hr, the level of DNA damage was equal to that in untreated cultures and there was no residual damage remaining.

4.4.4 The effect of X/XO and x-ray treatment on double-strand DNA damage and rejoining

Under neutral rather than alkaline conditions the comet assay is used to quantify DS DNA damage (Olive et al., 1991). X/XO at the same concentration used in the alkaline assay caused a marginal increase in DS DNA damage (average tail moment increase in both cultures was from about 3 in the untreated

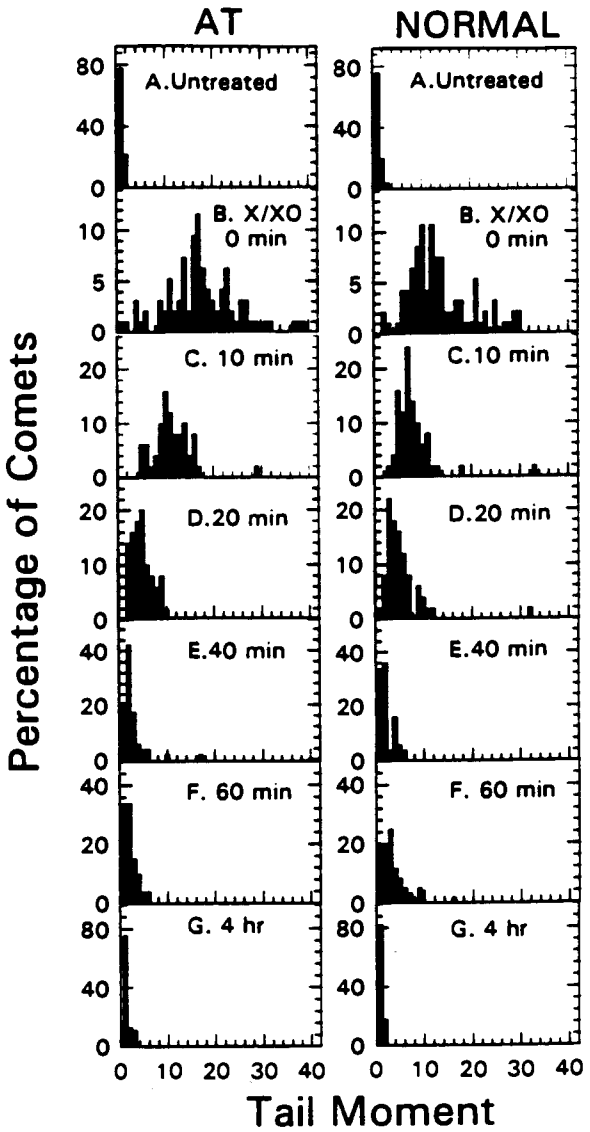

Figure 11. Heterogeneity in single-strand DNA damage and its rejoining in AT and normal cells after X/XO treatment as measured by the alkaline comet assay.

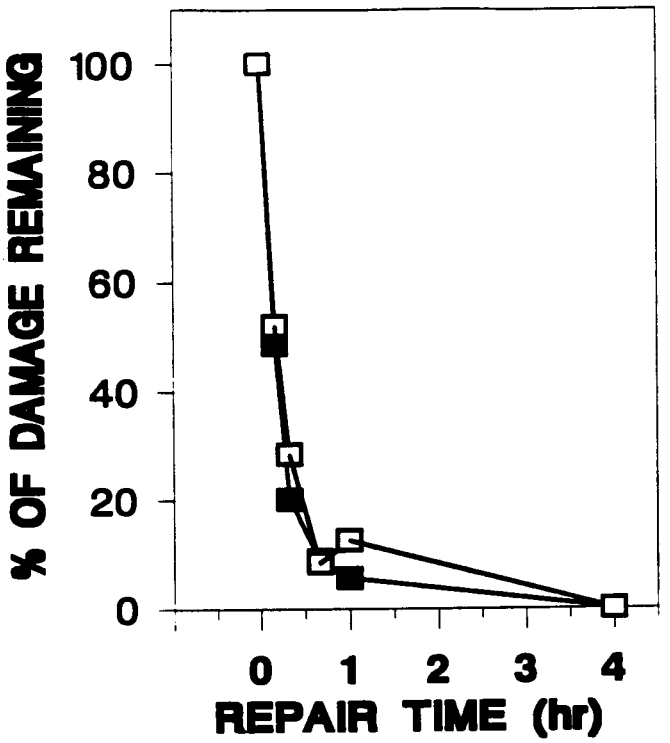

Figure 12. Rejoining of single-strand DNA damage induced by X/XO treatment with time in AT (closed symbols) and normal (open symbols) cultures. Values for untreated cultures: for AT, 0.7 ± 0.2; for normal, 0.6 ± 0.07.

controls to 5 at treatment). There was no significant difference between the AT and normal culture in the amount of DS DNA damage with treatment (P = 0.66). When the XO concentration was increased 10-fold the tail moment did not increase significantly. Urate formed during the X/XO reaction acts as an antioxidant by inactivating the XO (Gille and Joenje, 1992). This feedback mechanism was probably responsible for preventing an increase in DNA damage with higher XO concentrations. To increase the amount of DNA damage, and in so doing create enough damage to study the rejoining kinetics, AT and normal cultures were treated with x-rays. Cultures were exposed to a range of doses (data not shown) and 75 Gy was found to create a tail moment similar to that observed with SS DNA damage by X/XO in the alkaline assay (tail moment for AT culture: untreated cells, 3.4 $\pm$ 0.3; treated cells, 12.7 $\pm$ 1.8; for normal culture: untreated cells, 3.1 $\pm$ 0.2; treated cells, 12.3 $\pm$ 2.0). The initial level of DS DNA damage after treatment did not differ between the AT and normal cultures (P = 0.91). The two cultures exhibited similar rejoining kinetics (Figure 13). The rejoining half-time was less than 30 min. After 4 hr of rejoining the level of DNA damage remaining was similar for the AT and normal cells (for AT, 11%; for normal, 7%).

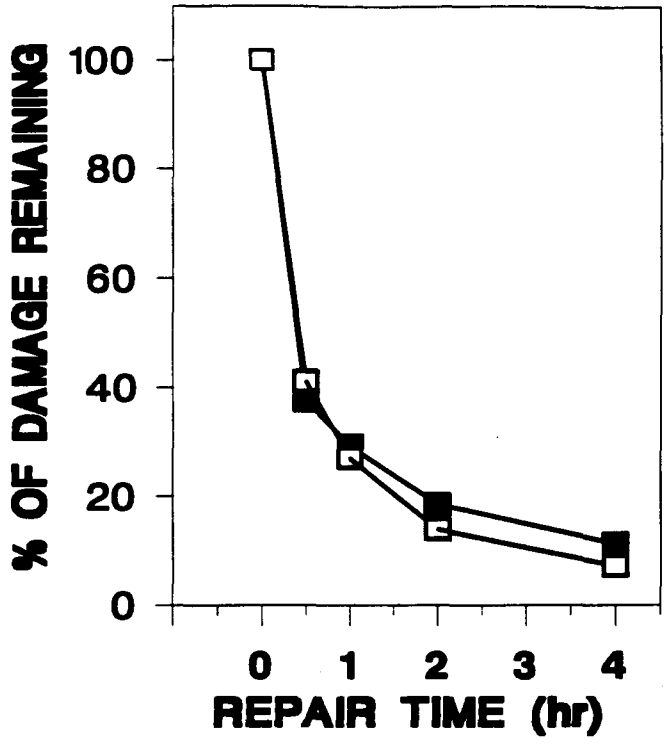

Figure 13. Rejoining of double strand DNA damage induced by X-ray treatment with time in AT (closed symbols) and normal (open symbols) cultures. Values for untreated cultures: for AT, 3.4 ± 0.3; for normal, 3.1 ± 0.2.

4.5 Discussion

Evidence suggests that activated inflammatory cells are capable of inducing DNA strand breaks, sister chromatid exchanges and mutation (Birnboim, 1982; Weitzman and Stossel, 1981; Yamashina et al., 1986; Shacter et al., 1988; Weitberg, 1989), and of promoting neoplastic transformation in co-cultured cells (Zimmerman and Cerutti, 1984; Weitzman et al., 1985). This study is the first to suggest that the genotype of the co-cultured cells affects their response to the DNA damaging agents of the inflammatory reaction. Our data show that AT cells are more sensitive than normal cells to chromosome damage when co-incubated with TPA-activated inflammatory cells. The difference in the AT response may be a result of elevated sensitivity to oxygen-derived free radical damage. This sensitivity is well-documented in the literature and is further supported by our data showing an increased induction of chromosomal damage in AT cells by X/XO treatments (Taylor, 1975; Shiloh et al., 1983; Morris et al., 1983; Yi et al., 1990; Sanford et al., 1990).

Both *in vitro* and *in vivo* evidence suggests that ROS are involved in the induction of DNA damage by inflammatory cells. Oxidative DNA damage is observed *in vitro* in cells co-incubated with TPA-activated neutrophils and macrophages (Birnboim, 1983; Dutton and Bowden, 1985; Frenkel and Chrzan, 1987; Dizdaroglu et al., 1993). Similar DNA modifications are observed in the epidermal DNA of mice receiving a topical exposure to TPA, a treatment which induces the infiltration and activation of inflammatory cells in this tissue (Wei and Frenkel, 1991, 1992). Further evidence in support of a role for ROS in the DNA damaging effects of inflammatory cells comes from studies showing that this damage is reduced by scavengers of ROS and inhibitors of the inflammatory response (Weitzman and Stossel, 1982; Frenkel, 1992).

The mechanism by which activated inflammatory cells cause DNA damage in a nearby cell is unknown. A favored hypothesis is that hydrogen peroxide (released during the respiratory burst) crosses the cytoplasmic and nuclear membranes and interacts with iron ions bound to the phosphate groups of nucleic acids in a metal-catalyzed Haber-Weiss reaction. A hydroxyl radical is produced that damages DNA (Gordon and Weitzman, 1988; Frenkel, 1992). Alternatively the DNA damage may result from hydroxyl radical generated by decomposition of a peroxynitrite radical formed by the coupling of nitric oxide and superoxide anion, both products of the inflammatory cell (Mulligan et al., 1991; Dizdaroglu et al., 1993). Finally, ROS released during the respiratory burst may indirectly cause DNA damage by stimulating lipid peroxidation in the cell membrane and inducing arachidonic acid metabolism in the co-cultured cell. A variety of potential genotoxic agents could be released including malonaldehyde and other breakdown products as well as lipid hydroperoxides (Khan and Emerit, 1985; Vaca et al., 1988; Harris, 1992). Although AT cells have been shown to sustain more chromosomal damage when exposed to hydrogen peroxide, their sensitivity to other products of the inflammatory response has not been studied.

A question left unresolved by this study is the nature of the defect in the AT cells that makes them more sensitive to treatment with TPA-activated neutrophils or X/XO. Our results with the comet assay indicate that the elevated chromosomal damage observed in treated AT cells is not due to a difference in the initial level of SS or DS DNA damage or in the rate of rejoining of strand breaks. Previous reports on the repair capacity of AT cells using x-irradiation as the DNA-damaging agent suggest that the AT defect is not in SS DNA breakage and rejoining (Forance and Little, 1980; Hariharan et al., 1981; Fornace et al., 1986; Cantoni et al., 1989) or in the initial level of DS DNA breakage (Coquerelle

and Weibezahn, 1981; Coquerelle et al., 1987; Radford and Hodgson, 1990; Pandita and Hittelman, 1992 a, b). However, there may be a difference in the way AT cells process DS DNA damage (Coquerelle and Weibezahn, 1981; Coquerelle et al., 1987; George and Cramp, 1987; Debenham et al., 1987; Mozdarani and Bryant, 1989; Radford and Hodgson, 1990; Blocher et al., 1991; Pandita and Hittelman, 1992 a, b). Our results indicate no difference in rejoining rate, or extent of rejoining. Since heavily damaged cells can be excluded from analysis, rejoining rates using the comet assay may be a more accurate indicator of repair. The fidelity of rejoining cannot be measured with this assay. Several recent papers have reported that AT cells show a significant increase in mis-rejoining of DNA breaks, possibly resulting from error-prone recombinational activity (Thacker, 1989; Powell et al., 1993; Ganesh et al., 1993).

