Effects of Cigarette Smoking and Vanillin concentration on Sister Chromatid Exchange and Chromosome Aberrations in Women aged 16-25.

A thesis presented in partial fulfilment of the requirements

for the degree
of Master of Science in Genetics
at Massey University.

Katherine Mary Harding

1996

ABSTRACT

The chromosomes of human peripheral blood lymphocytes were analysed for sister chromatid exchanges (SCEs) and structural aberrations and correlated to cigarette smoking habits of 15 individuals and to the concentration of vanillin, a flavour compound of cigarettes. An analysis of variance showed that there was a significant increase in the frequency of SCEs in smokers compared with non smokers. With non smokers had a mean SCE of 9.712 per cell whereas smokers had a mean of 12.771 SCEs per cell. Cigarette smoking showed no significant effect on the frequency of chromosome aberrations. *In vitro* studies also showed that an increase in vanillin concentration induced an increase in the number of SCEs per cell. Conversely there was no relationship between cigarette smoking and structural chromosome aberrations. The present studies indicate that cigarette smoking confers a genetic risk on the individual with vanillin contributing to such a risk.

ACKNOWLEDGEMENTS

First I would like to thank my supervisor Dr RE Rowland who has stood by me during the long period it took to complete this thesis. Al, thank you so much for all the patience and understanding during this period and I wish you all the best for the future.

I would also like to thank Elizabeth Nicklass who was an endless help in providing me invaluable advise on methods and equipment.

Thank you to the staff at Medical Diagnostics and Massey University Health Centre for providing blood collection facilities.

To my partner and best friend, Tony. Thank you so much for all your love, encouragement and support in finally getting to the final stage of my thesis. I have finally done it!

Thank you to my mum and dad who have supported me my whole life and encouraged me in anything I put my mind to. I love you both and can never thank you enough.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

Cigarette smoking is generally believed to be responsible for a substantial number of human health problems. However, the causal relationship between smoking, the induction of biological effects and the extent of health problems among smokers have not been fully documented. One of the biggest problems is the relationship between smoking and cancer. The carcinogenicity of tobacco tars and smoke in laboratory animals together with epidemiological evidence from man have clearly suggested that smoking causes most lung cancer. What has yet to be established is how cigarette smoke causes cancer. One method that has been used to investigate this carcinogenicity is cytogenetics.

The rationale for using cytogenetic analyses to detect mutagenic/carcinogenic exposure rests on both theoretical considerations and data obtained from animal experiments, *in vitro* tests, epidemiological findings, and studies of human cancer cells and hereditary disorders that predispose to cancer. Exposure to ionising radiation, benzene, ethylene oxide, styrene oxide, vinyl chloride, and cigarette smoking for example, all of which are known or suspected to increase human cancer risk, have repeatedly been shown to increase levels of cytogenetic damage in human peripheral lymphocytes (IARC 1987a, IARC 1987b).

Molecular genetic studies have considerably strengthened the cytogenetic evidence that genetic alterations are of the essence in tumorigenesis. Results point to the conclusion that DNA changes are an integral part of neoplastic development. Chromosome breakage must occur for chromosomes to be rearranged, so an increase in breakage must elevate the likelihood of producing such rearrangements and hence increasing the risk of tumour development (Heim *et al* 1989). Compatible with this reasoning is the observation that many physical and chemical carcinogens (e.g. X-rays) are also mutagenic or are clastogenic (i.e. chromosome breaking) (Lambert *et al* 1978, Jansson *et al* 1987). Thus environmental mutagens have

become a serious concern because of their carcinogenic potential. The supposition that higher levels of chromosome damage in peripheral lymphocytes may reflect increased cancer risk is based on the premise that although a limited number of genetic rearrangements are essential in neoplastic transformation, an overall increase in chromosomal aberrations makes it more likely that such neoplasia-inducing changes may occur (Heim, and Mitelman 1987). Some data indicate that aberration levels in lymphocytes correlate with levels in more important target tissues. For instance, the frequency of chromosomal aberrations in lymphocytes paralleled cytological abnormalities in bronchial cells in uranium miners, an occupational group at high risk of lung cancer (Brandom, etal 1978). However, no systematic attempts have so far been made to correlate, within the same group of persons in a prospective study, levels of cytogenetic damage in peripheral lymphocytes with cancer morbidity (Reuterwall 1990).

Two of the most widely used cytogenetic methods for measuring exposure to carcinogens is by examining chromosome aberrations and Sister Chromatid Exchanges (SCE). Chromosome aberrations analysis is a crude but simple method that basically looks at any chromosome abnormalities that involve a break or exchange of chromosomal material. They including breaks in the chromosome or chromatid, rearrangements, translocations, inversions etc. Data suggests that, on a population basis, increases in frequencies of chromosome aberrations are an indication of exposure, a factor which increases the risk to cancer and genetic ill health (Swierenga 1991).

SCE studies have been used extensively because of the advantages that the technique is reliable and relatively simple and the results provide a sensitive indication of exposure. An increase in the frequency of SCEs generally indicates that a compound is a mutagenic carcinogen. SCEs are believed to represent the interchange of DNA replication products at apparently homologous loci, and involve DNA breakage and reunion (Latt *et al* 1981). Although SCEs are readily observed experimentally, the exact mechanism(s) of SCE formation is not known, although several models have been proposed as outlined in the literature Review. It appears that SCEs are produced at or near the replication forks and most models generally provide

a rationale for strand switches relative to the replication fork, but differ in exact details. Giemsa banding (G-banding) is also a common cytogenetic tool used to detect chromosome damage. G-banding in conjunction with SCE is also possible. Although it has been attempted by a few researchers this area is fraught with pitfalls when interpreting results. For this reason G-banding plus SCE detection was not conducted in this experiment.

Previous studies looking at the relationship between cigarette smoking and the frequency of SCE have produced conflicting results mainly because of the diverse experimental procedures and the influence of environmental variables. Very few studies have been conducted in New Zealand on the effects of cigarette smoking on the genetic material leading to a paucity of information in this area. It is therefore important to repeat such experiments but improving them using the latest knowledge available.

From a level of around 2000 cigarette equivalents per adult between 1925 and 1935, tobacco products consumption rose steadily over the next 30 years to peak in 1963 at 3347 cigarette equivalents per adult (figures are calculated in number of cigarette equivalents per adult 15 years and over per year. By convention, one gram of loose tobacco is equivalent to one million manufactured cigarettes; thus one tonne of loose tobacco is equivalent to one million manufactured cigarettes). Between 1950 and 1975, total tobacco consumption in New Zealand remained relatively stable. The period was marked by a steady increase in the consumption manufactured cigarettes, accompanied by a decline in the consumption of loose tobacco (for pipes and 'roll your own' cigarettes). The overall level of cigarette and tobacco consumption in New Zealand has fallen sharply since 1975. In 1991, each adult aged 15 years and over smoked the equivalent of 1791 cigarettes per year, down 44% from 3219 in 1975. In 1983, proportionately more men than women smoked at all ages except for those in the 15-24 year age group (35% of women aged 15-24 smoke). By 1986, the sample population for women smoking was greater than that for men in the 15-24, 25-34, and 35-54 year age groups. For those sampled above the age of 54 years, the proportion of men who smoked continued to exceed the proportion of women (Dept. Health and Dept. Statistics 1991). Statistics cited in the Dominion

newspaper (1993) report that every year more than 19000 New Zealand teenagers become regular smokers. By 15 years, at least 33% of girls and 20% of boys are daily or occasional smokers, and about 80% of 15 year olds have tried tobacco. Considering the bewildering statistics on the young women smokers of New Zealand I felt it of great importance to target this group for my study (16-25 year old females).

Cigarette smoke is made up of thousands of chemicals and some of them are known to be mutagens and/or carcinogens. In terms of biological activity, cigarette smoke and its condensates have been shown to form adducts with DNA and protein and to induce gene mutations (Au etal 1991). One way to find out how cigarette smoke causes these mutations is by breaking down the ingredients of cigarettes and finding which individual chemicals are able to cause damage.

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a very common flavour compound found in cigarette smoke, foods and beverages. Vanillin has been shown to be converted to catechol by the intestinal microflora in the rat and catechol is also found in the urine after vanillin exposure (Jansson and Zech 1987). This may well indicate a genetic hazard, as catechol is known to be carcinogenic. Although as yet no data on vanillin metabolism in lymphocytes is available and any effect may be independent of metabolism.

Jansson etal 1986 found that vanillin is a potent inducer of SCE's which has invited further studies of the genotoxic effects of vanillin. Studies by Jansson and Zech in 1987 (Jansson and Zech 1987) found that vanillin may also have a low ability to induce chromosome aberrations. It has also been shown that many benzaldehydes induce SCE's. It appears that the benzaldehyde moiety of vanillin seems essential for the activity, since the analogue 4-hydroxy-3-methoxyaceptophenone does not induce SCE's. As vanillin is a common flavour compound in many products I feel the need to examine this area more.

Induction of chromosome aberrations and SCE by cigarette smoking in smokers has been extensively studied. Positive and negative results have

been found which shows how different experimental procedures and other various life style factors have contributed to the inconsistent observations. By trying to minimise these factors it may be possible to obtain more accurate results.

AIMS OF THESIS

The aims of this thesis are:

A To test the null hypothesis that cigarette smoking in women aged between 16-25 has no effect on chromosome aberrations or on SCE's.

B To test the null hypothesis that vanillin (4-hydroxy-3-methoxybenzaldehyde) does not have any effect on chromosome aberrations or on SCE's.

3. LITERATURE REVIEW

Tobacco is a dirty weed. I like it.
It satisfies no normal need. I like it.
It makes you thin, it makes you lean,
It takes the hair right off your bean.
It's the worst darn stuff I've ever seen.
I like it.

Tobacco 1915
Graham Lee Hemminger

3.1 Cancer and Cytogenetics

3.1.1 Somatic mutation theory of cancer

In 1914 Theodor Boveri formulated what became known as the *somatic mutation theory of cancer*. The hallmark of this hypothesis was the realisation that stable malignant transformation of previously normal cells could only be achieved by altering the cells genetic material, the chromosomes. It is now established beyond any doubt that most neoplasms, benign as well as malignant, do have genomic alterations that are detectable at the microscopic level and chromosome aberrations of tumour cells are not random. They occur in an orderly fashion, their distribution differs between neoplasms of different types and often the pattern of abnormalities is quite characteristic for the disease in question (Knudsen 1985).

There is increasing evidence that one prerequisite in the initiation of malignant transformation is an alteration in cellular DNA, and the findings

that most carcinogens react with DNA and that this reaction necessarily precedes transformation and that most carcinogens are mutagens, support the importance of somatic mutation (Hopkin and Evans 1980).

3.2 Carcinogens and Mutagens

3.2.1 Chromosome instability

It has been shown that certain chemicals can induce an alarming increase in the incidence of certain cancers compared with that in the human population at large, but not all persons exposed to the same chemicals in similar environments develop cancer. Also there are families who seem to genetically inherit a high probability of developing cancer suggesting a genetic predisposition to neoplasia. This genetic predisposition to cancer may be caused by genetic instability. Individuals with genetic instability may generate more cells with mutations or chromosomal aberrations than those with more stable genomes. One of these aberrant cells in a target tissue may happen to possess a genetic constitution equivalent to the first step of carcinogenesis (Heim *et al* 1989). Knudson (1985) suggested that a genetically determined elevated carcinogen sensitivity may be the underlying cause of many of the cancers that we now presume to be induced by environmental factors.

3.2.2 Environmental Parameters

It is also known that many environmental substances may contribute to cancer as mentioned earlier e.g. ionising radiation, cigarette smoke and ethylene oxide. An individuals tendency to develop chromosome breaks would therefore depend on two factors. The persons exposure to clastogens and his or her inherent chromosome stability including their ability to efficiently repair any damage that might have occurred to the DNA. So genetic and environmental parameters may combine, for example a person may not be genetically capable of responding or repairing genetic damage due to clastogenic or mutagenic challenges and hence develop a type of

cancer. Although environmental influence may sometimes be negligible as is the case with patients possessing autosomal dominant cancer syndromes; because of a genetic defect, these patients appear oblivious to exposure to a substance yet develop a cancer.

3.3 Smoking and Cancer

Our environment contains many potential carcinogens and mutagens that could facilitate the formation of cancerous cells. Cigarette smoking is presumed to be one of the major causes of lung cancer throughout the world. In New Zealand between 1940 and 1988, the lung cancer mortality rate in the 35-64 year old group increased markedly for both males and females (Dept. Health and Dept. Statistics 1991). The carcinogenicity of tobacco tars and smoke in laboratory animals together with epidemilolgical evidence from man have clearly suggested that smoking causes most lung cancer (De Marini 1983). 24 years ago, an international group of epidemiologists who worked on lung cancer, reached unanimous agreement that cigarette smoking was the cause for the rise in lung cancer (Reif 1958). Hoffmann and Wynder (1976) have shown that tobacco smoke's relative contribution to excess cancer deaths for specific sites is over 50% for cancer of the oral cavity, larynx and lung; 10-15% for cancer of the oesophagus, kidney and bladder; and 1-10% for cancer of the pancreas. In addition cigarette smoking is nearly the exclusive factor associated with emphysema (De Marini 1983). Epidemiological evidence also suggests that between 1/2 and 1/3 of all cigarette smokers die as a result of their smoking (Dept. Health and Dept. Statistics 1991). It has also been established that cigarette smoking causes almost all cases of anaplastic and squamous cell bronchial carcinomas (Royal college of Physicians 1977).