There are alternate hypotheses for the AT defect. It has been suggested that the increased DNA damage could result from an alteration in the capacity of these cells to recognize DNA damage. A common characteristic of AT cells is that they lack the inhibition of DNA synthesis which normally occurs after x-irradiation. This defect could allow the AT cells to enter mitosis without the DNA damage being repaired (Shiloh et al., 1983; Kastan et al., 1992; Gatti, 1993). However, this may not be the case since AT cells seem to show a G_2 block that prevents them from entering mitosis any sooner than normal cells (Smith et al., 1985; Bates and Lavin, 1989). A more likely explanation therefore could be that the AT cells bypass DNA damage during DNA replication and instead must undergo error-prone post-replication repair. A final possibility for the defect in AT cells is they have an impaired antioxidant status. A reduced catalase activity (Vuillaume, 1987) and a defect in glutathione metabolism (Meredith and Dodson, 1987) have both been reported for these cells, although this data is somewhat controversial (Sheridan and Huang, 1979; Dean and Jasper, 1988).

Our interest in using AT cells in this study was the fact that the AT locus is located on chromosome 11. AT cells show elevated chromosomal damage when they are irradiated with x-rays in the G_2-phase of the cell cycle (Shiloh et al., 1989; Sanford et al., 1990). Microcell-mediated transfer of a normal chromosome 11 into AT cells corrects this G_2-phase radiosensitivity to that observed in normal cells (Kodama et al., 1992). This sensitivity to x-irradiation during the G_2-phase is also observed in tumour cells. Sanford and co-workers have shown that there is an abnormally high frequency of chromatid breaks and gaps when human tumours are x-irradiated during the G_2-phase. Insertion of normal chromosome 11 into these tumor cells protects them from chromosome damage. They have suggested that many tumour cells may have a DNA repair defect on this chromosome (Parshad et al., 1992). We have recently published data showing that sensitivity to oxidative stress can be reduced by introduction of a normal chromosome 11 into a bladder tumour cell line. The insertion of this chromosome resulted in a cell line that was protected from chromosome damage by activated neutrophils or by X/XO treatment (Ward et al., 1993).

In summary, these studies suggest inflammation as a source of oxidative stress may play an important role in elevating the level of chromosomal damage found in cells from AT patients. The results of the comet assay indicate that the mechanism underlying this phenomenon is unlikely to be a rejoining defect. Furthermore, when this data is taken together with our previous studies with tumour cells, it supports the involvement of loci on chromosome 11 in determining a cell's ability to deal with oxidative DNA damage. The AT gene locus represents a possible candidate for the genes on chromosome 11 involved in this response.

4.6 References

Bates, P.R. (1989) Comparison of gamma radiation-induced accumulation of ataxia telangiectasia and control cells in G2-phase, Mutat. Res., 218, 165-170.

Birnboim, H.C., (1982) DNA strand breakage in human leukocytes exposed to a tumor promoter, phorbol myristate acetate, Science, 215,1247-1249.

Birnboim, H.C. (1983) Importance of DNA strand-break damage in tumor promotion, In: O.F. Nygaard and M.G. Simic (Eds.), Radioprotectors and Anticarcinogens, Academic Press, New York, pp. 539-556.

Blocher, D., D. Sigut and M.A. Hannan (1991) Fibroblasts for ataxia telangiectasia (AT) and AT heterozygotes show an enhanced level of residual DNA double-strand breaks after low dose-rate γ-irradiation as assayed by pulse field gel electrophoresis, Int. J. Radiat. Biol., 60 (5), 791-802.

Cantoni, O., P. Sestili, M. Fiorilli, M.P. Santoro, M.C. Tannoia, G. Novelli, F. Cattabeni and B. Dallapiccola (1989) Identification of 4 ataxia telangiectasia cell lines hypersensitive to γ-irradiation but not to hydrogen peroxide, Mutat. Res., 218, 143-148.

Coquerelle, T.M. and K.F. Weibezahn (1981) Rejoining of DNA double-strand breaks in human fibroblasts and its impairment in one ataxia telangiectasia and two Fanconi strains, J. Supramol. Struct. Cell. Biochem.,17, 369-376.

Coquerelle, T.M., K.F. Weibezahn and C. Lucke-Huhle (1987) Rejoining of double strand breaks in normal human and ataxia-telangiectasia

fibroblasts after exposure to ^{60}Co γ-rays, ^{241}Am α-particles or bleomycin, Int. J. Radiat. Biol., 51 (2), 209-218.

Dean, S.W. and N.G.J. Jasper (1988) Correspondence re: Michael J. Meredith and Marion L. Dodson. Impaired glutathione biosynthesis in cultured ataxia-telangiectasia cells, Cancer Res., 48, 5374-5375.

Debenham, P.G., M.B.T. Webb, N.J. Jones and R. Cox (1987) Molecular studies of the nature of the repair defect in ataxia-telangiectasia and their implications for cellular radiobiology, J. Cell Sci. Suppl., 6, 177-189.

Dizdaroglu, M., R. Olinski, J.H. Doroshow and S.A. Akman (1993) Modification of DNA bases in chromatin of intact target human cells by activated human polymorphonuclear leukocytes, Cancer Res., 53, 1269-1272.

Dutton, D.R. and G.T. Bowden (1985) Indirect induction of a clastogenic effect in epidermal cells by a tumor promoter, Carcinogenesis, 6 (9), 1279-1284.

Easmon, C.S.F., P.J. Cole, A.J. Williams and M. Hastings (1980) The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescence, Immun., 41, 67-74.

Fenech, M. and A.A. Morley (1985) Measurement of micronuclei in lymphocytes, Mutat. Res., 147, 29-36.

Fornace, A. J., T. J. Kinsella, P. P. Dobson and J. B. Mitchell (1986) Repair of ionizing radiation DNA base damage in ataxia-telangiectasia cells, Cancer Res., 46, 1703-1706.

Fornace, A. J. and J. B. Little (1980) Normal repair of DNA single-strand breaks on patients with ataxia telangiectasia, Biochim. Biophys. Acta, 607, 432-437.

Frenkel, K. (1992) Carcinogen-mediated oxidant formation and oxidative DNA damage, Pharmac. Ther., 53, 127-166.

Frenkel, K. and K. Chrzan (1987) Hydrogen peroxide formation and DNA base modification by tumour promoter-activated polymorphonuclear leukocytes, Carcinogenesis, 8, 455-460.

Ganesh, A., P. North and J. Thacker (1993) Repair and misrepair of site-specific DNA double-strand breaks by human cell extracts, Mut. Res., 299, 251-259.

Gatti, R. A. (1993) Candidates for the molecular defect in ataxia telangiectasia, Advances in Neurology, 61, 127-132.

George, A.M. and W.A. Cramp (1987) The effect of ionizing radiation on structure and function of DNA, Prog. Biophys. Molec. Biol., 50, 121-169.

Gille, J.J.P. and H. Joenje (1992) Cell culture models for oxidative stress: superoxide and hydrogen peroxide versus normobaric hyperoxia, Mutat. Res., 275, 405-414.

Gordon, L.I. and S.A. Weitzman (1988) The respiratory burst and carcinogenesis, In: A.J. Sbarra and R.R. Strauss (Eds.), The Respiratory Burst and Its Physiological Significance, Plenum, New York, pp. 277-298.

Hariharan, P. V., S. Eleczko, B. P. Smith and M. C. Paterson (1981) Normal rejoining of DNA strand breaks in ataxia telangiectasia fibroblast lines after low X-ray exposure, Radiat. Res., 86, 589-597.

Harris, M.L., H.J. Schiller, P.M. Reilly, M. Donowitz, M.B. Grisham and G.B. Bulkley (1992) Free radicals and other reactive oxygen metabolites in inflammatory bowel disease: cause, consequence or epiphenomenon?, Pharmac. Ther., 53, 375-408.

Kaplan, R.P. (1987) Cancer complicating chronic ulcerative and scarifying mucocutaneous disorders, Advances in Dermatol., 2, 19-46.

Kastan, M. B., Q. Zhan, W. S. El-Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett B. Vogelstein and A. J. Fornace (1992) A mammalian cell cycle checkpoint pathway utalizing p53 and GADD45 is defective in ataxia-telangiectasia, Cell, 71, 587-597.

Kensler, T.W., M.A. Trush and K.Z. Guyton (1992) Free radicals as targets for cancer chemoprotection: prospects and problems, In: V.E. Steel, G.D. Stoner, C.W. Boone and G.J. Kelloff (Eds.), Cellular and Molecular Targets for Chemoprovention, Ann. Arbor.,MI, CRC Press, pp. 173-191.

Khan, S.H. and I. Emerit (1985) Lipid peroxidation products and clastogenic material in culture media of human leukocytes exposed to the tumor promoter phorbol-myristate-acetate, J. Free Rad. Biol. Med., 1, 443-449.

Kodama, S., K. Komatsu, Y. Okumura and M. Oshimura (1992) Suppression of x-ray-induced chromosome aberrations in ataxi telangiectasia cells by introduction of a normal human chromosome 11, Mutat. Res., 293, 31-37.

Lewis, J.G. and D.O. Adams (1985) Induction of 5,6-ring saturated thymine bases in NIH-3T3 cells by phorbol ester-stimulated macrophages: role of reactive oxygen intermediates, Cancer Res., 45, 1270-1275.

Meredith, M.J. and J.L. Dodson (1987) Impaired glutathione biosynthesis in cultures human ataxia-telangiectasia cells, Cancer Res., 47, 4576-4581.

Morris, C., R. Mohamed and M. F. Lavin (1983) DNA replication and repair in ataxia telangiectasia cells exposed to bleomycin, Mutat. Res., 112, 67-74.

Mozdarani, H. and P. E. Bryant (1989) Induction and rejoining of chromatid breaks in x-irradiated A-T and normal human G2 fibroblasts, Int. J. Radiat. Biol., 56 (5), 645-650.

Mulligan, M.S., J.M. Hevel, M.A. Marletta and P.A. Ward (1991) Tissue injury caused by deposition of immune complexes is L-arginine dependent, Proc. Natl. Acad. Sci. USA, 88, 6338-6342.

Ochi, T and P.A. Cerutti (1987) Clastogenic action of hydroperoxy-5,8,11,13-icosatetraenoic acids on the mouse embryo fibroblasts C3H 10T1/2, Proc. Natl. Acad. Sci., USA, 84, 990-994.

Olive, P. L., J.P. Banath and R.E. Durand (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay, Radiat. Res., 122, 86-94.

Olive, P.L., D. Wlodek and J.P. Banath (1991) DNA double-strand breaks measured in individual cells subjected to gel electrophoresis, Cancer Res., 51, 4671-4676.

Pandita, T. K. and W. N. Hittelman (1992) Initial chromosome damage but not DNA damage is greater in ataxia telangiectasia cells, Radiat. Res., 130, 94-103.

Pandita, T. K. and W. N. Hittelman (1992) The contribution of DNA and chromosome repair deficiencies to the radiosensitivity of ataxia-telangiectasia, Radiat. Res., 131, 214-223.

Parshad, R., F.M. Price, M. Oshimura, J.C. Barrett, H. Satoh, B.E. Weissman, E.J. Stanbridge and K.K. Sanford (1992) Complementation of a DNA repair deficiency in six human tumor cell lines by chromosome 11, Hum. Genet., 88, 524-528.

Powell, S., S. Whitaker, J. Peacock and T. McMillan (1993) Ataxia telangiectasia: An investigation of the repair defect in the cell line AT5BIVA by plasmid reconstruction, Mutat. Res., 294, 9-20.

Preston-Martin, S., M.C. Pike, R.K. Ross, P.A. Jones and B.E. Henderson (1990) Increased cell division as a cause of human cancer, Cancer Res., 50, 7415-7421.

Radford, I. R. and G. S. Hodgson (1990) A comparison of the induction of DNA double-strand breakage and lethal lesions by x-irradiation in ataxia telangiectasia and normal fibroblasts, Radiat. Res., 124, 334-335.

Rosin, M.P. (1992) The use of the micronucleus test on exfoliated cell to identify anti-clastogenic action in humans: a biological marker for the efficancy of chemopreventative agents, Mutat. Res., 267, 665-276.

Rosin, M.P., W.A. Anwar and A.J. Ward (1994) Inflammation, chromosomal instability, and cancer: the Schistosomiasis model, Cancer Res., 54 (7), 1929-1933.

Rosin, M.P., S.S. El Din Zaki, A.J. Ward and W.A. Anwar (1993) Involvement of inflammatory reactions and elevated cell prolliferation in the development of bladder cancer in schistosomiasis patients, Mutat. Res., 305, 283-292.

Sanford, K.K., R. Parshad, F.M. Price, G.M. Jones, R.E. Tarone, L. Eierman, P. Hale and T.A. Waldmann (1990) Enhanced chromatid damage in blood

lymphocytes after G2 phase x-irradiation, a marker of the ataxia-telangiectasia gene, J. Nat. Can. Inst., 82 (12), 1050-1054.

Shacter, E., E.J. Beecham. J.M. Covey, K.W. Kohn and M. Potter (1988) Activated neutrophils induce prolonged DNA damage in neighboring cells, Carcinogenesis, 9, 2297-2304.

Sheridan III, R.B. and P.C. Huang (1979) Superoxide dismutase and catalase activites in ataxia telangiectasia and normal fibroblast cell extracts, Mut. Res., 61, 381-386.

Shiloh, Y., E. Tabor and Y. Becker (1983) Abnormal response of ataxia telangiectasia cells to agents that break the deoxyribose moiety of DNA via a targeted free radical mechanism, Carcinogenesis, 4, 1317-1322.

Shiloh, Y, R. Parshad, M. Frydman, K.K. Sanford, S. Portnoi, Y. Ziv and G.M. Jones (1989) G2 chromosomal radiosensitivity in families with ataxia telangiectasia, Hum. Genet., 84, 15-18.

Smith, P.J., C.O. Anderson and J.V. Watson (1985) Abnormal retention of x-irradiated ataxia-telangiectasia fibroblasts in G2-phase of the cell cycle: cellular RNA content, chromatin stability and the effect of 3-aminobenzamide, Inter. J. Radiat. Biol., 47, 701-712.

Taylor, A. M. R., D. G. Harnden, C. F. Arlett, S. A. Harcourt, A. R. Lehmann, S. Stevens and B. A. Bridges (1975) Ataxia telangiectasia: a human mutation with abnormal radiation sensitivity, Nature, 258, 427-429.

Thacker, J. (1989) The use of integrated DNA vectors to analyse the molecular defect in ionising radiation-sensitive mutants of mammalian cells including ataxia telangiectasia, Mutat. Res., 220, 187-204.

Vaca, C.E., J. Wilhelm and M. Harms-Ringdahl (1988) Interaction of lipid peroxidation products with DNA. A review, Mut. Res., 195, 137-149

Vuillaume, M. (1987) Reduced oxygen species, mutation, induction and cancer initiation, Mutat. Res., 186, 43-72.

Ward, A. J., P. L. Olive, A. H. Burr and M. P. Rosin (1993) A sensitivity to oxidative stress is linked to chromosome 11 but is not due to a difference in single strand DNA breakage or repair, Mutat. Res., 294, 299-308.

Wei, H. and Frenkel K. (1992) Suppression of tumor promoter-induced oxidative events and DNA damage *in vivo* by sarcophytol A: a possible mechanism of anti-promotion, Cancer Res., 52, 1298-2303.

Weitberg, A.B. (1988) Effect of combinations of antioxidants on phagocyte-induced sister-chromatid exchanges, Mut. Res., 224, 1-4.

Weitzman, S.A. and T.P. Stossel (1981) Mutation caused by human phagocytes, Science, 212, 546-547.

Weitzman, S.A., A.B. Weitberg, E.P. Clark and T.P. Stossel (1985) Phagocytes as carcinogens: malignant transformation produced by human neutrophils, Science, 227, 1231-1233.

Weitzman, S.A and T.P. Stossel (1982) Effect of oxygen radical scavengers and anti-oxidants on phagocyte-induced mutagenesis, J. Immunol., 128, 2770.

Weitzman, S.A. and L.I. Gordon (1990) Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis, Blood, 76 (4), 655-664.

Yamashina, K., B.E. Miller and G.H. Heppner (1986) Macrophage-mediated induction of drug resistant variants in a mouse mammary tumor cell line, Cancer Res., 46, 2396-2401.

Yi, M., M. P. Rosin and C.K. Anderson (1990) Response of fibroblast cultures from ataxia-telangiectaisia patients to oxidative stress, Cancer Lett., 54, 43-50.

Zimmerman, R. and P. Cerutti (1984) Active oxygen acts as a promoter of transformation in mouse embryo C3H/10T1/2/C18 fibroblasts, Proc. Natl. Acad. Sci. U.S.A., 81, 2085-2087.

CHAPTER V

A protective effect of chromosome 11 against DNA damage by TPA-activated neutrophils but not TPA acting alone

Submitted to Carcinogenesis for publication.

5.1 Abstract

Evidence from animal models suggests that TPA is capable of inducing genetic damage within a tissue although the mechanism underlying this response is unknown. A favored hypothesis is that the TPA is acting either by stimulating cells in the tissue directly to generate DNA-damaging agents or, by recruiting inflammatory cells to the tissue and stimulating them to release such agents. These agents include ROS such as hydrogen peroxide and superoxide anion as well as products generated during lipid peroxidation and arachidonic acid metabolism. It is not known whether significant alterations occur in the sensitivity of cells to TPA during the process of tumourigenesis. In this paper the capacity of TPA to induce chromosomal breakage (measured by micronuclei induction) was found to be elevated in bladder tumour cell lines compared to two normal cultures, a primary epithelial culture and a fibroblast culture. This effect was observed when cells were exposed to TPA directly or co-cultured with TPA-activated neutrophils isolated from human blood. In addition, we present evidence that loci on chromosome 11 may be involved in altering the response of cells to TPA. When chromosome 11 was inserted into a bladder tumour cell line, reduction in sensitivity to TPA-activated neutrophils was observed. The chromosome insert did not protect against damage induced by direct treatment with TPA alone. In another set of experiments, fibroblasts from a patient with AT (a syndrome localized to chromosome 11) were shown to have an elevated sensitivity to the chromosome-damaging action of TPA-activated neutrophils, but not to TPA alone. These results suggest that some of the alterations occurring in a tissue during tumourigenesis could have a significant impact on the responsiveness of cells to genetic damage by TPA. They also suggest that the damage induced by TPA in a cell may be different if a neutrophil is present.

5.2 Introduction

Historically, the definition of a tumour promoter has been that it is an agent that acts to increase tumourigenesis through epigenetic mechanisms and not by damaging DNA (Yuspa et al., 1987). However, recent evidence suggests that the classical promoting agent, TPA, does induce genetic change in a tissue (Wei and Frenkel, 1991, 1992). Frenkel and co-workers have shown that TPA can induce the formation of oxidized DNA bases in mouse epidermis. This response appears to involve two components. TPA may act directly on the tissue cells to stimulate the generation of ROS and other cellular mediators (Robertson et al., 1990; Wei and Frenkel, 1991). Among these products are chemotactic factors that recruit neutrophils to the tissue. A second component involves the activation of these neutrophils by the TPA, leading to the release of a complex array of products including more ROS. The involvement of both of these pathways in the induction of genetic damage is supported by *in vitro* observations in which cells were exposed directly to TPA (Hartley et al., 1985; Petrusevska et al., 1988; Farber et al., 1989) or co-cultured with TPA-activated neutrophils (Dutton and Bowden, 1985; Shacter et al., 1988, 1990; Chong et al., 1989).

Genetic alterations are continually occurring in a tissue that is undergoing carcinogenesis. It is not known how these changes affect the response of cells in the tissue to TPA. Initiated and tumourigenic cells could have a significant alteration in sensitivity to TPA compared with normal cells. This study was designed to begin to address this possibility. The approach used was to examine the ability of TPA to induce chromosomal breakage in the presence and absence of neutrophils in a series of cell lines. The micronucleus test was used to quantitate chromosomal damage. The cultures used included a combination of two bladder tumours (J82 and A1698) and two normal cultures, one a

fibroblast culture (GMO5757) and the other a primary epithelial culture (NHEK-267-1). In addition we selected two other cell cultures that have altered sensitivity to oxidative DNA damage based on earlier studies in this laboratory (Ward et al., 1993; Ward et al., in press). One cell line was a microcell-mediated hybrid of the bladder tumour cell line A1698 containing an insert of normal chromosome 11 (A1698 der[11]). This insert protects the cell line from chromosome breakage by X/XO mixture, hydrogen peroxide, and x-irradiation (Ward et al., 1993; Rosin et al., unpublished data). The second cell line was derived from a patient with AT (GMO1588). The gene(s) for this cancer-prone syndrome has been localized to chromosome 11 (Sanal et al., 1992).

These studies support the theory that induction of chromosome damage in tumour cells exposed to TPA occurs at a higher rate than in normal cultures. In addition they suggest that alterations to loci on chromosome 11 may play a role in tumour promotion by increasing sensitivity to DNA damaging agents released by activated neutrophils.

5.3 Materials and methods

5.3.1 Cell lines and culture

The bladder carcinoma cell line J82 (ATCC HTB1J82) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The bladder carcinoma cell line A1689 and its microcell hybrid clone, A1689 der(11), were obtained from Dr. O. Pereira-Smith, Baylor College of Medicine, Houston, TX. This hybrid contains an intact der(11), which consists of a derivative chromosome 11 from a normal donor which has a balanced X;11 translocation [der(11)t(X;11) (q25;q23)]. The der(11) is composed of most of chromosome 11 with a small piece of distal Xq containing the HPRT locus attached (Scott et al., 1979; Ning et al., 1991). Ning and co-workers have shown that this insertion results in a loss of tumorigenicity in the hybrid. The primary culture of normal human epidermal breast keratinocytes, NHEK-267-1, was obtained from Clonetics (San Diego, CA). Fibroblast cell lines were obtained from the National Institute of General Medical Sciences, Human Genetic Cell Repository (NIGMS, Camden, NJ). There was 1 culture from an AT patient (GM01588) and 1 culture from an apparently normal individual (GM05757).

Bladder carcinoma and fibroblast cell cultures were grown in Dulbecco's modified eagle media (D-MEM, 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 µg/ml). The medium for the hybrid was also supplemented with HAT (hypoxanthine 10^{-4} M, aminopterin 5 x 10^{-7}M and thymidine 10^{-5} M) to maintain selection for the der(11) insert. The keratinocyte culture was maintained in keratinocyte serum free medium supplemented with bovine pituitary extract (50 µg/ml) and epidermal growth factor (5 ng/ml) (K-SFM, Clonetics, San Diego, CA). All stock

cultures were maintained in 75 cm^2 culture flasks at 37°C in incubators with a 5% CO_2/95% air atmosphere.