Cigarette smoke also exerts a number of physiological effects upon the nonsmoker. Passive smoking has even been implicated as a causative factor in lung cancer among non-smokers (Trichopoulos 1981) although it has also been disputed (De Marini 1983).

It is equally well known, however, that many smokers do not develop lung cancer and, although the amount of tobacco smoked is undoubtedly the dominant risk factor, it seems possible that other factors may also be involved (Wynne Griffith 1976), for example chromosome instability.

3.4 Measuring Carcinogenicity and Mutagenicity

3.4.1 Human peripheral lymphocytes

Studies on exposed individuals and on cultured cells, have shown that the human peripheral blood lymphocyte is an extremely sensitive indicator of both *in vivo* and *in vitro* induced chromosome structural changes (Evans and O'riordan 1975). Chromosome aberrations and sister chromatid exchange (SCE) assays in these cells discussed later are cytogenetic measures of mutagenicity and DNA damage (Reuterwall 1990).

Advantages of using peripheral blood lymphocytes

a. Easy availability of large numbers of *human* cells: a few ml of peripheral blood can be easily and repeatedly obtained from an individual and each 1 ml of blood can contain $1\text{-}3\text{x}10^6$ small lymphocytes.
b. The lymphocytes are distributed throughout the body, circulate in all tissues and a proportion are long lived.
c. Virtually all the peripheral blood lymphocytes are a synchronised cell population in the same G_0 or G_1 stage of mitotic interphase, and in healthy individuals, these cells are only infrequently involved in mitotic proliferation *in vivo*.
d. A proportion of the lymphocytes can be stimulated by mitogens to undergo mitosis in culture. They are easy to culture and thus provide a ready source of dividing cells for scoring the chromosome aberrations.
e. There are excellent techniques available for making chromosome preparations from lymphocytes and the cells have a low spontaneous

chromosome aberration frequency.

Disadvantages of using peripheral blood lymphocytes

a. Although the lymphocytes are a synchronised population, there are different sub-populations of cells even within the same individual. These can have varying responses to different mitogenic agents and probably differing intermitotic times which may be further influenced by variations in culture conditions.
b. As in other cell systems, there is the possibility of non-uniformity of exposure and of preferential growth in culture of less heavily damaged cells.
c. It is now recognised that in man only T-lymphocytes, i.e. the thymic-derived cells, can be stimulated in culture by PHA to undergo mitosis. The T-lymphocyte is closely concerned with the immune response, thus, where an analysis of an *in vivo* exposure is to be undertaken, any recent previous exposure of an individual to an immunological stimulus may positively or negatively alter the number of cells with chromosome aberrations, depending on when the cells are withdrawn from the individual. It is therefore important to obtain detailed medical information before selection of participants.

These disadvantages relate largely to the use of lymphocytes for studying mutagen effects *in vivo* and are of less relevance to the use of lymphocytes for *in vitro* mutagen testing (Evans and O'riordan 1975).

3.4.2 Chromosome aberrations

Chromosome aberrations are microscopically visible changes in chromosome structure that involve a break or exchange of chromosomal material. They may be a complete break of a single chromatid or chromosome resulting in a loss or deletion of part of the chromosomal material, translocations, and other rearrangements. Gaps and achromatic lesions are also observed. Chromosomal aberrations are stained with Giemsa and can be detected in the first division following exposure of a cell

culture to a mutagenic compound (Swierenga 1991).

Principles of Chromosome Aberrations

The type of chromosomal structural alteration produced by physical and chemical agents depends on the lesions induced in the DNA and, therefore, upon the chemical structure of the genotoxic substance. Structural chromosomal aberrations result from breakage and rearrangement of whole chromosome into abnormal forms.

Chromosome aberrations are most efficiently induced by:

- substances that directly break the backbone of DNA (eg ionising radiation and radiomimetic chemicals)
- or substances thatsignificantly distort the DNA helix (intercalating agents).

Structural aberrations can be classified as either unstable or stable depending upon their ability to persist in dividing cell populations.

Unstable aberrations

These include:

- dicentrics
- rings
- acentric fragments
- and other asymmetrical rearrangements

Studies indicate that unstable aberrations will lead to the death of the cell, this is because the asymmetrical exchanges such as dicentrics and tri-radials can prevent the cell from dividing at mitosis or because the formation of these exchanges also results in chromosome fragments that can be lost at cell division, i.e. resulting in deletion of genetic material in progeny cells which is normally intolerable in actively dividing populations. In non-dividing cells the expression of genes involved in the aberration may be altered.

Stable aberrations:

These include:

- balanced translocations
- inversions
- other symmetrical arrangements

These are stable because they can be transmitted to progeny cells at division. Symmetrical rearrangements are formed with equal frequency to the asymmetrical arrangements.

Significance of Chromosome Aberrations

- Approximately 50% of all abortuses are chromosomally abnormal.
- Populations exposed to ionising radiation (atom bomb survivors) or to genotoxic chemicals (e.g. benzene) have increased frequencies of chromosome aberrations in their lymphocytes.
- Many types of human cancers are associated with specific or non-specific chromosome aberrations.
- Several human hereditary diseases (ataxia telangiectasia, Fanconi's anemia, and Bloom's syndrome) are associated with increased frequencies of chromosome aberrations and increased incidence of cancer.

These data and others not mentioned suggest that, on a population basis, increases in frequencies of aberrations are an indication of exposure, a factor which increases the risk to cancer and genetic ill health (Swierenga 1991).

Classification of Aberrations
(From Carrano and Natarajan 1988)

Chromatid-type aberrations

- chromatid gaps or achromatic lesions - unstained regions in the chromatid which are less than the width of the chromatid.

- chromatid breaks - breaks in a chromatid greater than the width of the arm.
- isochromatid-chromosome breaks - breaks involving both sister chromatids at the same position (these generally cannot be distinguished from acentric fragments).
- chromatid exchanges - exchanges between chromatids of different chromosomes.
- chromatid intrachange - exchange between the sister chromatids at non-homologous points.

Chromosome-type aberrations

- Acentric fragments (see isochromatid-chromatid breaks; also called minutes depending on size).
- dicentric chromosomes - exchanges between two chromosomes resulting in a structure with two centromeres and an associated acentric fragment.
- ring chromosome - exchanges within one chromosome. Centric rings associated with a fragment. Small acentric rings lacking a centromere are often indistinguishable from minutes.

Numerical aberrations

Due to nondisjunction events, metaphases with additional or missing chromosomes can arise. In view of the procedures involved in preparing metaphases for microscope analysis, especially hypotonic treatment to spread the chromosomes, cells with deviating chromosome numbers (aneuploid cells) can arise. Therefore, in practice, sporadic aneuploid cells are not scored as aberrant. By contrast, polyploid cells usually cannot be attributed to preparatory procedures and can easily be scored. All types of aberrations mentioned can eventually lead to the formation of micronuclei, as the acentric fragments which are lagging during anaphase-telophase movement can condense.

Balanced translocations and inversions

These aberration types are difficult to quantify without banding analysis and therefore are not usually scored in population monitoring studies. If banding analysis is performed these aberrations should be tabulated separately from the other aberration types.

3.4.2 Sister Chromatid Exchange (SCE)

Method

SCEs were first visualised by Taylor etal in 1957 (Taylor etal 1957) who used tritiated thymidine to differentially label the DNA of replicating cells and autoradiography to distinguish the silver grain pattern on the two sister chromatids. This method was eventually replaced by cytochemical methods for differentiating sister chromatids. Latt (1973) demonstrated that when the thymidine analogue, bromodeoxyuridine (BrdU) was incorporated into DNA, it could quench the fluorescence of the fluorochrome Hoechst 33258. Perry and Wolf(1974) found that incorporated BrdU also diminished the uptake of Giemsa stain into the chromatin causing one chromatid to be paler than its sister (Figure 3.1) In principle then, SCEs can be observed in any cell that has completed two, or the first of two, replication cycles in the presence of BrdU (Carrano and Natarajan 1988) and then stained with Hoechst 33258 and subsequently with Giemsa.

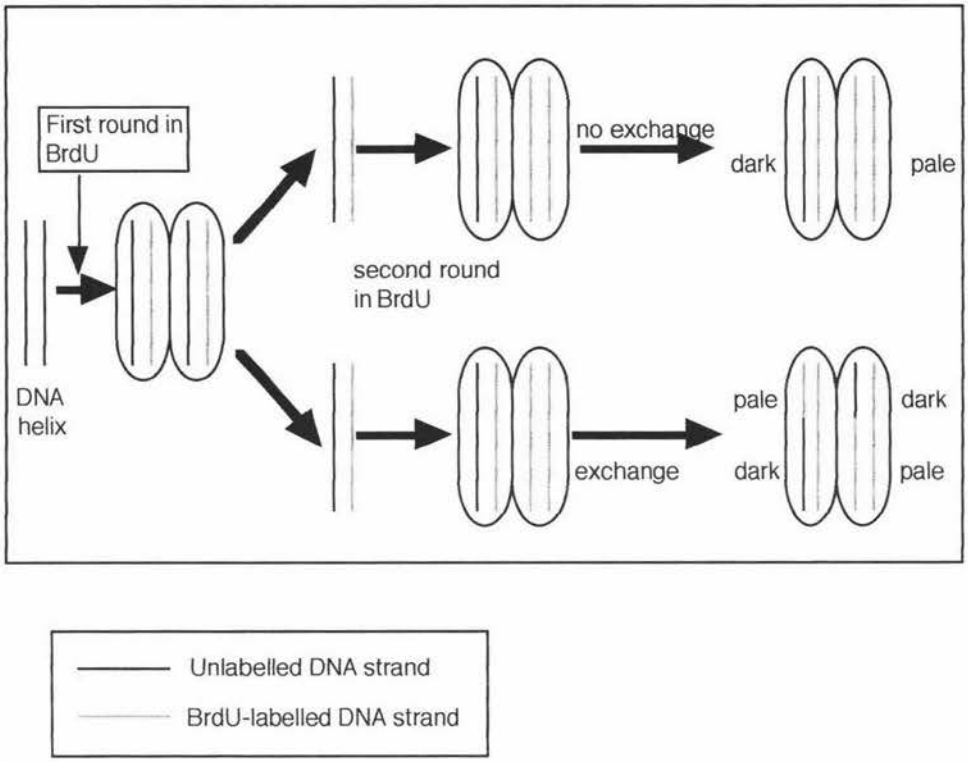


Figure showing how SCE staining method produces differentially stained chromatids by the use of incorporation of BrdU.

Figure 3.1

Genetic Basis

SCEs are now known to occur as a normal feature of cell division in mammalian tissues (Tice etal 1976; Tucker et al 1986). SCE studies have been employed extensively because the technique is reliable and relatively simple, the results provide a sensitive indication of exposure, and because sample acquisition is usually easy (Tucker etal 1986). SCEs are now widely believed to represent the interchange of DNA replication products at apparently homologous loci, and involve DNA breakage and reunion (Latt et al 1981). Although SCEs are readily observed experimentally, the exact mechanism(s) of SCE formation is unknown. It appears that SCEs are produced at or near the replication forks. Most models generally provide a rationale for strand switches relative to the replication fork, but differ in exact details.

Models for SCE formation

Convincing evidence suggests that cells have evolved repair systems to overcome genetic damage induced by environmental agents. The agents that provoke DNA repair also induce SCE, but attempts to correlate SCE with any known DNA repair processes have been unsuccessful. Sasaki (1982) discusses the relationship between SCE and cellular DNA repair and reports that all available evidence indicates that SCE might be a chromosomal manifestation of a damage-tolerating mechanism yet unidentified in molecular terms but operating when the damage is carried out during DNA replication. In general, SCE reflects the amount of damage which has occurred through the total period of DNA replication, and therefore, the cell's repair function for different types of damage may well be of importance in determining the SCE response of cells to mutagens. However, studies in repair-deficient human diseases suggest that the factors which are responsible for SCE induction do not represent a simple function of the total amount of damage subjected to replication bypass repair, but are also related to the type, nature, and conformational state of the damage, which will vary according to the causative agent and also during the course of repair (Sasaki 1982).

The interaction of chemicals and radiation with DNA, results in a variety of DNA damage and interferes with DNA function by altering the interaction of damaged sites with enzymes as in the case of base damage, or by DNA topological changes as in the case of chain breaks and interstrand or protein-DNA crosslinks. These damages can result in cytotoxicity, clastogenesis, mutagenesis, and carcinogenesis. But cells appear to have excellent systems to cope with the damage, such as those associated with the repair, replication, and recombination of DNA (Sasaki 1982).