5.3.2 Preparation of coverslip cultures

The micronucleus test was performed on cell cultures growing on sterile 22 mm^2 glass coverslips placed in 10 x 30 mm^2 tissue culture dishes. These coverslip cultures were prepared by seeding bladder and fibroblast cell cultures in D-MEM medium with 10% FCS and the keratinocyte cell culture in K-SFM. The epithelial cell cultures were seeded at 1.5 x 10^5 cells/dish and the fibroblast cell cultures were seeded at 6 x 10^4 cells/dish. At the time of treatment the cell density of all cultures was approximately 65% of a monolayer. This represented an average growth time of 2 days for AT and normal fibroblast cultures, 1 day for bladder carcinoma cells and $1^1/_2$ days for keratinocytes.

5.3.3 Studies involving exposure of coverslip cultures to TPA alone

The TPA treatment was performed as follows: The medium was removed by suction from the dishes and the cells were washed twice in 1 ml of medium minus FCS. Cultures were exposed for 1 h or 24 h at 37°C to medium containing TPA. Depending on the exposure criteria, the medium used for the treatment was either with or without FCS, and the dose of TPA ranged from 0.01 to 100 ng/ml. For the 1 h treatment, the medium containing TPA was removed by suction, the cells were washed twice and fresh culture media was added. Cytochalasin B was added to the medium in each dish (final concentration, 2μg/ml) 2 h after the initiation of the treatment. This drug prevents cytokinesis and thus cells undergoing karyokinesis after the treatment will appear binucleated (Fenech and Morley, 1985). This allows for identification of cells that

have passed through one division cycle after treatment. The cells were incubated for 24 h prior to harvest.

5.3.4 Co-incubation treatment with TPA-activated neutrophils

Neutrophils were isolated by placing 6 ml of heparanized human blood onto a Histopaque density gradient consisting of 3 ml of Histopaque #1077 overlaid on 3 ml of Histopaque #1119 (Sigma, St. Louis, MO). This gradient was centrifuged (700 x g) at room temperature for 30 min. The separated neutrophils were washed twice in PBS and resuspended at a cell concentration of 2×10^6 cells/ml in RPMI 1640 (Gibco Laboratories, Grand Island, NY). Using Wright Stain, these preparations were determined to consist of 95 $\pm$ 1.2 % neutrophils. Prior to co-culture, the activation capacity of each neutrophil isolation was monitored by measuring the luminol-enhanced chemiluminescence produced when the neutrophils were exposed to TPA (Easmon et al., 1980; Fischer et al., 1985). 75µl of the neutrophil suspension was added to a mixture consisting of 100µl of TPA (Sigma, St. Louis, MO, 25 ng/ml) and 75 µl of luminol (Sigma, St. Louis, MO, 0.1 mg/ml) prepared in RPMI medium and the chemiluminescence generated in the sample was quantitated on a Packard Model 6100 (Pico-lite) Luminometer. Preparations were used if the counts/sec were within the range of 7.6×10^4 to 1.6×10^5. The counts remained within this range for 17 $\pm$ 2 min after initial activation of the neutrophils, the maximum intensity occurred at 14 $\pm$ 2 min and the photon emission was complete within 1 h.

To co-incubate, a coverslip culture was removed from its tissue culture dish, washed by dipping 5 times in RPMI (without FCS) and placed in a fresh dish. A treatment mixture consisting of 75 µl of neutrophils, 100 µl of TPA and 75 µl of RPMI was prepared and immediately pipetted onto the centre of the coverslip. As the coverslip was wet from washing and the surrounding dish dry,

the drop of 250 µl was held in a crescent-shaped meniscus over the coverslip. As controls, coverslip cultures were exposed to either TPA or neutrophils in RPMI. Treatment was for 1 h in the dark at room temperature. Each coverslip was then removed from its dish, washed by dipping 5 times in RPMI, and placed into a fresh dish with 2 ml of D-MEM with FCS. Cytochalasin B (Sigma, St. Louis, MO.) was added after 1 h and the cells were harvested 24 h later for the epithelial cultures and 48 h later for the fibroblast cultures.

5.3.5 Harvesting and Scoring

Coverslip cultures were harvested for micronucleus analysis by removing the coverslips from the dishes, dipping them in PBS, air-drying and fixing them for 20 min with methanol/glacial acetic acid (3:1). The cellular DNA was stained with the Feulgen reaction as described in Yi et al. (1990). To delineate the cell cytoplasm, cells were counterstained for 15 sec with a 0.5% solution of fast green (Fisher Scientific, Fairlawn, NJ) prepared in 95% ethanol. The coverslip cultures were then dehydrated through an alcohol-xylene series and mounted in permount on glass slides.

The slides were blind-coded and a total of 500 binucleated cells per slide were analyzed for the presence of micronuclei using criteria described by Rosin (1992). In addition to calculating the percentage of binucleated cells with micronuclei, the nuclear division index was determined for each slide. This index is used as a measurement of toxicity and/or inhibition of cell growth in different cultures. It is calculated by dividing the percentage binucleated cells present in the treated culture by the percentage of such cells in the untreated culture and multiplying this ratio by 100.

5.3.6 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 Bonferroni multiple comparisons. Comparisons between different cell lines were performed using a split plot design ANOVA with Bonferroni multiple comparisons. The P-value chosen for significance in these studies was 0.05.

5.4 Results

5.4.1 The direct effect of TPA on bladder carcinoma cultures

MN induction (Figure 14A) and NDI (Figure 14B) were determined in the bladder carcinoma culture, J82 after exposure to TPA (1 - 20 ng/ml) in D-MEM. Two exposure times were used: 1 h and 24 h. Both exposure times resulted in the induction of MN; however, the 24 h treatment induced more micronucleated cells (effect of treatment for 1 h, $P = 0.03$; for 24 h, $P = 0.002$). The 24 h treatment also resulted in a larger drop in the NDI. The effect of TPA on MN frequencies and NDI was not dose-dependent at either of the exposure times. The data suggests that the response of bladder carcinoma cells to TPA was not dose-dependent since neither MN induction or NDI showed significant alteration with increasing doses. This effect may be due to the saturation kinetics of protein kinase C, the cell receptor to which TPA binds to induce cellular changes.

Table 5 shows the data obtained when a second bladder carcinoma culture, A1698, was exposed to the same dose range of TPA for 24 h. The induction of MN in this culture was not significantly different from that observed with J82 and the trend in the reduction of NDI was similar.

5.4.2 The effect of TPA on a primary epithelial culture

To test the hypothesis that the direct effects of TPA on the bladder carcinoma cell lines was due to alterations occurring in the cells during tumourigenesis, we exposed a normal epithelial culture (NHEK-267-1) to TPA. Since a primary bladder epithelial culture was not available, we used a commercially available breast keratinocyte culture prepared from a clinically normal individual. The treatment could not be carried out in D-MEM

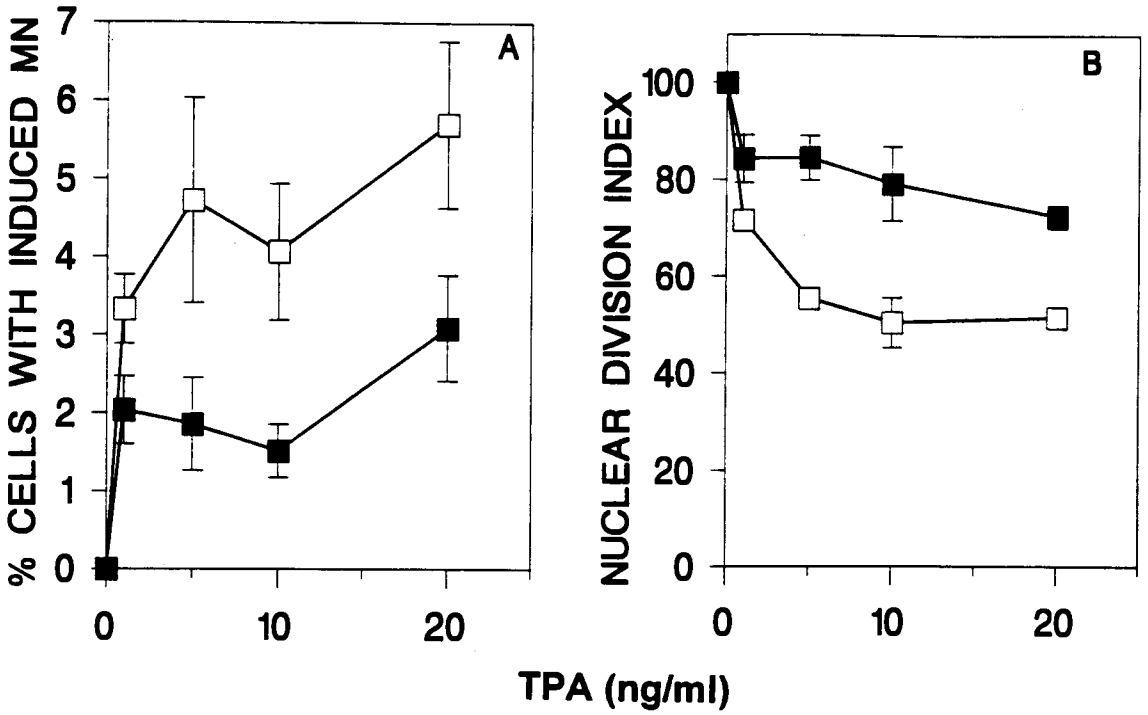

Figure 14. The effect of TPA treatment for 24 hours (open symbol) or 1 hour (closed symbol) on A) MN formation and B) NDI in a bladder carcinoma cell culture (J82). Values represent the mean of 3 separate experiments ± S.E. Induced MN, is the frequency of MN at treatment minus the spontaneous MN frequency. The spontaneous frequencies were: for 24 hours, 6.4 ± 0.9; for 1 hour, 6.4 ± 0.4.

Table 5. Effect of TPA alone on epithelial and fibroblast cell cultures[a]

Cell type	Induced % MN[b,c]				Nuclear Division Index (NDI)[d]			
	TPA (ng/ml)				TPA (ng/ml)			
	1	5	10	20	1	5	10	20
Epithelial								
J82	3.3 ± 0.4	4.7 ± 1.3	4.1 ± 0.9	5.7 ± 1.1	71.6 ± 1.9	55.5 ± 1.0	50.5 ± 5.0	51.6 ± 1.5
A1698	4.6 ± 1.0	4.6 ± 0.5	5.1 ± 0.7	4.2 ± 0.7	36.2 ± 4.0	30.2 ± 2.6	38.5 ± 7.2	31.5 ± 4.8
Fibroblast								
GMO1588 (AT)	-1.2 ± 1.6	-0.8 ± 1.4	-1.2 ± 0.9	0.1 ± 0.1	76.6 ± 4.1	54.7 ± 3.5	49.0 ± 0.2	63.6 ± 5.4
GMO5757 (N)	0.3 ± 0.1	0.2 ± 0.2	0.6 ± 0.7	0.2 ±0.4	52.9 ± 8.5	42.4 ± 9.7	35.0 ± 6.9	52.0 ±10.1

[a] Cultures were treated with TPA in D-MEM for 24 h at 37°C. Values are the mean of 3 experiments ± SEM.

[b] Induced % MN, is the frequency of MN at treatment minus the spontaneous MN frequency. The spontaneous frequencies were: J82, 6.4 ± 0.9; A1698, 4.3 ± 0.3; A1698 der(11), 3.9 ± 0.3; AT, 9.4 ± 0.2; normal, 1.5 ± 0.4.

[c] Significant induction of MN for all doses with epithelial cultures. Treated differ from untreated cultures at $P<0.05$.

[d] Significant reduction of NDI at all doses for both epithelial and fibroblast cultures. Treated differ from untreated cultures at $P<0.05$.

because the calcium in the medium stimulates differentiation of the cells and stops cell proliferation. The medium used was K-SFM with and without fetal calf serum (FCS). The FCS did not appear to alter the growth rate of the keratinocyte culture in the 24 h incubation time (P = 0.47).

Table 6 compares MN frequencies and NDI in the keratinocyte and the bladder carcinoma cell line A1698 after a 24 h treatment with TPA (10 and 100 ng/ml). In K-SFM with FCS there was no induction of MN or reduction in NDI for the keratinocyte culture with TPA treatment (comparison of untreated cultures with TPA-treated cultures: for MN, P = 0.6; for NDI, P = 0.45). A1698 did respond with both MN induction and NDI reduction at similar rates to those observed when this cell line was exposed to TPA in D-MEM (comparison of untreated cultures with TPA treated cultures: for MN, P = 0.01; for NDI, P = 0.01).

In the absence of FCS, an induction of MN occurred in both cultures (keratinocyte, P = 0.02; parent, P = 0.002). The induction in the keratinocyte culture was small, but consistent. A reduction in NDI also occurred in both cultures after TPA treatment, a larger drop occurring in the bladder cell line.

5.4.3 The effect of TPA treatment on fibroblast cultures

When fibroblast cells from a normal individual (GMO5757) and from a patient with the AT syndrome (GMO1588) were exposed to a 24 h treatment with TPA using conditions that induced MN in the bladder carcinoma cell lines, no induction of MN occurred in either culture (Table 5). However, TPA was inducing an effect on these cultures. A reduction in NDI was observed in treated cells, with both cultures responding with a similar drop in the index. The effect on NDI was not dose-dependent.

Table 6. Effect of TPA alone on epithelial cells in K-SFM media with and without FCS[a]

Cell type	TPA (ng/ml)	Induced % MN[b]		Nuclear Division Index (NDI)	
		K-SFM	K-SFM+ FCS	K-SFM	K-SFM+FCS
Normal (NHEK)	10	1.0 ± 0.4[c]	0.2 ± 0.2	74.5 ± 27.0	104.5 ± 8.0
	100	1.4 ± 0.07[c]	0.3 ± 0.3[d]	62.7 ± 10.0	109.7 ± 7.1[d]
Bladder carcinoma (A1698)	10	3.1 ± 0.3[c]	4.3 ± 0.6[c]	32.5 ± 2.0[c]	37.5 ± 3.8[c]
	100	5.1 ± 0.5[c]	4.6 ± 0.7[c]	27.7 ± 1.7[c]	50.7 ± 3.8[c,d]

[a] Cultures were treated with TPA (10 and 100 ng/ml) in K-SFM media with and without FCS for 24 hours at 37°C. Values are the mean of 3 experiments ± SEM.

[b] Induced % MN, is the frequency of MN at treatment minus the spontaneous MN frequency. The spontaneous frequency of MN for keratinocytes: in K-SFM, 1.4 ± 0.1; in K-SFM+FCS, 1.9 ± 0.1; for A1698: in K-SFM, 2.9 ± 0.5; in K-SFM+FCS, 3.8 ± 0.8.

[c] Differs from untreated culture at $P<0.05$.

[d] Significantly differs from treatment in K-SFM without FCS.

5.4.4 The effect of chromosome 11 insertion in A1698 on sensitivity to direct effects of TPA

In order to determine whether the insertion of a normal chromosome 11 would decrease the induction of MN in cultures exposed directly to TPA, a comparison was made of the response of A1698 and its microcell hybrid, A1698 der(11), to a 24 h treatment with TPA (1 and 20 ng/ml). The two cultures did not differ significantly in their response to treatment (Table 7, comparison of MN induction: $P = 0.73$; of NDI, $P = 0.37$).

In order to be sure that a difference in response to TPA was not occuring at doses below the saturation dose, the response of A1698 and its microcell hybrid were further studied at lower doses of TPA (Figure 15). Although a dose response was observed for both MN and NDI when lower doses were used (0.01 - 0.5 ng/ml), the two cultures did not differ significantly in their responses.

5.4.5 The effect of co-incubation of A1698 with TPA-activated neutrophils

A shorter exposure time of 1 h was required in co-culture to induce the same level of genetic damage observed in exposure to TPA treatment alone. Table 8 shows the data obtained when A1698 was co-incubated for 1 h with TPA-activated neutrophils. As controls, cultures were exposed for 1 h to unstimulated neutrophils and to TPA alone. Cultures co-incubated with activated neutrophils showed a significant induction of micronucleated cells ($P = 0.0001$) concurrent to a reduction in the NDI. Cultures treated with TPA alone showed a slight induction of MN ($P = 0.02$) and a similar decrease in NDI. Cultures co-

Table 7. Effect of TPA alone on a bladder carcinoma cell line and its microcell-mediated hybrid [A1698 der(11)][a]

Cell type	Induced % MN[b,c]				Nuclear Division Index (NDI)[d]			
	TPA (ng/ml)				TPA (ng/ml)			
	1	5	10	20	1	5	10	20
A1698	4.6 ± 1.0	4.6 ± 0.5	5.1 ± 0.7	4.2 ± 0.7	36.2 ± 4.0	30.2 ± 2.6	38.5 ± 7.2	31.5 ± 4.8
A1698der(11)	4.3 ± 0.7	3.4 ± 0.5	3.7 ± 0.8	4.0 ± 0.5	57.5 ± 7.0	42.3 ± 6.9	47.5 ±10.7	49.5 ±14.2

[a] Cultures were treated with TPA in D-MEM for 24 h at 37°C. Values are the mean of 3 experiments ± SEM.

[b] Induced % MN, is the frequency of MN at treatment minus the spontaneous MN frequency. The spontaneous frequencies were: A1698,4.3 ± 0.3; A1698 der(11), 3.9 ± 0.3.

[c] Significant induction of MN and reduction in NDI for both cultures. Treated cultures differ from untreated cultures at $P < 0.05$.

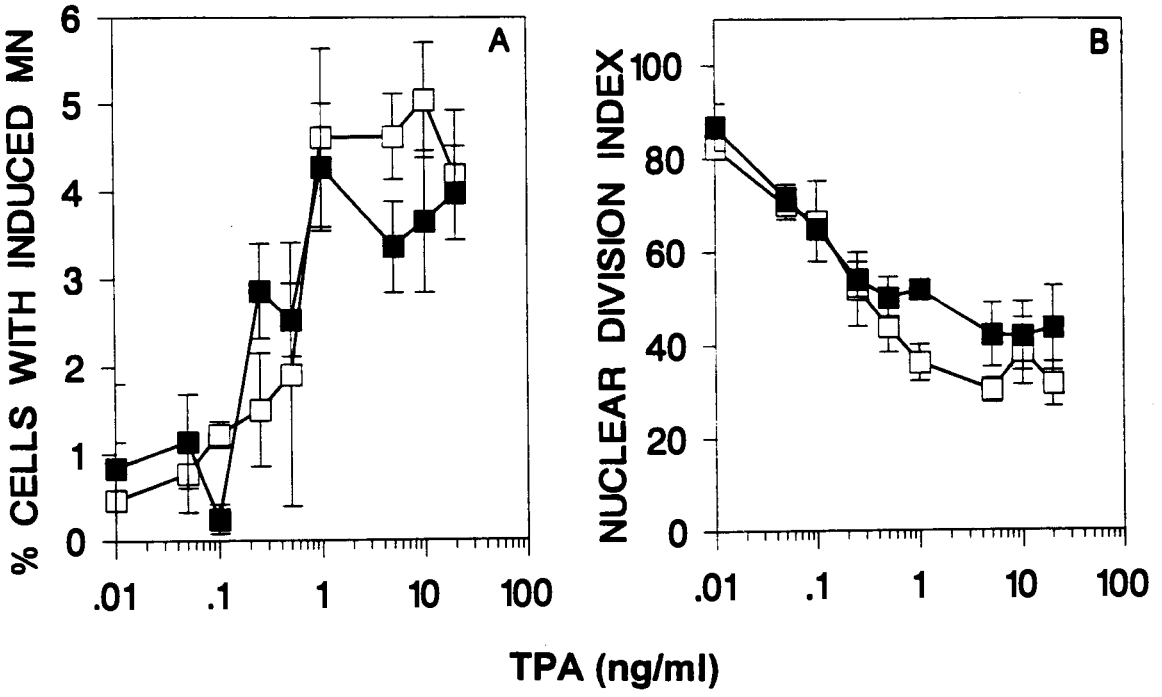

Figure 15. A log plot of the effect of TPA treatment for 24 hours on A) MN formation and B) NDI in a bladder carcinoma culture (A1698, open symbol), and its hybrid culture containing a normal chromosome 11 insert (A1698der[11], closed symbol). Induced MN, is the frequency of MN at treatment minus the spontaneous MN frequency. The spontaneous frequencies were: for A1698, 4.0 ± 0.4; for A1698der(11), 3.7 ± 0.3. Values represent the mean of 3 separate experiments ± S.E.

incubated with unstimulated neutrophils had no significant change in MN frequencies or in NDI.

5.4.6 The effect of chromosome 11 insertion in A1698 on sensitivity to TPA-activated neutrophils

The microcell hybrid of the A1698 culture, A1698 der(11), was exposed to similar co-culture treatments (Table 8). There was no induction of MN in cells co-incubated with activated neutrophils ($P = 0.34$). Furthermore MN frequencies were not altered in cells treated with TPA alone ($P = 0.31$). In contrast, there was a reduction in the NDI in cells co-incubated with activated neutrophils; however, the decrease was smaller than that observed in the parent A1698 culture (NDI, for hybrid, 80%; for parent, 42%). The decrease in NDI in the hybrid culture receiving TPA alone was also less than that observed in the parent A1698 culture. Unstimulated neutrophils had no effect on MN frequencies or NDI in the hybrid culture. These data suggest that the chromosome 11 insert is protecting against MN induction as well as reducing the toxicity and/or growth inhibition induced by this agent.

5.4.7 The effect of TPA-activated neutrophil treatment on fibroblast cultures

The response of an AT and a normal fibroblast culture to co-incubation with TPA-activated neutrophils is compared in Table 8. MN were induced only in the AT culture ($P = 0.0006$). However, a significant decrease in the NDI was observed in both cultures, the drop being slightly greater in the AT culture (index for AT, 42%; for normal, 58%; $P = 0.02$). Unstimulated neutrophils and TPA alone caused no significant induction of MN, but did result in a drop in the NDI in the AT culture (for unstimulated neutrophils, $P = 0.03$; for TPA alone, $P = 0.01$).

Table 8. Effect of TPA-activated neutrophils on epithelial and fibroblast cell cultures[a]

Cell type	Induced % MN[b]			Nuclear Division Index (NDI)		
	PMN	TPA	TPA+PMN	PMN	TPA	TPA+PMN
Epithelial						
A1698	-0.7 ± 0.2	1.4 ± 0.4 [c]	5.5 ± 0.7[c]	90.9 ± 3.2	39.0 ± 3.5[c]	41.8 ± 4.6[c]
A1698der(11)	-0.3 ± 0.4	0.5 ± 0.7	0.5 ± 0.6	91.3 ± 4.8	55.4 ± 3.4[c]	80.1 ± 8.8[c]
Fibroblast						
AT (GMO1588)	0.7 ± 0.6	0.5 ± 0.4	4.5 ± 0.5[c]	86.7 ± 2.4[c]	83.8 ± 2.9[c]	42.2 ± 3.9[c]
Normal (GMO5757)	0.5 ± 0.3	-0.5 ± 0.5	0.1 ± 0.5	106.0 ± 1.1	95.8 ± 2.5	58.0 ± 0.7[c]

[a] Cultures were co-incubated with TPA-activated (10ng/ml) neutrophils for 1 hour at room temperature. Values are the mean of 3 experiments ± SEM.