The rejoining of DNA strand breaks by means of ionising radiation, photo-reactivation, excision repair, and post-replication repair constitutes several major classes of repair systems.

- Photoreactivation is specific for pyrimidine dimers induced by ultravioted light. It is found in most prokaryotes and lower eukaryotes

and shows declining efficiency from birds, amphibia, and marsupials to placental mammals.

- Excision repair is the most universal system of DNA repair, and represents a complex pathway involving many enzymes with various degrees of specificity to DNA damage.

Despite its error free nature, excision repair in mammalian cells is often a slow process, sometimes incomplete before the cells enter the DNA replication (S) phase. The damage, thus, circumvents the excision repair system, leading to chromosome damage and, hence, cell death. However, the cells have evolved another type of repair or damage tolerating process, which can function in conjunction with the process of semiconservative DNA relication. It is called post-replication repair, and its function is reflected in the ability of the cell to tolerate the DNA damage when it is carried over into the replication process. Although it is the least understood, the process is thought to be the one most probably linked to mutagenesis and carcinogenesis (Sasaki 1982).

In order to assign a possible role for these DNA repair mechanisms in SCE formation, numerous attempts have been made to establish the relationship experimentally. Xeroderma pigmentosum (XP) is a rare human genetic disease associated with defective DNA repair and has provided many important keys to the understanding of the relation between DNA repair and SCE. The cells from XP patients have a partial or total defect in the ability to excise DNA damage produced by UV and some chemical mutagens. The defect lies in the endonucleolytic incision step of the nucleotide excision repair process. Another class of XP patients, called XP variant, show no defect in excision repair but are defective in the ability to carry out post-replication repair (Lehman et al 1975). Wolff et al (1975) and Kato and Stich (1976) studied the spontaneous level of SCE in cultured skin fibroblasts of these groups of patients and found no significant deviation from normal. Similar results were also obtained in cultured peripheral blood lymphocytes from XP patients. These findings indicate that there is no common pathway between these types of repair processes and SCE formation. However it is now clear that the cells ability to excise DNA damage plays an important role

in the induction of SCE when DNA damage is introduced exogenously. The excision repair defective XP cells have been demonstrated to be abnormally and highly sensitive to the induction of SCE by UV-irradiation (De Weerd-Kastelein et al 1977). Since SCE induction requires the coincident occurrence of DNA damage and replication, it is probable that the unexcised UV-induced damage constitutes an integral component of the high SCE response of XP cells to UV-irradiation (Sasaki 1982).

The close association between DNA replication and SCE suggests that SCE might arise by a replication-mediated process when the DNA damage is carried over to semiconservative replication. These characteristics favour the idea that SCE is a cytological manifestation of a post-replication recombination repair.

All available evidence indicates that for the induction of SCE by mutagens it is necessary for the DNA damage to pass through replication during which SCE can arise as a consequence of a replication-mediated process. This also holds true for the induction of chromosome structural aberrations in cells exposed to UV and chemical mutagens. Therefore, the efficiency of the damage in inducing SCE, as well as chromosome aberrations, must be dependent on the type of damage and the cell's capacity to repair this damage. The relative efficiency in inducing SCE as compared with that for chromosome structural aberrations may largely differ according to the type of DNA damage (Sasaki 1982).

Following are two proposed models for the formation of SCE.

Alternative Replication Bypass Model

Shafer (1982) discusses the possible mechanism of the alternative replication bypass model (RBM). There are different alternative replication bypass mechanisms proposed all of which are based on the hypothesis that different SCE mechanisms may be required for different DNA lesion states and conditions. The principle being that SCEs occur as ongoing replication processes encounter intact or partially repaired DNA lesions. These lesions

arise as the advance of a leading strand of nascent DNA toward a crosslink site creates distortional stress near the crosslink resulting in a parental-strand break or incision. Since the leading nascent strands would approach the crosslink along opposite parental strands, two resultant breaks or incisions would occur contralaterally on opposite parental strands above and below the crosslink. Due to convergent unwinding processes, the free ends produced in the first stage could become terminally aligned irrespective of their opposite polarity. Completion of an SCE could then be accomplished by either of two rejoining processes - terminal ligation of the free ends via nascent Okazaki pieces or heteroduplex complementation by overlapping of the free ends (Figure 3.2). This bypass mechanims would allow replication to continue past a crosslink, leaving it intact, but would also result in a switching of parental strands and their attached incomplete nascent strands above and below the crosslink site, producing an apparent exchange between sister chromatids.

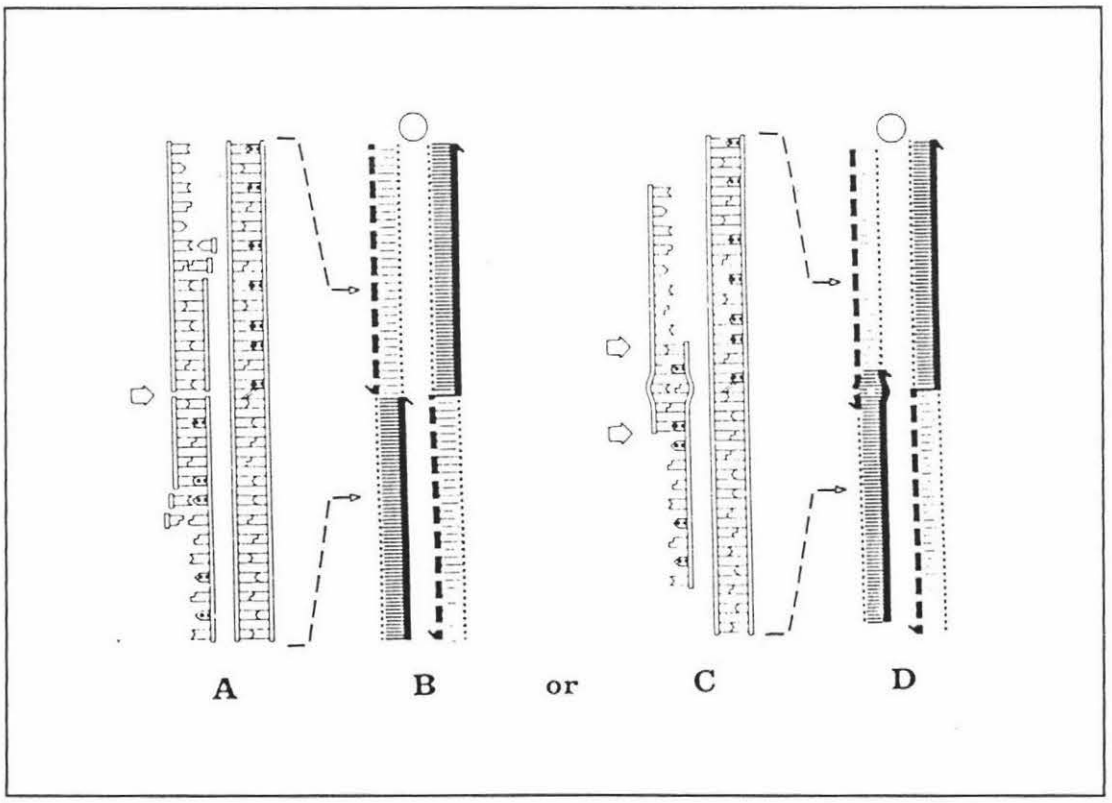


Figure 3.2 Alternate rejoining processes. Rejoining may occur by terminal ligation (A, B) or by heteroduplex complementation (C,D), in which the free "sticky" ends overlap. The later process would occur more readily in repetitive DNA regions and would result in mismatched bases or base loss in one daughter cell in the next generation (Shafer 1982).

It is proposed that most SCEs are induced by a few repair intermediates that are common to multiple repair pathways The most prevalent intermediates being either an endonuclease incision in one strand or the pre-incision binding of an endonuclease at the lesion site. Some SCEs may also occur by direct lesions such as DNA crosslinks and single-strand breaks, although the former may be converted to large monoadducts and the latter are usually repaired too rapidly to induce SCEs. It is also proposed that SCE may be indirectly influenced by the metabolic imbalance of nucleotide pools and the inhibition of replication fork progress leading to similar replication bypass SCE processes. The latter mechanisms can largely account for "spontaneous" SCE levels. Several hypothetical mechanisms are proposed by which various single-strand lesions result in two parental-strand incisions and thus replication bypass-induced SCEs. Overall, the proposed mechanisms suggest that SCEs may be error-prone processes but may occur with far less frequency than is suggested by background, so-called "spontaneous," SCE levels. These mechanisms also suggest that SCE processes can result in the fixation of mutagenic coding alterations and may also participate in the oncogenic transformation of cells by genetic inactivation or alteration of negative repressors for oncogenes or "growth" genes.

A common thread to the mechanisms is not the outcome - the SCE effect - but their association as a cellular bypass process for overcoming various lesions or their repair intermediated that disrupt normal DNA replication.

Main points of RBM - cross-links and SCE induction:

- Mechanism by which SCEs are produced due to a series of sequenctial events which might occur as bidirectional replication encountered a crosslink
- Considered the primary mechanism by which SCEs occur
- Other non-xlinking lesions could also induce SCEs e.g. single strand lesions may mimic RBM for cross-links, by inducing alternate lesions on both strands and recieving similar parental-strand exchange during replicaiton.

Several lines of evidence support three principle conclusions relevant to the general replication bypass model and to the specific crosslink induction mechanism:

- Crosslinks appear to be a potent SCE induction lesions though other lesions also induce SCES
- SCE induction is linked to the bypass of DNA lesions at the replication forks.
- SCE induction is additionally related to the repair capacity of the cells and/or the rate and mechanism of repair for different types of lesions.

Therefore, the completion of lesions repair prior to the arrival of replication forks will prevent SCE induction, while the retention of lesions up to that same point will induce an SCE bypass. If these conclusions are correct, there may indeed be a number of replication bypass mechanisms specific for different types of lesions in much the same manner as there are multiple mechanisms of DNA repair. It might then be unlikely that any global SCE mechanism can be decsribed. Rather,SCE could reflect a category of several replication bypass processes that may use different enzymes and different patterns of strand transfer and, therefore, may also have different genetic consequences (Figure 3.3 and Figure 3.4).

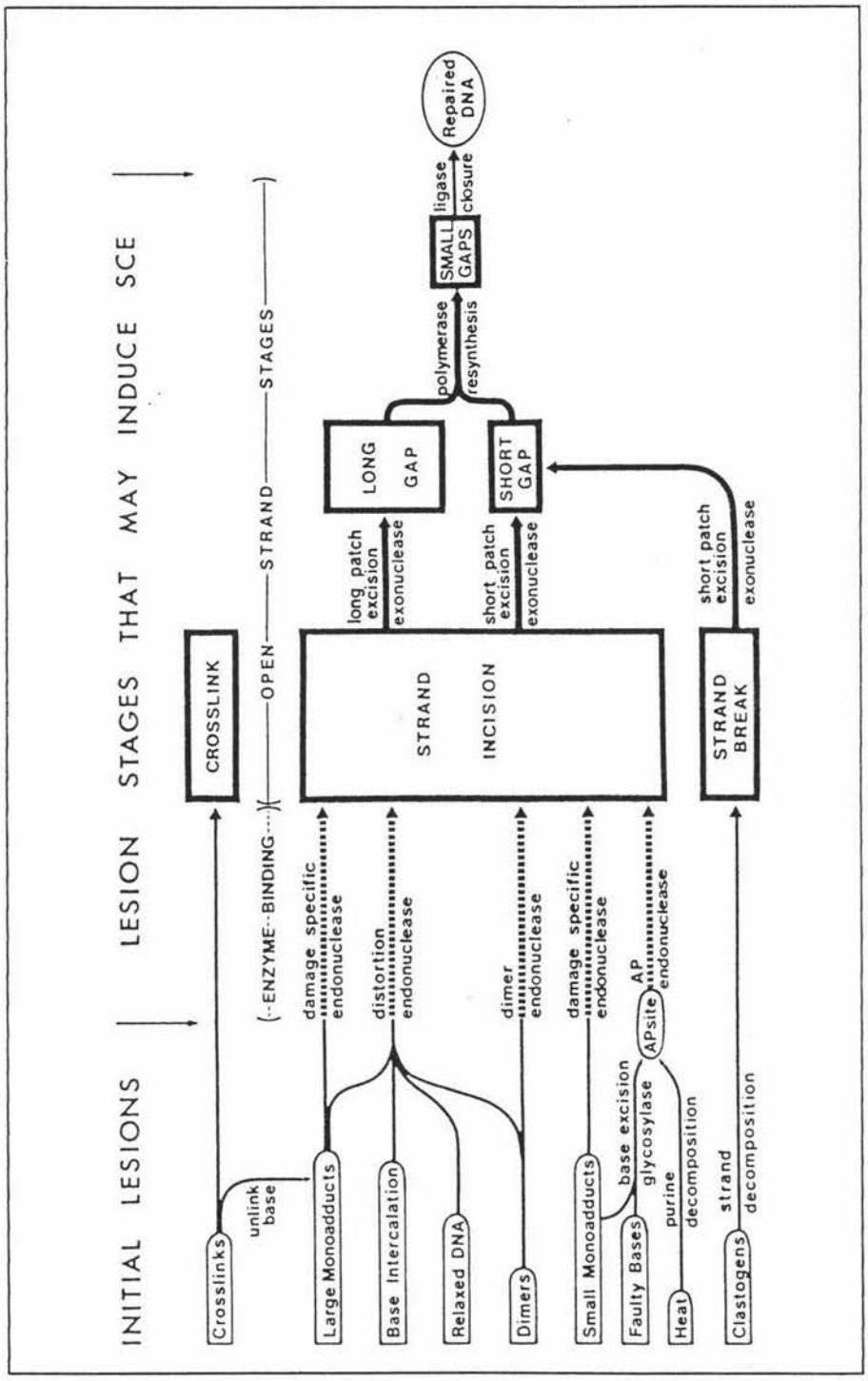


Figure 3.3. Multiple lesion pathways leading to a small number of SCE-inducing lesion states. Some lesions such as crosslinks and strand breaks may cause SCE directly, thought the latter lesion type has only a short time span. Most other lesions are converted by a variety of repair processes to a similar strand incision stage and subsequent repair intermediate stages that may induce SCE by a single or several SCE mechanisms. Prior to completion of repair any one or more of these open-strand stages may induce SCEs. Alternatively, SCE induction with such lesions may be caused if replication encounters a lesion in the process of endonuclease binding (Shafer 1982).