[b] Induced % MN, is the frequency of MN at treatment minus the spontaneous MN frequency. The spontaneous MN frequencies were: A1698, 2.4 ± 0.3; A1698der(11), 4.1 ± 0.5; AT, 8.8 ± 0.7; normal, 2.0 ± 0.1.

[c] Differs from untreated culture at $P<0.05$.

5.5 Discussion

When TPA is applied to the skin of an animal during a classical tumour promotion study, a complex series of events is initiated. An active area of research over the last decade has been on the ability of this agent to recruit inflammatory cells to a tissue and to stimulate the respiratory burst (Birmboim, 1983; Lewis et al., 1986; Wei and Frenkel, 1991,1992). This event has been associated with the induction of genetic damage in the non-phagocytic cells within the treated tissue (Dutton and Bowden, 1985; Lewis and Adams, 1985; Shacter et al., 1988, 1990; Chong et al., 1989). The data presented in this paper suggest that the ability of TPA to induce chromosomal damage in these cells may be dependent on whether a normal or tumour cell is exposed to the TPA and whether a neutrophil is present in close proximity to the cell. We also present evidence for the involvement of loci on chromosome 11 in altering the sensitivity of a cell specifically to the chromosome-damaging effects of TPA when a neutrophil is present.

To our knowledge there are no studies that have directly compared the relative sensitivity of tumour and normal cells to genetic damage by TPA. In this study, micronuclei were induced in the bladder carcinoma culture A1698 by co-culture with TPA-activated neutrophils and by TPA treatment alone. In contrast, micronuclei were not induced in the normal fibroblast culture in either treatment. These results could represent a difference in the sensitivity of epithelial and mesodermal cultures, or more likely, they could be due to an alteration that occurs in cells during tumourigenesis. Although we have no data from normal bladder cultures, our results using keratinocyte primary cultures suggest that they have a reduced sensitivity to TPA exposure. A slight induction of micronuclei occurred in the keratinocyte culture after exposure to TPA; this effect was dependent upon the absence of fetal calf serum in the media. We did not

co-culture the keratinocyte cultures with TPA-activated neutrophils due to technical difficulties. However, we have compared the sensitivity of the bladder tumour culture and the keratinocyte culture to a mixture of xanthine and xanthine oxidase, a model system commonly used to simulate the oxidative burst of activated inflammatory cells. This treatment results in the production of superoxide anion and hydrogen peroxide. Micronuclei were induced only in the bladder tumour culture and not in the keratinocytes, although both cell cultures showed a similar effect to the treatment in toxicity and growth inhibition (Ward et al., 1993). It is of interest to note that our results with keratinocytes and TPA exposure are similar to those reported by Haesen and co-workers (1993) in their work with keratinocytes isolated from the skin of mice chronically exposed to TPA. *In vivo* rates of chromosomal breakage were assayed directly in these cell isolates by using the micronucleus test. The results showed no significant elevation in MN frequencies. Taken together, these data support the involvement of an alteration in cellular response to TPA as a critical component in the promotion of cells to tumours.

At this point we can only speculate on the nature of the changes that make cells more sensitive to TPA. One aspect of this altered sensitivity is that tumour cells sustain elevated chromosomal breakage in response to TPA itself, without a neutrophil being present. As ROS have been strongly implicated in the induction of genetic damage by TPA (Lewis and Adams, 1985; Dutton and Bowden, 1985; Frenkel and Chrzan, 1987; Bhimani and Frenkel, 1991; Frenkel, 1992), one possible explanation for this response of tumour cells is that there is an increased production of ROS by these cells in response to TPA. Although it is well known that neutrophils respond to TPA treatment with the production of hydrogen peroxide and superoxide (Phillips et al., 1986; Gordon and Weitzman, 1988), this phenomenon also appears to occur in other cell types including

keratinocytes, fibroblasts and tumour cells (Fisher and Adams, 1985; Robertson et al., 1990; Meier et al., 1990; Frenkel, 1992). The mechanism by which ROS are produced in these non-phagocytic cells is not clearly established. However, there are indications in the literature that the kinetics of production of ROS species in non-phagocytic cells are quite different from that observed with neutrophils. For example, normal human fibroblasts release very low amounts of both hydrogen peroxide and superoxide when stimulated with TPA, with the rate of production of these reactive oxygen species 2000- to 4000-fold less than that observed with an equal number of TPA-activated neutrophils (Meier et al., 1990). Furthermore, this production occurs over a 4 h interval whereas the oxidative burst in activated neutrophils peaks in 1 h and then begins to decline. A hypothesis that could be generated from these observations is that the production of ROS in tumours may be significantly greater than that observed in normal fibroblasts or keratinocytes, and be at levels that are beyond the protective capacity of the cells. Currently, there is insufficient data in the literature to allow for a direct comparison to be made of the rate of ROS production in tumour and normal fibroblast or keratinocyte cultures after TPA stimulation.

This may be a rather simplistic explanation, however, since a wide array of products are released by cells exposed to TPA including not only ROS but also arachidonic acid and its metabolites as well as lipid peroxidation products (Lewis et al., 1986; Ochi and Cerutti, 1987; Gordon and Weitzman, 1988; Frenkel, 1992). These have all been implicated as mediators of the DNA damage in cells directly exposed to TPA by studies in which specific agents were used to inhibit arachidonic acid metabolism or remove ROS (Emerit et al., 1983; Emerit and Maghani, 1989; Fischer and Adams, 1985; Snyder, 1985; Petusevska et al 1988). Similar studies have implicated these products as

mediators of DNA damage in cells co-cultured with activated inflammatory cells (Chong et al., 1989; Paul et al., 1989; Dutton and Bowden, 1985; Shacter et al., 1988; Birnboim and Kanabus-Kaminska, 1985). Although all these agents are potential mediators of DNA damage, their relative contribution to the damage is not known. Nor is it known whether the proportion of these products is altered in a tumour compared with a normal cell or if it changes when a neutrophil is present during the exposure.

A second possible explanation for the altered response of tumour cells to TPA is that among the genetic alterations occurring during tumourigenesis is the acquisition of an elevated sensitivity to products generated during TPA exposures. These products could be produced by the cell itself or alternatively by the nearby neutrophil. The two models that we examined in this study only provide evidence for an alteration in sensitivity to DNA damage produced during co-cultures. In the first model, insertion of chromosome 11 was shown to protect a bladder tumour culture from chromosomal breakage during co-cultures. In the second model, a comparison was made of the sensitivity of fibroblast cultures from a normal individual and an AT patient. The AT culture showed an increased sensitivity to micronuclei induction by TPA-activated neutrophils compared with the normal cells. The AT defect has been localized to chromosome 11q22-23 (Sanal et al., 1990).

It is not known whether the same mechanism is involved in protecting the normal fibroblast and hybrid cells from the activated neutrophils. Of interest is the fact that previous studies with the bladder tumour model have shown that the chromosome 11 insert protects the hybrid from chromosomal damage by agents that produce oxidative damage: X/XO mixture, x-irradiation and hydrogen peroxide (Ward et al., 1993; unpublished data). Similarly, AT cells show an elevated sensitivity when compared with normal fibroblasts to agents which act

via free radical mechanisms such as x-irradiation, hydrogen peroxide and X/XO (Yi et al., 1990; Shiloh et al., 1983,1989; Sanford et al., 1990; Ward et al., in press). Therefore, one explanation for the protective effect of chromosome 11 is that it is playing a role in defending the cells from oxidative damage by the ROS released from the TPA-activated neutrophil. Unexplained by this hypothesis is why the insert failed to protect the bladder tumour cell line from the effects of TPA alone, assuming that the latter response was primarily due to ROS. One could speculate that during co-culture most of the DNA damage results from the rapid release of copious quantities of ROS generated during the respiratory burst. Treatment with TPA alone may increase the relative contribution of lipid peroxidation and arachidonic metabolism to the DNA damage. A corollary of this hypothesis would be that the chromosome 11 insert did not protect against chromosomal breakage induced by the latter pathways. We have not yet tested the bladder carcinoma cell line and its hybrid or the AT and normal fibroblasts for relative sensitivity to lipid oxidation products.

In summary, these studies support the hypothesis that TPA acts directly on cells in a tissue as well as acting through the mediation of inflammatory cells. They show that the relative contribution of these two pathways of DNA damage is dependent on a number of factors and suggest that, with tumourigenesis, cells may become more sensitive to the action of TPA. This elevated sensitivity is seen in two ways: an alteration in response to TPA alone and an alteration in response to TPA-activated neutrophils. The latter process appears to involve alterations to loci on chromosome 11.

5.6 References

Bhimani, R. and K. Frenkel (1991) Suppression of H_2O_2 production and oxidative DNA damage in HeLa cells by (-) epigallocatechin gallate (EGCG), Proc. Am. Assoc. Cancer Tes., 32, 126.

Birnboim, H.C. (1983) Importance of DNA strand-break damage in tumor promotion, In: O.F. Nygaard and M.G. Simic (Eds.), Radioprotectors and Anticarcinogens, Academic Press, New York, pp. 539-556.

Birnboim, H.C. and M. Kanabus-Kaminska (1985) The production of DNA strand breaks in human leukocytes by superoxide anion may involve a metabolic process, Proc. Natl. Acad. Sci. USA., 82, 6820-6824.

Chong, Y.C., G.H. Heppner, L.A. Paul and A.M.Fulton (1989) Macrophage-mediated induction of DNA strand breaks in target tumor cells, Cancer Res., 49, 6652-6657.

Dutton, D.R. and G.T. Bowden (1985) Indirect induction of a clastogenic effect in epidermal cells by a tumor promoter, Carcinogenesis, 6, 1279-1284.

Easmon, C. S. F., P.J. Cole, A.J. Williams and M. Hastings (1980) The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescence, Immun., 41, 67-74.

Emerit, I. and M. Lahoud-Maghani (1989) Mutagenic effects of TPA induced clastogenic factor in chinese hamster cells, Mutat. Res., 97-104.

Emerit, I., A. Levy and P. Cerutti (1983) Suppression of tumor promoter phorbolmyristate acetate-induced chromosome breakage by antioxidants and inhibitors of arachidonic acid metabolism, Mutat. Res., 110, 327-335.

Farber, B., R.T. Petrusevska, N.E. Fusenig and V. Kinzel (1989) Cytogenetic effects caused by phorbol ester tumour promoters in HeLa cells: mechanistic aspects, Carcinogenesis, 10 (12), 2345-2350.

Fenech, M. and Morley, A.A. (1985) Measurement of micronuclei in lymphocytes, Mutat. Res., 147, 29-36.

Fischer, S.M. and L.M. Adams (1985) Suppression of tumor promoter-induced chemiluminescence in mouse epithelial cells by several inhibitors of arachidonic acid metabolism, Cancer Res., 45, 3130-3136.

Frenkel, K. and K. Chrzan (1987) Radiation-like modification of DNA and H_2O_2 formation by activated human polymorphonuclear leukocytes (PMNs), In: Anticarcinogenesis and Radiation Protection, Plenum Press, New York and London, pp.97-102.

Frenkel, K. (1992) Carcinogen-mediated oxidant formation and oxidative DNA damage, Pharmac. Ther., 53, 127-166.

Gordon, L.I. and S.A. Weitzman (1988) The respiratory burst and carcinogenesis, In: A.J. Sbarra and R.R. Strauss (Eds.), The Respiratory Burst and Its Physiological Significance, Plenum Press, New York, pp. 277-298.

Phillips, B.J., D. Anderson and S.D. Gangolli (1986) Influence of phagocyte-derived active oxygen species in tissue responses to tumour promoters and irritants, Fd. Chem. Toxic., 24, (6/7), 681-683.

Haesen, S., M. Timmermans and M. Kirsch-Volders (1993) Induction of micronuclei and karyotype aberrations during *in vivo* mouse skin carcinogenesis, Carcinogenesis, 14 (11), 2319-2327.

Hartley, J.A., N.W. Gibson, L.A. Zwelling and S.H. Yuspa (1985) Association of DNA strand breaks with accelerated terminal differentiation in mouse epidermal cells exposed to tumor promoters, Cancer Res., 45, 4864-4870.

Lewis, J.G. and D.O. Adams (1985) Induction of 5,6-ring saturated thymine bases in NIH-3T3 cells by phorbol ester-stimulated macrophages: role of reactive oxygen intermediates, Cancer Res., 45, 1270-1275.

Lewis, J.G., T. Hamilton, and D.O. Adams (1986) The effect of macrophage development on the release of reactive oxygen intermediates and lipid oxidation products, and their ability to induce oxidative DNA damage in mammalian cells, Carcinogenesis, 7, 813-818.

Meier, B., H.H. Radeke, S. Selle, G.G. Habermehl, K. Resch and H. Sies (1990) Human fibroblasts release low amounts of reactive oxygen species in response to the potent phagocyte stimulants, serum-treated zymosan, N-formyl-methionyl-leucyl-phenylalanine, leukotriene B4 or 12-O-tetradecanoylphorbol-13-acetate, Biol. Chem. Hoppe-Seyler, 371, 1021-1025.

Ning, Y. (1991) Tumor suppression by chromosome 11 is not due to cellular senescence, Exp. Cell Res., 192, 220-226.

Ochi, T. and P.A. Cerutti (1987) Clastogenic action of hydroperoxy-5,8,11,13-icosatetraenoic acids on the mouse embryo fibroblasts C3H 10T1/2, Proc. Natl. Acad. Sci, USA, 84, 990-994.

Paul, L.A., A.M. Fulton and G.H. Heppner (1989) Reactive oxygen-mediated damage to murine mammary tumor cells, Mutat. Res., 215, 223-234.

Petrusevska, R.T., G. Furstenberger, F. Marks and N.E. Fusenig (1988) Cytogenetic effects caused by phorbol ester tumor promoter in primary

mouse keratinocyte cultures: correlation with the convertogenic activity of TPA in multistage skin carcinogenesis, Carcinogenesis, 9, 1207-1215.

Robertson, F.M., A.J. Beavis, T.M. Oberyszyn, S.M. O'Connell, A. Dokidos, D.L. Laskin, J.D. Laskin and J.J. Reiners Jr. (1990) Production of hydrogen peroxide by murine epidermal keratinocytes following treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, Cancer Res., 50, 6062- 6067.

Rosin, M.P. (1992) The use of the micronucleus test on exfoliated cells to identify anti-clastogenic action in humans: a biological marker for the efficacy of chemopreventive agents, Mutat. Res., 267, 265-276.

Sanal, O., E. Lange, M. Telatar, E. Sobel, J. Salazar-Novak, F. Ersoy, A. Morrison, P. Concannon, A. Tolun and R.A. Gatti (1992) Ataxia-telangiectasia: linkage analysis of chromosome 11q22-23 markers in Turkish families, FASEB, 6, 2848-2852.

Sanford, K.K., R. Parshad, F.M. Price, G.M. Jones, R.E. Tarone, L. Eierman, P. Hale and T.A. Waldmann (1990) Enhanced chromatid damage in blood lymphocytes after G2 phase x-irradiation, a marker of the ataxia-telangiectasia gene, J. Nat. Can. Inst., 82 (12), 1050-1054.

Shacter, E., E.J. Beecham, J.M. Covey, K.W. Kohn and M. Potter (1988) Activated neutrophils induce prolonged DNA damage in neighboring cells, Carcinogenesis, 9, 2297-2304.

Shacter, E., R.L. Lopez, E.J. Beecham and S. Janz (1990) DNA damage induced by phorbol ester-stimulated neutrophils is augmented by extracellular cofactors, J. Biol. Chem., 265 (12), 6693-6699.

Shiloh, Y., E. Tabor, and Y. Becker (1983) Abnormal response of ataxia-telangiectasia cells to agents that break the deoxyribose moiety of DNA via a targeted free radical mechanism, Carcinogenesis, 4, 1317-1322.

Shiloh, Y., R. Parshad, M. Frydaman, K.K. Sanford, S. Portnoi, Y. Ziv, and G.M. Jones (1989) G2 chromosomal radiosensitivity in families with ataxia-telangiectasia, Hum. Genet., 84, 15-18.

Snyder, R.D. (1985) An examination of the DNA damaging and repair inhibitory capacity of phorbol myristate acetate in human diploid fibroblasts, Carcinogenesis, 6, 1667-1670.

Ward, A.J., P.L. Olive, A.H. Burr and M.P. Rosin (1994) Response of fibroblast cultures from ataxia-telangiectasia patients to reactive oxygen species generated during inflammatory reactions, Environ. Mol. Mutagen., in press, Vol. 24, 2 or 3.

Ward, A.J., P.L. Olive, A.H. Burr and M.P. Rosin (1993) A sensitivity to oxidative stress is linked to chromosome 11 but is not due to a difference in single-strand DNA breakage or repair, Mutat. Res., 294, 299-308.

Wei, H. and K. Frenkel (1991) *In vivo* formation of oxidized DNA bases in tumor promoter-treated mouse skin, Cancer Res., 51, 4443-4449.

Wei, H. and K. Frenkel (1992) Suppression of tumor promoter-induced oxidative events and DNA damage *in vivo* by sacophytol A: A possible mechanism of anti-promotion, Cancer Res., 52, 2298-2303.

Yi, M., M.P. Rosin, and C.K. Anderson (1990) Response of fibroblast cultures from ataxia-telangiectasia patients to oxidative stress, Cancer Lett., 54, 43-50.

Yuspa, S.H., H. Hennings, T Sako, G.R. Pettit, J. Hartley and P.M. Blumberg (1987) Tumour promotion: a problem of differential response of normal and neoplastic cells to trophic stimuli, In: P.A. Cerutti, O.F. Nygaard and M.G. Simic (Eds.), Anticarcinogenesis and Radiation Protection, New York and London, Plenum Press, pp. 169-174.

CHAPTER VI

DISCUSSION

6.1 Significance of research

This thesis provides the first evidence for the involvement of chromosome 11 in determining cell sensitivity to DNA-damaging agents released by activated inflammatory cells. This relationship was demonstrated in two independent model systems: the first involving AT fibroblast cultures and the second a bladder tumour cell line. Although the location of the gene(s) that is involved in this altered sensitivity is unknown, the AT gene(s) is likely to be involved in the sensitivity of the AT cells to this treatment. Whether this gene(s) is also responsible for the same response in the bladder tumour cell line has yet to be determined.

These studies are unique because they represent the first time that AT cells have been examined with respect to sensitivity to inflammatory cells. This is also the first occasion in which micronuclei have been assayed in co-cultures. Finally, the results that were obtained highlight the importance of extending cell culture studies to include epithelial cells rather than limiting them to the traditional fibroblast cultures. For example, X/XO treatment induced micronuclei in the normal fibroblast cultures but not in the primary epithelial culture that was examined.

The thesis was based on the widely accepted premise that cells in a tissue are constantly undergoing genetic change during carcinogenesis with some of these changes resulting in alterated sensitivity to exogenously and endogenously produced agents (reviewed by Weitzman and Gordon, 1990; Cerutti and Trump, 1991; Frenkel, 1992; Guyton and Kensler, 1993). The studies support the possibility that alterations could be occurring in the response of cells to products of activated neutrophils and more specifically to ROS. It is not known whether this altered sensitivity is specific to the cell lines that were used or represents a common alteration in many tumours. Also unresolved is

the impact that this genetic alteration(s) will have on a tissue, although we hypothesize that the change will accelerate tumourigenesis. Assuming that the altered sensitivity occurs in many tumours, it would be important to determine if it were present in earlier stages such as in premalignant lesions. This would then suggest that the altered sensitivity could be a driving force for carcinogenesis rather than a chance event occurring in a genetically unstable tumour cell.

The focus of all the studies described in this thesis has been at the cellular level. The discussion to follow will incorporate these studies into a broader framework that encompasses tissue interactions with inflammatory cells.

6.2 The relevance of inflammation in tissues to carcinogenesis

The inflammatory response is a highly complex process that involves an interplay of many cell types (e.g. mast cells, basophils, neutrophils, macrophages, eosinophils, platelets and lymphocytes) and the release of numerous mediators, both from the tissue and from inflammatory cells (Abramson et al., 1991; Haines et al., 1993). We have focused on one segment of this response: the interaction of neutrophils and epithelial or fibroblast cells. We have also focused on genetic damage as one aspect of this interaction, and have explored the possible role of ROS in inducing this damage. There are other components of inflammation that are beyond the scope of the model systems used in this thesis. For example, there is evidence to suggest that inflammation may alter the expression of growth and differentiation genes and play a critical role in clonal expansion by encouraging the selective outgrowth of specific cell populations (Hartley et al., 1987; Muehlematter et al., 1988; Crawford et al., 1989; Kulesz-Martin et al., 1980; Guyton and Kensler, 1993). This clonal expansion could be induced by oxidative mechanisms and/or involve growth factors and mediators released during inflammation.

We started investigating inflammation because of its association with cancer in humans (Chen and Mott, 1989; Simmonds et al., 1992; Correa, 1992) and also because promoters had been shown to induce inflammation in animal models, with some evidence that this process may be critical to the action of the promoter (Birmboim, 1983; Wei and Frenkel, 1991, 1992). However, inflammation is a normal tissue response to a variety of stimuli and a critical defence against disease. As cancer is not always associated with inflammation, 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 classic example is the involvement of the schistosomiasis infections with bladder cancer in Egypt, a condition that often involves a life-long inflammation in the urinary bladder (Chen and Mott, 1989; Rosin et al., 1994). In addition, the impact of the inflammation on the tissue may depend on extent of the inflammation (the numbers of inflammatory cells within the tissue) and on the capacity of cells in the tissue to prevent or repair damage induced by products released by inflammatory cells. The micronuclei data obtained suggest that cells can have altered susceptibilities to the products of inflammatory cells, though the underlying mechanism responsible for this alteration is unknown.

Using the comet assay we were unable to attribute the increased sensitivity to oxidative stress in AT and parent bladder tumour cells to a DNA rejoining mechanism defect. This and other unresolved issues will complete the discussion of this thesis and provide possible research goals for future consideration.

6.3 Unresolved issues in the AT and bladder models

6.3.1 Is there a common defect in the AT and bladder tumour cell lines?

Although the studies described in this thesis show many phenotypic similarities between AT and bladder cell lines in their responses to oxidative stress, they do not provide direct evidence that the same loci are involved. Nor do they show that the AT gene is directly involved, even in the AT cells, although this is probable. One possible approach to obtaining further information on the loci involved would be to use microcell hybrids containing recombinant chromosomes covering different regions of chromosome 11. These microcell hybrids already exist for AT cell lines (Ejima et al., 1991). Since it is known that the AT loci is located at the q23 region of chromosome 11 (Sanal et al., 1992), only chromosome inserts containing this region should alter the sensitivity to oxidative stress if this gene is indeed involved in the response of either or both of these models.