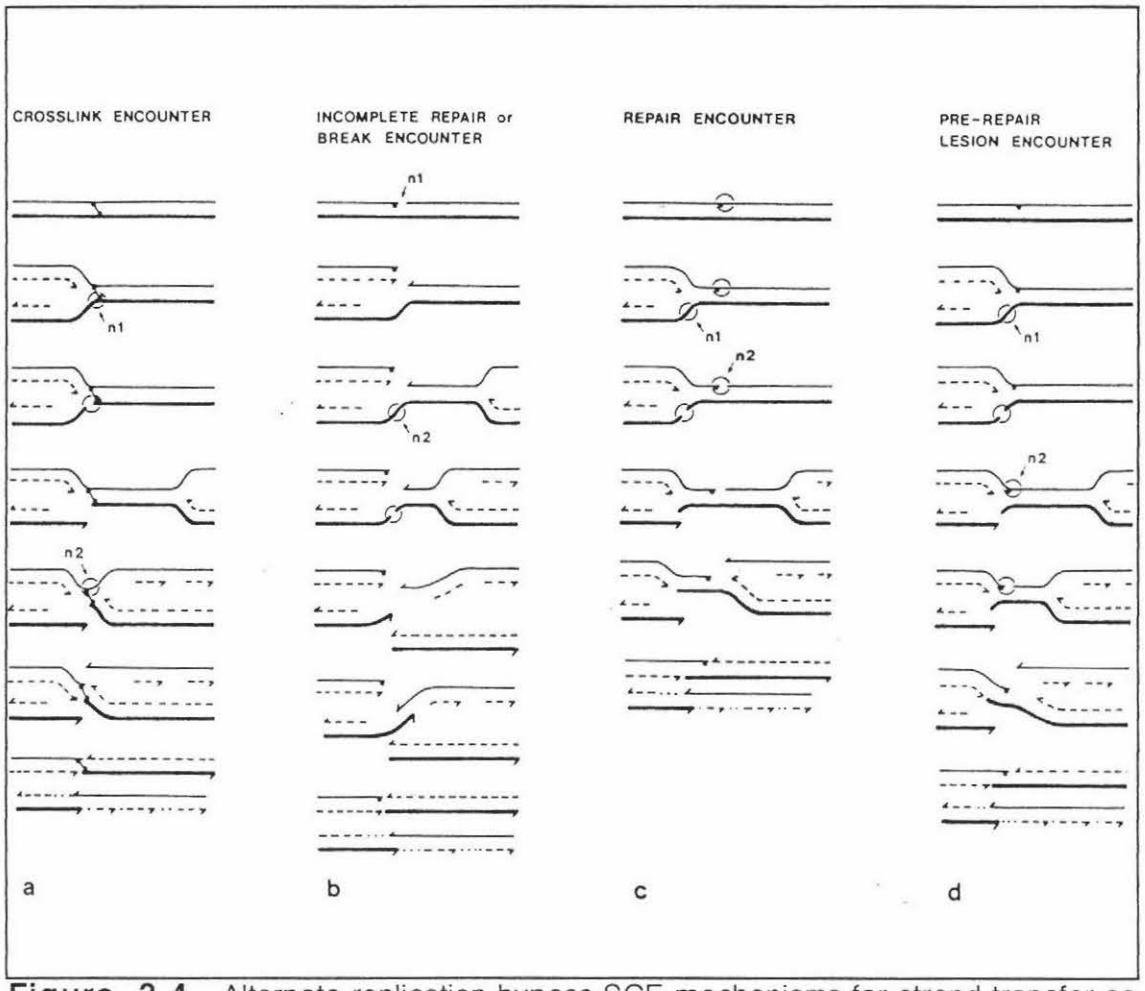


Figure 3.4. Alternate replication bypass SCE mechanisms for strand transfer as described in the text. Only important stages are depicted and thus the time courses of the different mechanisms are not comparative. The sites of first- and second-strand incisions or nicks are indicated by n1 and n2. The globular objects indicate the different repair endonucleases. The crosslink induction mechanism (a) indicates incisions by damage-specific endonucleases adjacent to the lesions, but it could also occur by incisions from single-strand-recognizing endonucleases a few bases further form the lesion. The incomplete repair or strand break mechanism (b) is initiated by an open parental strand lesion arising by multiple pathways as indicated in figure 1. Rejoining by this mechanism could be faulty, leading to breaks rather than SCEs. The repair encounter (c) and pre-repair encounter (d) mechanisms are somewhat similar. In the first case the enzyme binding itself may inhibit fork progress (perhaps inhibiting the progress of topoisomerase unwinding enzymes preceding the replication fork). In the second case, the lesion itself may inhibit or block fork progress, also leading to secondary incisions and SCEs. Neither of these last two mechanisms are as prone to break induction, though the time constraints for SCE induction maybe more critical. In all four mechanisms, the secondary rejoining of free ends is ambiguously depicted as either heteroduplex overlapping or terminal ligation (Shafer 1982).

Painter's Replication Model for SCE

Painter's model (Painter 1980) is based on the idea that double-strand breaks are generated at junctions between a completely duplicated replicon cluster. Painter proposed that specific supercoiled subunits of the chromosome, which are physically separated from one another, somehow allow the DNA in the region to be susceptible to double-strand break formation during replication of the adjoining clusters.

Damage to the DNA that involves slowing or stopping of growing points disrupts the timing of the program for replication of contiguous clusters so that the DNA in junctions remains unreplicated for a long time. This increases the probability of double-strand breaks after one cluster has finished replication (Figure 3.5).

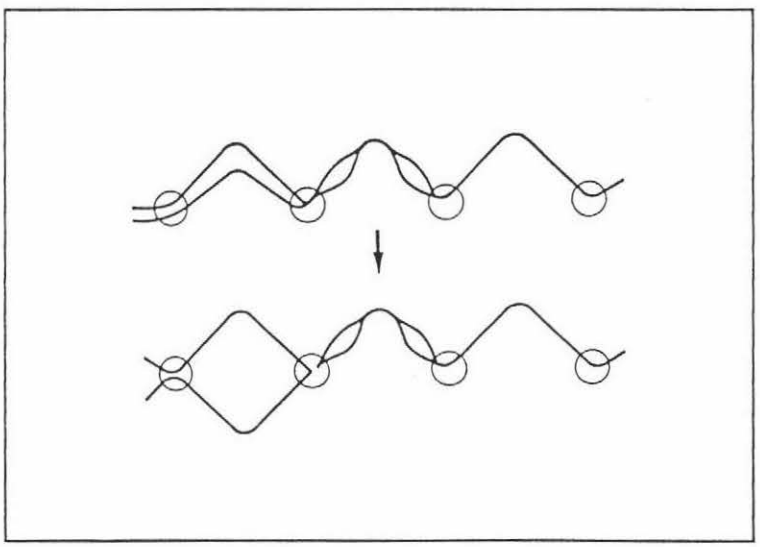


Figure 3.5. Model for possible effects of DNA-damaging agents on cluster replication and segregation. An array of these clusters is depicted; the one on the left is completely replicated and the one on the right is unreplicated. In the centre, damage has blocked fork progression so that the cluster is only partially replicated. Lines represent the super-supercoiled Watson-Crick DNA double helices. Junctions where RNA and/or proteins maintain the separate domains of clusters are indicated by circles. In the lower half of the diagram, segregation in the completely replicated cluster is accompanied by a double-strand break at the junction between this cluster and the partially replicated cluster (Painter 1980).

If this happens, both a daughter and a parental strand of each polarity would be available for ligation with the DNA of the unreplicated adjacent cluster (Figure 3.6) and there would be a finite probability that the daughter strands, rather than the parental ones, will join with the unreplicated strands. When replication of the other cluster finishes, the normal ligation process at the junction will complete the exchange.

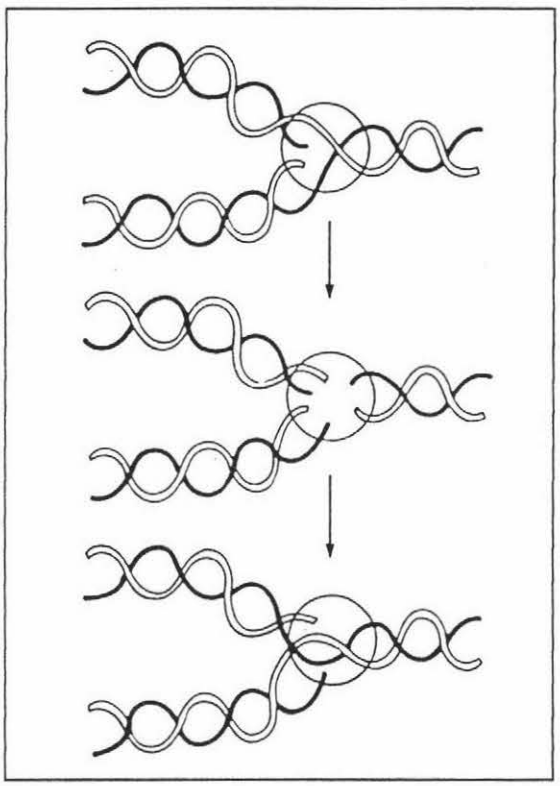


Figure 3.6. Double-strand recombination at the junction between replication clusters. The exchange shown in the bottom diagram is between the newly formed strands of the replicated cluster and the parental strands of the unreplicated cluster. The system responsible for joining DNA at the junction will later complete the SCE (Painter 1980).

This hypothesis may also explain the high number of spontaneous SCEs observed in cells from individuals with Bloom's syndrome. The average rate of DNA-fork displacement is about 30% slower in these cells than in normal cells. An examination of their data show that this average decrease is due to a large fraction of replicons whose fork-displacement rate is about two-thirds that of normal cells and a smaller fraction whose average fork-displacement rate is the same as in normal cells. In Bloom's syndrome cells, partially

defective polymerase or another component of DNA replication may exist in a fraction of the clusters that are in operation at any one time; the rate of chain elongation in these clusters would be slower than in nearby clusters containing normal components. The slowly replicating clusters would not finish their duplication at the same time as adjacent clusters, resulting in the same effects seen in mutagen-damaged cells.

The hypothesis Painter suggests does not implicate any repair process in the formation of SCEs; the only role for repair is to reduce the number of lesions before the damaged DNA replicates and thereby to lower the frequency of SCEs.

Role of Topoisomerases

There is a possible role of topoisomerases in the induction and rejoining of DNA strand breaks which may lead to SCE formation (Liu *et al* 1980; Dillehay *et al* 1989). Cleaver (1981) found that SCE frequency depends on the size of the replicon, i.e. there is increased SCE formation with larger replicons. It may be that small replicons, which would be easier for topoisomerases I and II to unravel, replicate their DNA into separate daughter helices faster, which would present fewer opportunities for error and SCE formation.

Several more recent studies support the role of topoisomerases in SCE formation. Topoisomerase I and or II may be involved in breaking the DNA strands at points of supercoil stress near replication forks, and, therefore, may be involved in SCE formation (Pommier *et al* 1985; Holden *et al* 1989; Dillehay *et al* 1989). The rejoining of the DNA strands after replication may either restore the original order of the strands or result in a spontaneous SCE if the original ends are switched upon rejoining. DNA damage by chemicals would presumably increase SCE formation by blocking movement of the replication fork, this might result in a greater chance of error when corrective measures were taken to replicate past the damaged portion of the DNA strand. Novobiocon, which inhibits the activity of topoisomerase II, has been shown to decrease SCE formation following treatment with the clastogen

mAMSA (Dillehay et al., 1987). This suggests that the action of topoisomerases during replication may be causally related to the formation of SCEs.

Location of SCE

Some attempts have been made to localise SCE to specific chromosome bands (Schubert and Reiger 1981). SCE appear to occur preferentially at the junctions of Q-positive and Q-negative regions or in between Q-negative bands in human chromosomes (Schuler and Latt 1978). SCEs have been reported to occur preferentially in heterochromatic regions of human chromosomes (Schnedl *etal* 1976), as well as in clusters at the junctions between heterochromatic and euchromatic regions in several rodent species (Bostock and Christie 1976; Hsu and Pathak 1976).

Genetic and environmental influences on Baseline SCE

age

The effects of donor age on baseline SCE have been investigated by several groups and the results are as yet still unclear. Galloway and Evans (1981) found no difference in SCE between new borns and seven adults ranging in age from 26-35 years. Morgan and Crossen (1977), in an extensive study encompassing newborns to 85-year-old individuals, likewise found that donor age did not affect baseline SCE. Hedner *et al* (1982) also found no relationship between SCE and age, although they did find that older individuals have significantly more structural aberrations than younger individuals. Bender *et al* (1989) found a statistically significant increase in raw mean SCE frequencies with age but when cigarette smoking status was taken into account, there was no significant age relationship. However, Funes-Craviota *et al* (1977) found that a control group of children (mean age 2.4 years) had a lower SCE rate than a control group of adults (mean age 23.5 years). Furthermore, Ardiot *et al* (1980) and Seshardri (1982) found that infants had a lower SCE rate than their mothers. Husum *et al* (1986) also found that age contributed to a small but significant, 17% of the inter-personal

variation among females and males and therefore should not be disregarded when planning studies using the SCE test. They also suggest that because SCEs would only be expected to increase 0.17 SCE/cell per ten years of age, other more important sources of variation may have "drowned" the influence of age *per se* in the previous negative studies. Many other groups have had similar results with a statistically significant increase in SCE due to age.

Gender

Results of most studies on SCE in males and females have been consistent, with no group demonstrating a difference. Although Hedner *et al* (1982) did find a significant difference between the sexes, in that both chromosomal aberrations and SCEs were higher in females. Hedner *et al* concluded that any sex difference observed can only be speculated upon at present, e.g. an increased rate of SCE has been reported in oral contraceptive users (Bala Krishna Murphy *et al* 1979). Bender *et al* (1992a) also found that females have a slightly elevated mean SCE frequency which is believed to be a consequence of the increased chromosome length (DNA content) represented by the second X-chromosome in females versus the smaller Y-chromosome in males and the consequently larger 'target' size.

Culture Media

Various culture media are used for culturing lymphocytes, and these have a small but significant effect on baseline SCE. Lymphocytes cultured in McCoy's 5A medium had an SCE rate significantly lower than those grown in T.C. 199. The amount of thymidine in the various media may be partly responsible for the variation, since it would compete with the BrdU leading to a lower SCE rate in these cultures. However, the amount of thymidine in the medium in the medium cannot be the entire explanation for the lower SCE rate because there was found to be no difference between Ham's F10, which does contain thymidine, and RPMI 1640 and Dulbecco's MEM, which does not (Morgan and Crossen,1981). The culture medium can also affect lymphocyte proliferation as Obe *et al* (1975) found that metaphases occur

earlier and in greater numbers in Ham's F10 than TC199.

Serum

SCE frequency can also be influenced by the serum used to supplement the culture medium. Lymphocytes cultured in autologous plasma are reported to show a lower SCE rate than those cultured in fetal calf serum from an unspecified source or non-heat-inactivated human AB serum (Gosh *et al* 1979).

State of Health of Donor

The role of viral infections and vaccination in influencing baseline SCE in human lymphocytes is uncertain. Patients with herpes simplex, infectious hepatitis, and unspecified cold and infulenza viral infections showed a significantly increased SCE when compared with normal controls (Kurvink *et al* 1978). An increased frequency of SCE was reported in ten women 7 days after small pox vaccination (Knuutila *et al* 1978). Measles vaccination has a remarkable effect on SCE. Two weeks after vaccination two subjects showed a marked decrease in SCE, which coincided with the clinical vaccination symptoms (Lambert *et al* 1979). Surprisingly, by 1 month the SCE level had risen to 25% greater than the prevaccination level. A third subject showed a 50% increase in SCE both at 2 weeks and at 1 month following vaccination. The significance of these most interesting findings remains to be determined.

Time

Several studies have investigated changes in the SCE frequency as a function of time in normal healthy individuals. Carrano *et al* (1980) found that SCE frequencies varied from 11% to 23% in 3 donors examined for up to 6 months. In another study of 5 donors examined over a 3-year period (Carrano 1982), differences were found ranging from 24% to 61%. Tucker *et al* (1987) quantified the amount of variation in the SCE frequency on both a daily and twice-weekly basis. This indicates that differences in the SCE frequency increases as the time between samples increases. The data also

suggest an association with the menstrual cycle, but most variation could not be explained (Tucker *et al* 1988).

White Blood Cell Count

Bender *et al* (1992b) found that the total lymphocyte inoculum in the cultures contributed to the overall variance in SCEs. As the number of lymphocytes increased the number of SCEs decreased. It is therefore necessary to standardize the lymphocyte concentration when carrying out SCE experiments.

Coffee Drinking Status

Hirsch *et al* (1992) showed that coffee drinking status has a significant effect on SCE rates. A step wise multiple regression analysis showed that together, smoking and coffee drinking status entered at the first step accounted for 21% of the observed variance in SCE. Their twin analysis showed that after adjustment of the data set for smoking and other significant predictors, genetic factors accounted for approximately 30% of the variation in SCE rates.

Dietary Habits

A study by Wulf *et al* (1985) on members of the Seventh-day Adventist Church, who are ovolacto-vegetarians, non smokers and do not drink coffee, tea or alcohol, were found to have significantly lower SCE rates than matched controls (5.54 ± 0.07 SCE/cell vs. 8.00 ± 0.15 SCE/cell), indicating that dietary habits might be an important source of inter-personal variation in SCE rates.

Reproductive Hormones and Biological Rhythms

In 1993, Taubes wrote an article in the Science magazine attacking a study of Canadian smokers, which was published in the American Journal of Epidemiology, which stated that smoking may be two or three times more

hazardous for women than for men. Although Richard Peto, co-director of the cancer studies unit of Oxford University believes that "it's simply not true that females have bigger risks of smoking than males." Harvey Risch, a biomathemetician at Yale University found that women had a risk 27.9 times as great as non smoking women. In contrast, the risk for men who smoked regularly was only 9.6 times greater than that for male non smokers (Taubes 1993).

Different frequencies of SCEs during various stages of the menstrual cycle have previously been observed. Joseph-Lerner *et al* (1993) looked at women on hormone treatment i.e. ovulation induction for *in vitro* fertilisation treatment. They found an increased SCE frequency around ovulation time in the controls and around the time of human chorionic gonadotropin administration in the group undergoing ovulation induction. However in the latter group, SCE frequency was significantly higher. SCE frequency was positively correlated with the level of testosterone and FSH in the ovulation induction group, and positively correlated with the estradiol level in both levels.

D'Souza *et al* (1988) observed a higher rate of cellular genetic damage in terms of increased frequencies of SCEs and chromosome aberrations (including gaps, breaks, and deletions) during "ovulatory" and "estrogenic" stages as compared with those of "progestogenic" stage of the menstrual cycle in women and the reported fluctuations in SCEs as a function of time in men. Indicating that chronobiologic considerations are essential in the design of studies of humans and animal experiments. It seems that female subjects are more influenced by biologic rhythms because of specific hormonal cycles and are sensitive to genetic damage during ovulatory/estrogenic stages of the menstrual cycle.

Recently, an enhanced frequency of SCEs and chromosome aberrations has also been reported in women during advanced stages of pregnancy, and this has been attributed to the high levels of sex steroids present during the last trimester of pregnancy (Sharma and Das 1986).

Bromodeoxyuridine (BrdU) Concentration

The ability of BrdU to induce SCE has been extensively studied in a number of different cellular systems (Schvartzman and Tice 1982). Although it is not clear whether all of the SCEs observed at low BrdU levels are induced or spontaneous, most of the SCEs observed at high levels of BrdU concentration are clearly BrdU-dependent events. Mazrimas and Stetka (1978) determined that a linear relationship existed between the extent of BrdU incorporated into DNA (as BrdU/base pair) and SCE frequency. Schvartsman *et al* (1979) after examining the ratio of non-symmetrical to symmetrical SCE in third generation metaphase cells after differing periods of BrdU-exposure, also concluded that the frequency of SCE was closely related to the degree of BrdU substitution. However, it appears that SCE can be induced by BrdU independently of its incorporation into DNA as well. Davidson *et al* (1980) and San Sebastial *et al* (1980) have both shown that, at fixed levels of BrdU-substitution, increasing the concentration of BrdU in the medium significantly contributes to the induction of SCE.

Strengths of SCE Analysis

(Latt *et al* 1981)

- It is excellent for detecting compounds e.g. alkylating agents such as ethyl methanesulfonate and mitomycin C, that produce DNA adducts.
- It is extremely sensitive as it can detect SCEs at far lower concentration of compounds than are needed to produce ordinary chromosome aberrations.
- It can detect both direct-acting compounds and those that require metabolic activation.
- It is a relatively easy and rapid method (especially with *in vitro* systems).
- It is a mammalian test system that can be used both for *in vitro* and *in vivo* studies, the latter permitting analysis of multiple tissues, including germ cells.
- The assay can be used in a variety of non-mammalian organisms, e.g. for testing environmental pollutants in special ecological situations (in both plants and animals).

- The assay is also applicable to human population monitoring.
- A positive result for SCE inducation generally indicates that a compound is a mutagenic carcinogen, i.e. the test gives few false positive results.

Weaknesses of SCE Analysis

- It is relatively insensitive to compounds (e.g. bleomycin), that are capable of inducing double-strand DNA breaks.
- The mechanism of SCE formation is unknown. SCEs may represent a "signature" that DNA damage has occurred but may reflect a very small proportion of that damage. This proportion may vary greatly among agents. Information about this variation is needed.
- Related to above, a positive SCE response has greater significance than does a negative response.
- It might conceivably be insensitive to carcinogens requiring repeated chronic exposure unless the test is modified (e.g. to involve SCE analysis after multiple exposures).
- Inadequate activation of substance reactivity before reaching the target tissue may give a false negative response. Subtle variation in the assay system might thereby produce conflicting results in different analyses of effects due to a particular compound (e.g. diethylstilbestrol).
- Numbers of non-mutagenic carcinogens may be negative for SCE induction e.g. diethylstilbestrol in some but not all systems; the test is subject to false negatives.
- *In vivo* systems posses excellent possibilities for metabolic activation of compounds. However, there is also the possibility that a highly reactive compound will dissipate its effectiveness before reaching the tissue selected for analysis.

Chromosome Aberrations and SCEs

Hedner *et al* (1982) looked at SCEs and chromosome aberrations in 100 individuals. They found no correlation between SCE frequency and the frequency of the different structural chromosome aberrations. Only gross

genetic damage is detectable by looking at chromosome aberrations, and the absence of chromosome aberrations does not exclude other types of DNA damage caused by environmental genotoxicants. Whereas SCE is potentially a more sensitive and rapid indicator of DNA damage than conventional chromosome aberrations.

Major limitations of chromosome aberratoin assay is that a high degree of skill is required on the part of the scorer, and inter-scorer and inter-laboratory variations in scoring criteria. Also, the method is laborious and time-consuming, and the capacity for extensive analyses in existing laboratories is limited.

It appears that mechanisms leading to the formation of SCE are heterogeneous and fundamentally different from those that cause structural chromosome aberrations (Gerbhart 1981). For example, agents that produce severe chromosome breakage, such as X-rays, have little or no effect on SCE frequency (Perry and Evans 1975). Also, among patients with hereditary chromosome instability disorders, such as ataxia telangiectasia, Bloom's syndrome and Fanconi's anemia, characterised by a markedly increased frequency of spontaneous chromosome aberrations, only patients with Bloom's syndrome display an increase in SCE (Changanti *et al* 1974) while patients with ataxia telangiectasia and Fanconi's anemia have a normal frequency (Galloway and Evans 1975; Latt *et al* 1975).

3.4.4 Microgel Electrophoresis Assay (Comet test)

Microscopic examination of individual human lymphocytes embedded in agarose, subjected to electrophoresis and stained with a fluorescent DNA-binding dye, can be used to measure DNA damage as an extent of migration of DNA fragments, mainly single-strand breaks. It requires only a small number of cells and the results can be obtained in a relatively short time, i.e. a few hours. Moreover the tail length of the comet is positively correlated with the amount of DNA breakage in a cell, particularly at low dose exposure (Betti 1994).

Betti (1994) found that the extent of DNA migration was found to be significantly increased by smoking and that the effect of smoking was more significant in men than women and that DNA migration was similar in the young and in the older people. SCE analysis did not reveal any significant effect of smoking, sex or age in the same population, suggesting a higher responsiveness of the comet test to DNA-damaging agents.