6.3.2 Could the observed sensitivity to oxidative stress result from a catalase defect?

There are several reasons to suspect that decreased catalase activity could be involved in the elevated sensitivity to oxidative stress. The catalase gene is located at 11p13 (Smith et al., 1993). A marked deficiency in catalase activity has been reported for AT cells (Villaume et al.,1983, 1986), and also for many tumours and some pre-neoplastic lesions (Rabilloud et al., 1990; Villaume et al., 1991; Hoffschir et al., 1993), although these observations have been limited to a single laboratory. Finally, the area of the catalase gene locus is very commonly altered during bladder carcinogenesis. From 60-70% of bladder tumours have a loss of heterozygosity at this site (Shipman et al., 1993).

The data presented in this thesis do not support a catalase defect. The results obtained with the comet assay show that there was no significant difference between the parent and hybrid or between the AT and normal cell lines in X/XO-induced strand breakage. If the catalase hypothesis was correct, strand breakage should have been greater in the parent than in the hybrid culture, and in the AT compared to the normal culture.

Furthermore, other unpublished observations would suggest the insertion of chromosome 11 in the hybrid does not correct a catalase defect (Rosin, unpublished data). The parent and hybrid cultures have been assayed for alterations to chromosome number after hydrogen peroxide treatment by using fluorescent *in situ* hybridization techniques with centromere-specific DNA probes to identify chromosome loss or gain. Alterations to chromosome number are primarly due to disruption of the mitotic spindle. Although the hydrogen peroxide caused significant chromosome loss and gain in both cultures, there was no significant difference between the two cultures. As a more direct indication of catalase activity, the rate of hydrogen peroxide detoxification is now being measured using spectrophotometry. The hybrid cell line showed a faster rate of conversion of hydrogen peroxide to water and oxygen than the parent culture. However, the difference in the rate of conversion was too small to adequately explain the much greater differences that are observed in MN frequencies in the two cultures after hydrogen peroxide or X/XO treatment.

6.3.4 Is DNA repair of oxidative damage defective ?

Only one component of DNA repair was examined in this thesis: the ability of cells to rejoin single- and double- strand breaks. This endpoint appeared to be an appropriate point at which to begin to examine these cells for a DNA repair defect because alterations in this repair capacity would lead to micronuclei.

However, neither the AT nor bladder tumour cell lines were defective in this aspect of repair.

ROS induce a wide variety of DNA damage products including base modifications, base free sites, strand breaks and cross-links. Several enzymes are involved in the repair of these lesions (Breimer, 1991; Pacifici and Davies, 1991; Reid and Loeb, 1993; Epe et al., 1993; Satoh et al., 1993). Many of these enzyme activities represent possible sites for defective repair in these cell lines. Another possibility is that the fidelity of the repair process is altered. Recent evidence suggests that although strand breaks are rejoined at the same rate in AT cells, the rejoining may be incorrect and generate deletions and other types of mutation (Thacker, 1989; Powell et al., 1993; Ganesh et al., 1993). In addition an alteration in fidelity of repair has also been observed for a bladder tumour cell line (Powell et al., 1992). These possibilities need to be explored.

6.4 Conclusion

In summary, the results of this thesis highlight the importance of cell interactions during tumour promotion and suggest that they may play a critical role in determining genetic instability in tissues during this stage in carcinogenesis. They suggest two important possibilities; that tumours are altering in sensitivity to DNA-damaging agents released during such interactions, and that there may be populations of individuals that have an inborn susceptibility to tumour promotion. Further studies to explore these possibilities could be crucial to the development of preventative measures that would suppress tumourigenesis in humans.

6.5 References

Abramson, S.B., J. Leszczynska-Piziak and G Weissmann (1991) Arachidonic acid as a second mesenger: Interaction with a GTP-binding protein of human neutrophils, J. Immuno., 147, 231-236.

Birnboim, H.C. (1983) Importance of DNA strand-break damage in tumour promotion, In: O.F. Nygaard and M.G. Simic (Eds.), Radioprotectors and Anticarcinogens, Academic press, New York, pp. 539-556.

Breimer, L.H. (1991) Repair of DNA damage induced by reactive oxygen species, Free Rad. Res. Comms., 14(3), 159-171.

Cerutti, P.A. and B.F Trump (1991) Inflammation and oxidative stress in carcinogenesis, Cancer Cells, 3(1), 1-7.

Chen, M. and K. Mott (1989) Progress in assessment of morbidity due to *Schistosoma haematobium* infection: a review of the recent literature, Trop. Dis. Bull., 84, 1-30.

Correa, P. (1992) Human gastric carcinogenesis: a multistep and multifactorial process-First American Cancer Society award lecture on cancer epidemiology and prevention, Cancer Res., 52, 6735-6740.

Crawford, D., P. Amstad, D.D.Y. Foo and P.A. Cerutti (1989) Constitutive and phorbol-myristate-acetate regulated antioxidant defences of mouse epidermal JB6 cells, Mol. Carcinog., 2, 136-143.

Ejima, Y., M. Oshima and M.S. Sasaki (1991) Determination of the chromosomal site for the human radiosensitive ataxia telangiectasia gene by chromosome transfer, Mutat. Res., 250, 337-343.

Epe, B., M. Pflaum, M. Haring, J. Hegler and H. Rudiger (1993) Use of repair endonucleases to characterize DNA damage induced by reactive oxygen species in cellular and cell-free systems, Toxicol. Letters, 67, 57-72.

Frenkel, K. (1992) Carcinogen-mediated oxidant formation and oxidative DNA damage, Pharmac. Ther., 53, 127-166.

Ganesh, A., P. North and J. Thacker (1993) Repair and misrepair of site-specific DNA double-strand breaks by human cell extracts, Mutat. Res., 299, 251-259.

Guyton, K.Z. and T.W. Kensler (1993) Oxidative mechanisms in carcinogenesis, Brit. J. Med. Bull., 49 (3), 523-544.

Haines, K.A., S.L. Kolasinski, B.N. Cronstein, J. Reibman, L.I. Gold and G. Weissmann (1993) Chemoattraction of neutrophils by substance P and transforming growth factor β 1 is inadequately explained by current models of lipid remodeling, J. Immuno., 151 (3), 1491-1499.

Hartley, J., N. Gibson, A. Kilkenny and A. Yuspa (1987) Mouse keratinocytes derived from initiated skin or papillomas are resistant to DNA strand breakage by benzoyl peroxide: a possible mechanism for tumour promotion by benzoyl peroxide. Carcinogenesis, 8, 1827-1830.

Hoffschir, F., M. Vullaume, L. Sabatier, M. Ricoul, L. Daya-Grosjean. S. Estrade, R. Cassingena, R. Calvayrac, A. Sarasin and B. Dutrillaux (1993) Decrease in catalase activity and loss of the 11p chromosome arm in the course of SV40 transformation of human fibroblasts, Carcinogenesis, 14 (8), 1569-1572.

Kulesz-Martin, M.F., B. Koehler, H. Hennings and S.H. Yuspa (1980) Quantitative analysis for carcinogen altered differentiation in mouse epidermal cells, Carcinogenesis (Lond.), 1, 995-1006.

Muehlematter, D. R. Larsson and P. Cerutti (1988) Active oxygen induced DNA strand breakage and poly ADP-ribosylation in promotable and non-promotable JB6 mouse epidermal cells, Carcinogenesis, 9, 239-245.

Pacifici, R.E. and K.J.A. Davies (1991) Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of aging revisited, Gerontology, 37, 166-180.

Powell, S., S. Whitaker, J. Peacock and T. McMillan (1993) Ataxia telangiectasia: An investigation of the repair defect in the cell line AT5BIVA by plasmid reconstruction, Mutat. Res., 294, 9-20.

Powell, S.N., S.J. Whitaker, S.M. Edwards and T.J. McMillan (1992) A DNA repair defect in a radiation-sensitive clone of a human bladder carcinoma cell line, Brit. J. Cancer, 65(6), 798-802.

Rabilloud, T., D. Asselineau, C. Miquel, R. Calvayrac, M. Darmon and M. Vuillaume (1990) Deficiency in catalase activity correlates with the appearance of tumour phenotype in human keratinocytes, Int. J. Cancer, 45, 952-956.

Reid, T.M. and L.A. Loeb (1993) Effect of DNA-repair enzymes on mutagenesis by oxygen free radicals, Mutat. Res., 289, 181-186.

Rosin, M.P., S.S. El Din Zaki, A.J. Ward and W.A. Anwar (1994) Involvement of inflammatory reactions and elevated cell proliferation in the development of bladder cancer in schistosomiasis patients, Mutat. Res., 305, 283-292.

Sanal, O., E. Lange, M. Telatar, E. Sobel, J. Salazar-Novak, F. Ersoy, A. Morrison, P. Concannon, A. Tolum and R.A. Gatti (1992) Ataxia-telangiectiasia: linkage analysis of chromosome 11q22-23 markers in Turkish families, FASEB, 6, 2848-2852.

Satoh, M.S., C.J. Jones, R.D. Wood and T. Lindahi (1993) DNA excision-repair defect of xeroderma pigmentosum prevents removal of a class of oxygen free radical-induced base lesions, Med. Sci., 90, 6335-6339.

Shipman, R., P. Schraml, M. Colombi, G. Raefle and C.U. Ludwig (1993) Loss of heterozygosity on chromosome 11p13 in primary bladder carcinoma, Hum. Genet., 91, 455-458.

Simmonds, N.J., R.E. Allen, T.R.J. Stevens, R.N.M. Van Someren, D.R. Blake and D.S. Rampton (1992) Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease, Gastroenterology, 103, 186-196.

Smith, M.W., S.P. Clark, J.S. Hutchinson, Y.H. Wei, A.C. Churukian, L.B. Daniels, K.L. Diggle, M.W. Gen, A.J. Romo, Y. Lin, L. Selleri, D.L. McElligott and G.A. Evans (1993) A sequence-tagged site map of human chromosome 11, Genomics, 17, 699-725.

Thacker, J. (1989) The use of integrated DNA vectors to analyse the molecular defect in ionizing radiation-sensitive mutants of mammalian cells including ataxia telangiectasia, Mutat. Res., 220, 187-204.

Vuillaume, M., Y. Decroix, R. Calvayrac, J.B. Truc, b.J. Paniel, P. Vincens, P. Tarroux and P. Poitout (1991) Catalase-associated abnormalities and hydrogen peroxide increase in pre-neoplastic lesions of the human lower

female genital tract and their near adjacent epithelia, Biomed. Pharmacother., 45, 435-444.

Vuillaume, M. R. Calvayrac, M. Best-Belpomme, P Tarroux, M. Hubert, Y. Decroix and S. Sarasin (1986) Deficiency in the catalase activity of xeroderma pigmentosum cell and SV40-transformed human cell extracts, Cancer Res., 46, 538-544.

Vuillaume, M. Y. Decroix, Y. Calvayrac, R. Vallot and M. Best-belpomme (1983) Xeroderma pigmentosum: hydrogen peroxide formation and catalatic activity during the evolution of the skin cell: application to an early diagnosis and to a tentative treatment, C.R. Acad. Sci. (Paris), 845-850.

Wei, H. and K. Frenkel (1991) *In vivo* formation of oxidized DNA bases in tumour promoter-treated mouse skin, Cancer Res., 51, 4443-4449.

Wei, H. and K. Frenkel (1992) Suppression of tumour promoter-induced oxidative events and DNA damage *in vivo* by sacophytol A: A possible mechanism of anti-promotion, Cancer Res., 52, 2298-2303.

Weitzman, S.A. and L.I. Gordon, (1990) Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis, Blood, 76(4), 555-663.