3.4.5 A modified immunochemical assay

Exposure to physical or chemical agents can induce a large variety of lesions in the DNA, including single- and double-strand breaks, and damage to bases and sugar residues not leading to a break. It is important to be able to quantify the different types of DNA damage in order to get information about their persistence, which may provide more insight into the importance of the various lesions for cell death and/or mutation induction. The most abundant lesions induced in DNA by ionizing radiation are the single-strand breaks, whereas the double-strand breaks are thought to be mainly responsible for the cell killing.

Timmerman *et al* (1995) report on an immunochemical assay to detect damage in DNA has been modified to a so-called sandwhich ELISA. With this assay DNA damages can be detected that give rise to a certain level of single-strandedness in DNA of white blood cells during partial unwinding of cellular DNA under alkaline conditions. The modified method is a series of the following steps: incubation of alkali-treated whole blood in the wells of mocrotiter plates precoated with antibody directed against single-stranded DNA (ssDNA), which results in selective binding of ssDNA, and the subsequent detection of bound ssDNA by incubation with anti-ssDNA antibody alkaline phosphatase conjugate. With this method Timmerman *et al* (1995) detected the amound of damage induced by ionizing radiation in DNA in cells of human blood within 1 hour after doses as low as 0.2 Gy. The precoating of microtiter plates with anti-ssDNA antibody enables the detection of ssDNA fragments directly in alkali-treated blood samples, isolation of the nucleated cells from the blood is not necessary. Because the DNA is released somewhat faster from lymphocytes than from granulocytes

upon alkali treatment, it is even possible to discriminate between the effect of the radiation on these cell types in the same blood sample.

The advantages of the method are; that only small amounts of sample, e.g. a few microliters of blood, are required without any (pre)purification; damage can be detected after radiation doses as low as 0.2 Gy, which gives rise to about $5x10^{-11}$ single strand breaks per dalton DNA, which corresponds to about 200 single strand DNA breaks per cell; the assay takes less than 1 hour after collection of blood and can be applied simultanously on a large number of samples; and finally other types of cells that can be obtained in suspension can be assayed.

3.5 Smoking

Two of the most common ways to look at the effects of cigarette smoke is by looking at cigarette smoke condensate (CSC) in an *in vitro* manner or by looking at the organism itself in the presence of cigarette smoke in various test systems. The following is a summary of the effects of CSC and cigarette smoke from various research groups in various test systems.

3.5.1 Chromosome aberrations

In vitro

The first report of the mutagenicity of CSC was by Venema (1959). He used an aqueous emulsion of CSC that had been extracted with ether, which removed most of the polycyclic hydrocarbons. This aqueous fraction of CSC was shown to induce chromosome aberrations in the root-tips of onion (*Allium cepa*). Chromosome lagging, sticky chromosomes, and acentric fragments were observed. In addition, the mitotic cycle was altered in such a way that prophase was decreased, and telophase was increased. Later Leuchtenberger *et al* (1973) showed that there was an increase in DNA content and lagging and breakage of chromosomes in cultured human lung cells that were exposed to cigarette smoke or to the gas phase of cigarette

smoke. In addition, the gas phase of cigarette smoke was shown by Pandey *et al* (1978) to induce chromosome aberrations in the root-tips of garlic.

Nakayama *et al* (1985) found that CSC induces considerable numbers of DNA single stranded breaks (SSB) in cultured human cells, and that such strand breaks may be ascribed to active oxygen generated from cigarette smoke. The active oxygen is generated mostly from polyphenols, such as catechol, its methyl derivatives and hydroquinone, which occur in abundance in cigarette smoke. This is consistent with previous findings that the co-carcinogenic fraction in CSC contains catechol and hydroquinone and that catechol shows potent co-carcinogenic action in benzo (*a*) pyrene carcinogenesis (Van Duuren and Goldschmidt 1976). This may imply a causal significance of SSB in carcinogenesis, because the SSB in DNA were generated by active oxygen from active metabolites of naphthylamines. In these experiments, one cigarette produced approximately 10^4 SSB per cell. Although in the body, most SSB caused by cigarette smoke are expected to be efficiently repaired, some would remain unrepaired and the accumulation of such SSB over a long period may have serious consequences, especially for heavy smokers.

In vivo

Rees *et al* (1973) did not find an increase in chromosomal aberrations in rats injected with CSC. Obe and Herha (1978) found greater frequency of gross chromosomal aberrations in the lymphocyte chromosomes of heavy smokers than in those of non smokers, as did Kier *et al* (1974), who found that lymphocytes from heavy smokers show a significant elevation of exchange-type aberrations of the chromosome (dicentric and ring chromosomes) and chromatid (chromatid interchanges) types. Tawn and Cartmell (1989) found a significant increase in total aberrations in smokers compared to non-smokers. They also found an increase in dicentrics in smokers but this was statistically insignificant.

Kao-Shan *et al* (1987) found that the lymphocytes of smokers demonstrated a significantly higher frequency of fragile sites, an increased number of

metaphases with extensive breakage and elevated expression of fragile sites at the cancer breakpoints 3p14.2, 11q13.3, 22q12.2, and 11p13-p14.2 and the oncogene sites *bcl 1, erb B, erb A, and sis*.

Alternatively, Nordenson *et al* (1978) found that there was no difference in chromosome aberrations between smokers and non-smokers. Although smoking appeared to have a synergistic effect with arsenic, as smokers who were exposed occupationally to arsenic had a greater frequency of chromosome aberrations than did nonsmokers who were not exposed to arsenic.

3.5.2 Sister-chromatid exchange

In vitro

De Raat (1979) has investigated the ability of CSC to induce SCEs in Chinese hamster ovary (CHO) cells. He found that CSC induced a significant increase in SCEs compared to the control. In a similar study, Hopkin and Evans (1979) studied the ability to CSC to induce SCEs in human lymphocytes *in vitro*. They found that equivalent amounts of tar from the three brands induced equal numbers of SCEs. The pooled data show that exposure of a 10 ml culture containing $1x10^6$ lymphocytes to 0.5 mg of condensate results in an SCE frequency two - to threefold that in controls. They found that CSCs, produced by a cigarette-smoking machine, interact with the DNA in human cells *in vitro* and that this interaction can be detected at exposure levels that seem surprisingly small: the SCE frequency was detectably increased by 0.1 mg of condensate and more than doubled by 0.5 mg, these doses representing 1/400th and 1/80th, respectively, of a single high tar cigarette.

Hopkin and Evans (1979) emphasise that although there may be qualitative similarities between the response to tobacco tar of lymphocytes *in vitro* and bronchial epithelium *in vivo*, we cannot know whether the cells respond in the same way or to the same degree. Furthermore, although the SCEs they observed in the chromosomes of exposed cells reflected induced DNA

damage, these may not in themselves be mutational events, although their incidence in mammalian cells in culture was correlated with the incidence of mutations.

In vivo

There have been many *in vivo* studies done on the effects of smoking on SCEs, all with conflicting results. Lambert *et al* (1978), Husum *et al* (1982), Husgafvel-Pursiainen *et al* (1980) are included in the groups that have found a significant effect of smoking on SCE. Tucker *et al* (1988) reported such an observation and also reported that SCE frequencies in smokers do not decline for at least 12 months when smoking is stopped. They also reported a significant seasonal variation in the SCE frequency among smokers. Ghosh and Ghosh (1987) found that cigarette smokers had a mean SCE per cell of 8.15 ± 1.62, which is significantly higher that the mean value of 5.48 ± 1.29 found in controls. Higher frequencies of SCE were also observed in individuals who smoked more than 10 cigarettes per day, compared with people who smoked less. Individuals who smoked cigarettes for more than 10 years also showed an increased frequency of SCE as compared with those who smoked cigarettes for less than 10 years. Kao-Shan *et al* (1987) found that in both bone marrow cells and peripheral lymphocytes, there was a significant increase in SCE frequency in smokers with a 5 or more cigarette pack year history, but not those who smoke less than 5 pack years (1 pack of cigarettes smoked per day x number of years smoked; 1 pack = 20 cigarettes). Ozkul *et al* (1995) even looked at a new smokeless tobacco (Maras Powder) which is widely used instead of cigarettes in the South Eastern region of Turkey. They still found that the average SCE per metaphase and total SCEs were significantly increased among both smokeless tobacco users and smokers compared to nonsmokers. However the effect is significantly lower in smokeless tobacco users than in smokers.

Although, there are groups that have reported conflicting results. Hedner *et al* (1983) found no correlation between SCE frequency and smoking habits in their total material. Hollander *et al* (1978) also found no increase in SCEs in the lymphocytes of heavy smokers relative to non-smokers. An analysis of

35 smokers and 85 non-smokers by Crossen and Morgan (1980) also revealed no significant difference between the SCE frequencies of smokers and non-smokers. They also found no difference between heavy (greater than 10 cigarettes per day) and light (less than 10 per day) smokers. Compared to results obtained by Murthy (1979), who demonstrated that adult men who smoked more than 10 cigarettes per day had a greater average SCE frequency than did those who smoked less than 10 cigarettes per day. Furthermore, Murthy (1979) showed that the mean SCE frequency of those who smoked for over 10 years, irrespective of the number of cigarettes, was greater than that of people who had smoked for less than 10 years.

The dose response observed in the studies on SCEs in humans is important in view of the epidemiological evidence for a dose response between the number of cigarettes smoked and the risk of lung cancer and heart disease (DeMarini 1983). However, Lambert *et al* (1978) have shown a dose response for SCEs among moderate (less than 10 cigarettes per day) and heavy smokers (10 or more cigarettes per day). There was a stepwise increase of about 15% in the average frequency of SCEs among moderate smokers compared to non-smokers and among heavy smokers compared to moderate smokers.

The lack of agreement between the SCE studies is, perhaps, due partly to the difficulty of sampling human subjects because of the many uncontrollable variables inherent in such subjects (De Marini 1983).

It has been proposed that smokers are more sensitive than non-smokers (measured by the frequency of SCEs) to *in vitro* challenge by various compounds. Lundgren and Lucier (1985) demonstrated that a-Naphthoflavone (ANF) is a weak inducer of SCEs in human lymphocytes *in vitro* and that lymphocytes from smokers are more sensitive than lymphocytes from non-smokers. They proposed that this may be that lymphocytes from smokers might have an altered metabolic capacity leading to an increased formation and/or retention of genetically-active metabolites.

Salmonella

There have been many studies done on the mutagenicity of CSC and cigarette smoke in Salmonella. Each study varying in the type of CSC used, the type of tissue and inducer used for metabolic activation, and the strains of Salmonella employed (Kier *et al* 1974; Hutton and Hackney 1975; Sato *et al* 1979; Yoshida and Matsumoto 1980; DeMarini 1983).

Kier *et al* (1974) were the first to demonstrate the ability of CSC to induce point mutations. They showed that CSC form nitrate-treated cigarettes contained direct-acting mutagens, i.e., compounds that are mutagenic in the absence of metabolic activator S9. All other mutagenic CSCs tested required metabolic activation.

The CSC from high-charcoal filter cigarettes was shown to be mutagenic, indicating that such filters do not prevent the passage of certain mutagens into the lungs of smokers. Kier *et al* (1974) also demonstrated that CSC is a highly potent mutagen. The CSC from less than one hundredth of one cigarette produced detectable mutagenicity. The results of testing 12 Swain fractions of CSC, (CSC that was fractionated according to the procedure of Swain *et al* (1969)), showed that the basic fractions and some acidic fractions were the most mutagenic, and that the neutral fractions were weakly mutagenic.

Wynder and Hoffmann (1967) and Bock *et al* (1971) have found tumor-promoting activity in acidic fractions of CSC, and Bock et al (1971) have found some slight tumor-promoting activity in the basic fraction of CSC. However, the acidic and basic fractions are the most mutagenic fractions in Salmonella. In addition, several basic fractions, two acidic fractions, and two neutral fractions have been found to have *in vitro* transforming activity (Rhim and Huebner 1973; Benedict *et al* 1975).

Sato *et al* (1977) investigated mutagenicity of CSC from various sections of cigarettes. They found that the mutagenicity was almost the same among condensates collected from either the first, middle or last third of cigarettes.

In addition, the authors showed that the filters on most filter cigarettes reduce the amount of CSC by approximately 12%. However, the specific mutagenic activity of CSCs was almost the same whether the CSCs were from filter or nonfilter cigarettes. Thus, filters do not eliminate much, if any, of the specific mutagenic activity of CSCs. They also investigated the mutagenicity of smoke condensates from cigars and pipe tobacco as well as cigarettes. They found that the specific mutagenicity of cigar tobacco was greater than that of cigarettes, and that of cigarettes was greater than that found for pipe tobacco.

The mutagenicity of CSC in relation to the chemical composition to tobacco leaves has been studied in TA98 by Mizusaki et al (1977a). Among the nitrogenous components examined, the amounts of total nitrogen, protein nitrogen and soluble nitrogen were positively and significantly related to mutagenic potency. However, the amount of nicotene and nitrate were not related to mutagenic potency.

Mizusaki *et al* (1977b) also found that the age of tobacco leaves was shown to have an influence on mutagenic potency. CSC made from old leaves (those that are low on the stalk) was less mutagenic than CSC prepared from young leaves (those high on the stalk). Also, CSC from tobacco with a high sugar content was less mutagenic than CSC from tobacco with a low sugar content. Sato *et al* (1979) then studied the effect of exogenously added sugar to tobacco on the mutagenicity of the CSC prepared from such tobacco. They found that a high sugar content per se in tobacco seems to result in reduced mutagenicity of the CSC. The addition of glucose, fructose, galactose, sorbitol, sucrose or lactose increased the total amounts of CSC per cigarette. However, the mutagenicities of the CSCs were decreased by all of the sugars - the lowest value being 35% (high tar cigarettes) and 36% (low tar cigarettes) of that of CSCs from cigarettes without added sugar. Of the sugars tested, fructose and sorbitol exerted the greatest reduction of CSC mutagenicity. The mechanism by which sugars reduce mutagenicity is not known.

Mizusaki *et al* (1977b) have shown that CSC prepared from cigarettes with high draw resistance is more mutagenic than CSC made from cigarettes with

low draw resistance. This suggests that high draw resistance may provide favourable conditions for the formation of mutagens during tobacco pyrolysis. In addition, these authors demonstrated that the storage of CSC at room temperature for over 24 hours markedly reduced the mutagenicity of CSC. This may indicate that CSC contains 2 types of mutagens - one that is labile and another that is stable.

Various eukaryotic tests

Yeasts

Gairola (1982) exposed *Saccharomyces cerevisiae* D7 to fresh cigarette smoke and found that fresh cigarette smoke induced mitotic gene conversion, reverse mutation, and reciprocal mitotic recombination. The author also noted that the nicotine content of the cigarette did not affect the genetic activity of the smoke, and that the smoke from a lettuce cigarette also possessed genetic activity.

Neurospora

CSC has been shown to be mutagenic in a forward-mutation test at the *adenine-3* region in resting conidia of *Neurospora crassa* in the presence or absence of S9 (DeMarini 1981b). The mutagenic potency of CSC was similar in a repair-sufficient and a nucleotide excision repair-deficient strain of *N. crassa* and CSC killed the conidia by a cytoplasmic, rather than by a nuclear mechanism. In addition, direct-acting mutagenic activity was found in an enriched polycyclic aromatic hydrocarbon fraction (EPAH) and a basic fraction of CSC made from the University of Kentucky Reference Cigarette 1R1 (DeMarini 1981a).

Drosophila

The ability of cigarette smoke and CSC to induce sex-linked recessive lethals (SLRL) in *Drosophila melanogaster* was investigated by Pescitelli (1979). Wildtype (Oregon-R) males were exposed to the agent and mated to *Basc*

females, and the frequencies of SLRL were determined the the F_2. In 2 experiments, males that were fed CSC equivalent to six-hundredths of one cigarette per 10 ml of food as larvae had a significantly higher frequency of SLRL than did the control flies. The same was true for larvae that were exposed to 2-3 puffs of cigarette smoke per day for 7 days. However, treatment of adult males with either cigarette smoke or CSC did not result in a significant increase of SLRL over that of the control.

Urine

Yamasaki and Ames (1977) used XAD-2 resin to concentrate some of the nonpolar molecules, and glucuronide and sulphate conjugates of nonpolar molecules, that may be present in the urine of smokers and non-smokers. Using this procedure, the authors were able to add the equivalent of 25ml of urine per petri plate. At this concentration, all but 2 of 37 urine samples from nonsmokers were not mutagenic in strain TA1538. All 7 of the smokers examined who inhaled and smoked ordinary cigarettes had mutagenic urine. However, smokers who did not inhale did not have mutagenic urine. Interestingly, all of the urine concentrates that were mutagenic required the addition of Aroclor-1254-induced rat-liver S9. Also, urine collected in the evening was more mutagenic than that collected in the morning. This suggests, perhaps, that mutagenic metabolites present in the urine are absorbed by the body during the night and, thus, are no longer present in the urine. However, another explanation could be that the mutagenic metabolites resulting from cigarette smoke appear in the urine in a relatively short period of time. Thus most metabolites would be voided upon retiring in the evening. Because no further exposure to cigarette smoke occurs while the individuals sleep, the morning urine is relatively less mutagenic than the evening urine. Kinetic studies need to clarify this issue (Yamasaki and Ames 1977).

Guerrero *et al* (1979) investigated the ability of urine from smokers and nonsmokers to induce SCEs. Following a 48 hour incubation in 5-20% urine from smokers, the frequency of SCEs in human diploid (WI-38) cells was significantly higher than that from cells incubated in urine from non-smokers.

DNA repair

Human DNA repair-deficient diseases such as xeroderma pigmentosum confer an increased risk for certain types of cancer upon affected individuals. Thus, the effects, if any, of cigarette smoke on DNA repair could have important consequences for cancer induction by cigarette smoke (De Marini 1983). Rasmussen *et al* (1981) studied DNA repair in lungs of mice chronically exposed to cigarette smoke. Freshly-excised lung was treated with methyl methanesulfonate (MMS), and DNA repair was quantified by measuring [^{3}H]thymidine incorporation while inhibiting replicative DNA synthesis with hydroxyurea. After 1-12 weeks of exposure to unfiltered smoke, a 50% decrease in DNA repair activity occurred, and persisted even after smoke exposure ceased. When [^{14}C]MMS was injected intracheally, the specific radioactivity of total lung DNA after 24 hours *in vivo* decreased more than 50% in control lung while remaining unchanged in smoke-exposed lung. These results suggest that alkylated DNA was less subject to repair in lungs of smoke-exposed mice relative to control mice. Thus cigarette smoke reduced DNA repair.

Sperm Morphology

Evans *et al* (1981) showed that there were significantly more sperm abnormalities among 43 nonsmokers. However, a study of 75 smokers and 74 non-smokers by Godfrey (1981) revealed no significant differences in sperm morphology. This topic obviously requires further investigation.

Teratogenesis (effects on foetus)

Adverse foetal effects of maternal smoking have been extensively documented including intrauterine growth retardation, an increase in perinatal mortality and premature births (National Research Council 1986; Surgeon General 1986). The risk of these effects is further increased if the mother is 35 years old or older (Cnattingius *et al* 1988).

Amniotic fluid has a rapid turnover, is swallowed by the foetus from early pregnancy and contains foetal urine. Thus it may, in theory, serve as an indicator of foetal exposure to the environmental agents which are excreted via the urinary tract. This is supported by the present results and some earlier reports, in which biochemical measurements of amniotic fluid of smoking mothers demonstrate the transplacental passage of cigarette smoke constituents (VanVunakis *et al* 1974; Andresen *et al* 1982; Lambert *et al* 1982). Direct exposure of the foetus by maternal smoking is further supported by analyses of foetal blood (Bottoms *et al* 1982) and the umbilical cord of infants (Husgafvel-Pursianinen *et al* 1988). Cotinine is considered the most reliable indicator of tobacco smoke. The half-life of nicotine in the body is short (1-2H), compared with cotinine (20 h), which makes cotinine more suitable for assessing chronic exposure. Lähdetie *et al* (1993) studied the effect of maternal smoking in cotinine levels and showed a dose dependent increase of cotinine concentration in second trimester amniotic fluid with smoking.

Lähdetie *et al* (1993) also showed a correlation between cotinine concentration and SCE induction, suggesting that both indicate exposure to cigarette smoke. Measurement of cotinine concentration is useful in verifying the smoking status since, due to antismoking attitudes, there is reason to suspect denying under reporting of smoking, as was observed in one case in this study.

It is quite difficult to prove the teratogenic effects of cigarette smoke. There are several experimental observations that indicate that cigarette smoke might have a harmful effect on the foetus (Bridges *et al* 1979; Wynder and Hoffmann 1979). Cole *et al* (1972) proved that smoking by pregnant women substantially raises the carboxyhaemoglobin concentration of fetal blood.

There are numerous epidemiological studies on the relationship between smoking and teratogenesis. Mau and Netter (1974) have shown in a study of 5200 pregnancies that there was a significant increase in perinatal mortality when the fathers smoked more than 10 cigarettes per day. The frequency of still-births was found to increase with heavier paternal smoking habits.

Interestingly, the frequency of major congenital malformations of infants was increased with increased consumption of cigarettes by fathers, but was independent of maternal smoking habits.

Kelsey *et al* (1978) found that women who smoked 20 or more cigarettes daily had a relative risk of 1.6 for congenital malformations in the offspring compared to nonsmokers. This risk was greatest for abnormalities of the digestive system (2.0), heart valves (2.0), and skin (1.9), and for neural tube defects and chromosome abnormalities (1.8).

Although most spontaneous abortions possess chromosomal abnormalities, several studies have found an increased number of karyotypic or morphological anomalies among either the abortuses or live-born children of smokers (Underwood *et al* 1967). In fact, there are data that show an unusually large proportion of normal karyotypes among spontaneous abortions in smokers (Alberman *et al* 1976). Thus, there is some evidence for the direct toxic effects of cigarette smoke. If the excess abortions among cigarette smokers are not malformed or anomalous in other ways, then these foetuses are the first consequence of smoking - a lost pregnancy that probably would have produced a normal, healthy child (Hollinshead 1979).

Ammenheuser *et al (1994)* used an assay for somatic cell mutation to evaluate the in utero effects of exposure to maternal cigarette smoking. They looked at the frequency of lymphocytes containing mutations at the hypoxanthine phosphoribosyltransferase (*hprt*) locus. They found that mothers who smoked had a higher mean frequency of mutant cells than non smoking mothers. Newborns of smoking mothers also had a higher frequency of mutant cells than those with non smoking mothers.

Cell transformation

The ability of CSC to transform cells *in vitro* was studied first by Lasnitzki (1958), who used organ cultures from human fetal lung. All of the four neutral fractions of CSC tested, induced hyperplasia of the lining epithelium of the bronchioli. They also showed that an enriched hydro carbon fraction of CSC

was a potent inducer of hyperplasia and other cytological changes indicative of malignant transformation (Lasnitzki 1968). Davies *et al* (1975) treated primary cell cultures of hamster lung cells with cigarette smoke itself. They noted that cultures treated with smoke that was high in gas vapor-phase components displayed an abnormal mitotic index and DNA content, and were tumorigenic when injected into nude mice. However, such changes were not observed in cultures treated with smoke that was low in gas vapor-phase components.

Reactive Oxidants

Cigarette smoking can lead to chronic inflammation in the lungs (Cone *et al* 1971) and elevated levels of phagocyte-derived reactive oxidants (Anderson *et al* 1974). The hyperractive phagocytes of smokers release more oxidants than non-smokers. These highly reactive oxidants have mutagenic and DNA-damaging properties. They are potent inducers of DNA strand breaks and chromosomal aberrations (Birnboim and Kanabus-Kaminska 1985) and can act as tumour promoters (Cerutti 1985). Chronic inflammatory diseases lead to an increased risk of cancer (Zajicek 1985).

van Rensburg *et al* (1989) found a highly significant correlation between SCE levels and increased LECL (luminol-enhanced chemiluminescence) responses in a group of young smokers. This indicates that reactive oxidants released from phagocytes may be responsible for the increased SCE frequencies in cigarette smokers. A correlation was also found between LECL and smoking history. SCEs, however did not correlate with smoking history. Their results also indicated that normal antioxidant levels may be inadequate to protect cells against DNA-damage caused by the oxidant overload generated by the hyperactive phagocytes of smokers. In conclusion the results of this study suggest that phagocyte-derived oxidants are possible mediators of SCEs *in vivo*.

Signs of Damage by Free Radicals

An article by Csillag and Aldhous (1992) reported on how over the past few years, a growing band of researchers have pointed to free radicals as major culprits in health problems ranging from cancer to heart disease and even aging. Since cigarette smoke contains a cocktail of free radicals - highly reactive chemical species with one or more unpaired electrons that oxidize many biological molecules including DNA - there's been growing speculation that the increased cancer risk faced by smokers may in large part be due to the insidious effect of these agents. A handful of *in vitro* experiments have lent support to this speculation by showing that tobacco smoke can oxidize isolated DNA.

Researchers from Copenhagen University, Århus University, and the Danish Cancer Registry report in the December issue of Carcinogenesis (cited by Csillag and Aldhous 1992) that they have found that the urine of smokers contains larger quantities of a tell-tale indicator of DNA oxidation than that of nonsmokers. The Danish researches who carried out the study are now trying to determine whether giving smokers large doses of antioxidants can reduce the signs of oxidative damage. But they worry that die-hard smokers may erroneously believe that such measures can make smoking safe. The group found that smoking greatly increases the rate at which DNA is oxidized. This could be caused directly by the free radicals present in cigarette smoke. But Copenhagen University pharmacologist Steffen Loft, a member of the Danish team, believes that the fact that smokers' metabolic rates are typically 10% to 15% higher than those of nonsmokers also plays a role. He suspects that the higher rate of cellular respiration in smokers is largely due to the enhancement of one particular metabolic pathway that results in the formation of free radicals.

In spite of the growing interest in free radials, most researchers investigating the mechanisms by which smoking causes cancer have, until now, concentrated on the binding to DNA of the polyaromatic hydrocarbons found in tobacco smoke. More work must be done before it is possible to estimate the relative importance of the two processes in causing cancer among

smokers.

These results raise the question, can antioxidants such as vitamin C or beta-carotene reduce the risk of developing cancer? But this interest into studying antioxidants as potential preventative agents against cancer may in fact allow smokers to be lulled into a false sense of security, thinking that they can ward off tumors by dosing up on antioxidants. Because cigarette smoke contains so many noxious chemicals, it is far too early to say that DNA oxidation by free radicals is the single most important factor underlying the high rate of cancer among smokers (Csillag and Aldhous 1992).

4. MATERIALS AND METHODS

4.1 SMOKING STUDY

4.1.1 Participants

Human female participants in this study included 5 non smoking controls and 10 smoking participants. Each participant (including non smokers) were required to fill out a detailed questionnaire (Appendix 1). The 10 smoking participants between the ages of 16 to 25 were chosen with similar lifestyles, medical history and smoking habits.

4.1.2 Specimen Collection

Participants were required to complete a consent form (Appendix 2) allowing blood to be withdrawn for analysis by the nurse at Massey University Health Centre. Blood was collected into two different tubes from each participant; one EDTA tube which was then used for performing a leucocyte count and one heparanised tube which was used for the blood culture.

4.1.3 Preparation of Blood Films

For each cultures to have a standard number of lymphocytes, a lymphocyte count was required for each participant. A blood film was prepared using anticoagulated blood collected into an EDTA tube. This was done by placing a small drop of blood approximately 1-2 cm from the end of the slide. Using a bevelled smooth-edged spreader, the blood was spread over the slide to produce a film with an even surface, free from ridges, waves and holes (Figure 4.1). The slide was labelled with the participants code using a

diamond pen.

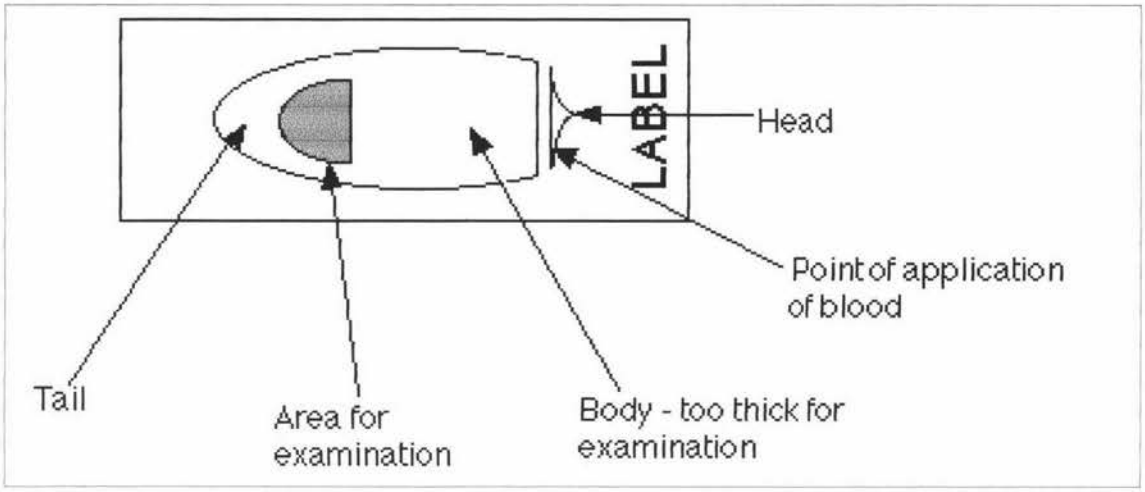


Figure 4.1

4.1.4 Staining of Blood Films

Air-dried slides were fixed in methanol for 10 minutes and then stained immediately in May-Grunwald stain for 5 minutes. Slides were counter stained in 10% Giemsa for 20 minutes and washed rapidly in three changes of buffered water (pH 6.5), allowing to stand in the last wash for 2 minutes to allow for differentiation to take place.

4.1.5 Leucocyte Counts

Once the slides were dried a leucocyte count was performed. A 1 in 20 dilution of blood was made by adding 20 μl of blood to 0.38 ml of dilution fluid (a solution, containing 2% acetic acid coloured pale violet with gentian violet, which lyses the red cells).

A Neubauer counting chamber was used to perform a count using a 4mm objective and a x10 eyepiece. At least 100 cells were counted in as many 1mm^2 areas (0.1 μl in volume) as necessary. The ruled area in an improved Neubauer chamber consists of nine of these areas.

$$\text{Count } (/l) = \frac{\#\text{ of cells counted}}{\text{volume counted } (\mu l)} \times \text{dilution} \times 10^6$$

Thus, if N cells are counted in 0.1 μl:

$$\text{Leucocyte count per litre} = N \times 10 \times (\text{dilution}) \times 10^6$$
$$N \times 200 \times 10^6 / l$$
$$(N \times 200 \text{ per } \mu l)$$

The slide was read only in the area where the cells were evenly spaced, i.e. not over-lapping each other.

Having established the total leucocyte count per litre, it was then necessary to perform a differential count to find the percentage of lymphocytes in the total leucocyte population. The total leucocyte count was multiplied by the percentage lymphocytes to obtain the total number of lymphocytes per litre.

1×10^6 lymphocytes per culture were required at the end of culture. Because cell doubling time is 72 hours, $0.5 \times (1 \times 10^6) = 5 \times 10^5$ lymphocytes were added to the culture medium.

4.1.6 Lymphocyte Cultures

Four culture tubes were set up per participant, two for analysis of chromosome aberrations and two for the analysis of SCE.

For chromosome aberration analysis each culture tube contained:

Medium 199	5.05 ml
Foetal calf serum	1.00 ml
Phytohaemagglutinin (PHA)	0.10 ml

For SCE analysis each culture tube contained:

Medium 199	5.00 ml
Foetal calf serum	1.00 ml
PHA	0.10 ml
10^{-2}M BrdU	0.05 ml

Each tube was wrapped in foil to protect the cultures from light. The cultures were inoculated with $0.5x10^5$ lymphocytes in a 0.3 ml dilution with medium 199. Each culture was incubated at 37°C for 72 hours. Tubes were then removed from the incubator and mixed gently. 0.1 ml of 0.05% colchicine was added and the cultures were incubated for a further 1 hour.

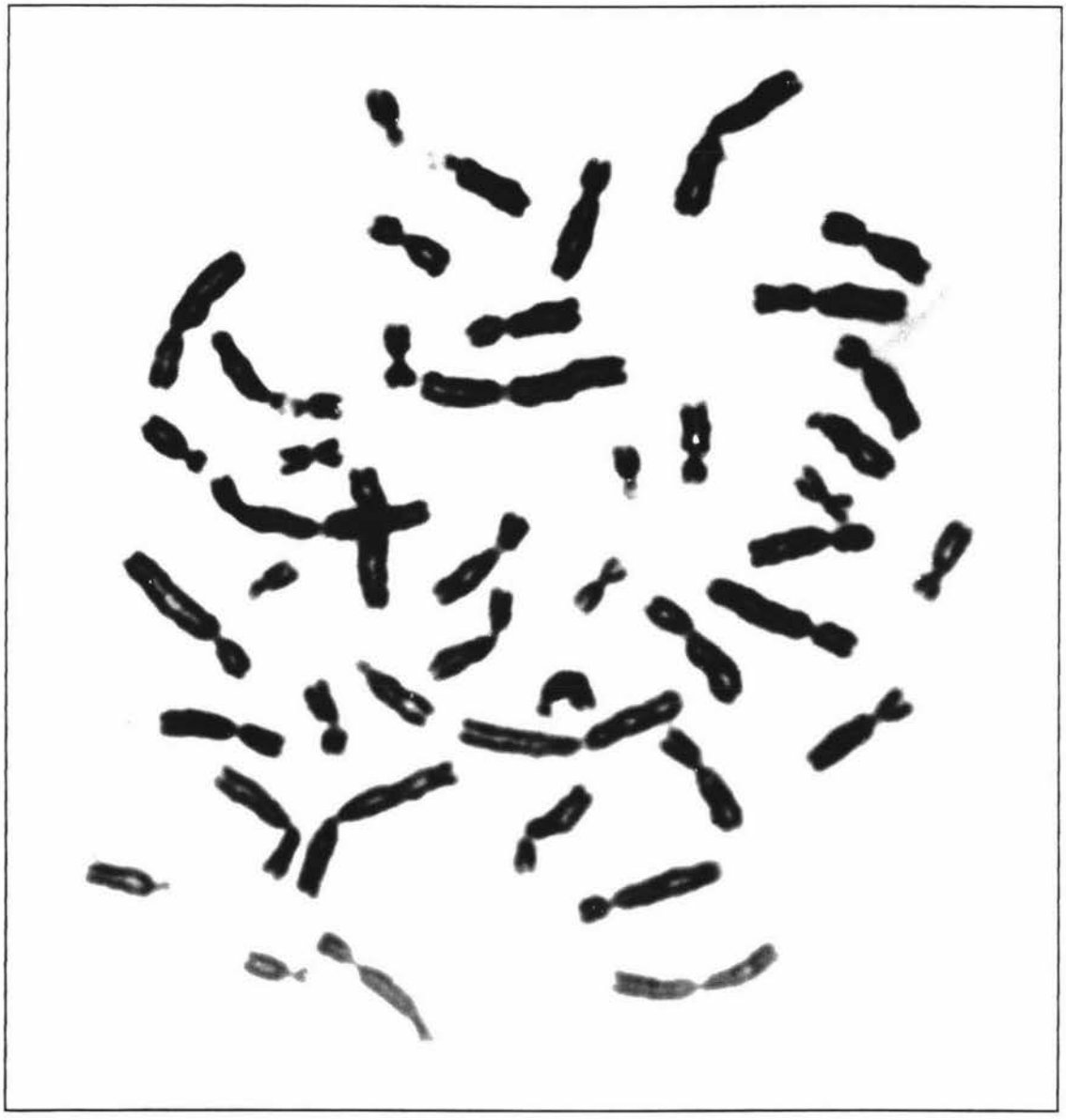
4.1.7 Harvesting

Culture tubes were mixed gently and centrifuged at 1000 rpm for 10 minutes after which the supernatant was removed to leave approximately 1 ml of culture. Cultures were mixed gently and resuspended in 6 ml of hypotonic solution (0.4% KCl and 0.4% sodium citrate) at 37°C and incubated for 15 minutes at 37°C. Tubes were centrifuged again for 10 minutes then all but 1 ml of supernatant removed. Cultures were mixed gently and resuspended in 5 ml 6% acetic acid under constant agitation and left for 5 minutes at room temperature. Cultures were once again centrifuged and the supernatant removed. Cultures were resuspended in 9 ml of cold fixative and centrifuged immediately and the supernatant removed. Cells were then dropped onto slides previously washed in acid alcohol (10ml of 1 NHCl in 500 ml of 95 % ethanol) and air dried.

4.1.8 Staining

Giemsa Block Stain

Slides were stained in 10% Giemsa (in Sorrensen's buffer) for 3-8 minutes and them rinsed twice in Sorrensen's buffer for 1-2 minutes. Figure 4.2 shows an example of a human chromosome complement which is block stained.



Block stained human chromosome preparation.

Figure 4.2

Fluorescence-plus-giemsa Stain (SCE Stain)

Slides were soaked in phosphate buffer solution (PBS) (pH 7.0) for 5 minutes and subsequently stained with Hoechst (0.5 g/ml PBS) and rinsed briefly in PBS and distilled H_20. Slides were mounted with McIlvaine's buffer and irradiated with UV for 25 minutes. The coverslips were removed and the slides rinsed in distilled H_20 and incubated at 65°C in 2xSSC for 15 minutes. Slides were finally stained for 5-10 minutes with fresh 10% giemsa.

4.1.9 Scoring

Each slide was coded and then scored in a random order. For each participant, 30 metaphase spreads were analysed to establish the frequency of sister chromatid exchange and 100 metaphase spreads for the presence of chromosome aberrations.

Giemsa banded preparations were examined for abnormalities in banding patters such as translocations, inversions and deletions.

Slides were coded and scored blind, the coded slides were scanned under low magnification (100 x) and selected for scoring on the basis of good staining and chromosome number. Only cells with 46 chromosomes were scored for chromosome aberrations. For SCEs, well differentiated metaphases were accepted for scoring. SCEs were expressed per cell so it was necessary for a full complement of 46 chromosomes to be present. Figure 4.3 illustrates the method used to score SCEs.

The exchange of stain had to be reciprocal, i.e. a darkly stained region on one chromatid must have been accompanied by a lightly stained region in the reciprocal part of the sister chromatid. It was not necessary that the entire chromatid width be involved in an exchange. Exchanges of stain occurring at the centromere were included as an SCE unless there was an obvious twist of the chromatids.

The following guidelines, as stated in Swierenga et al (1991), were followed when scoring slides.

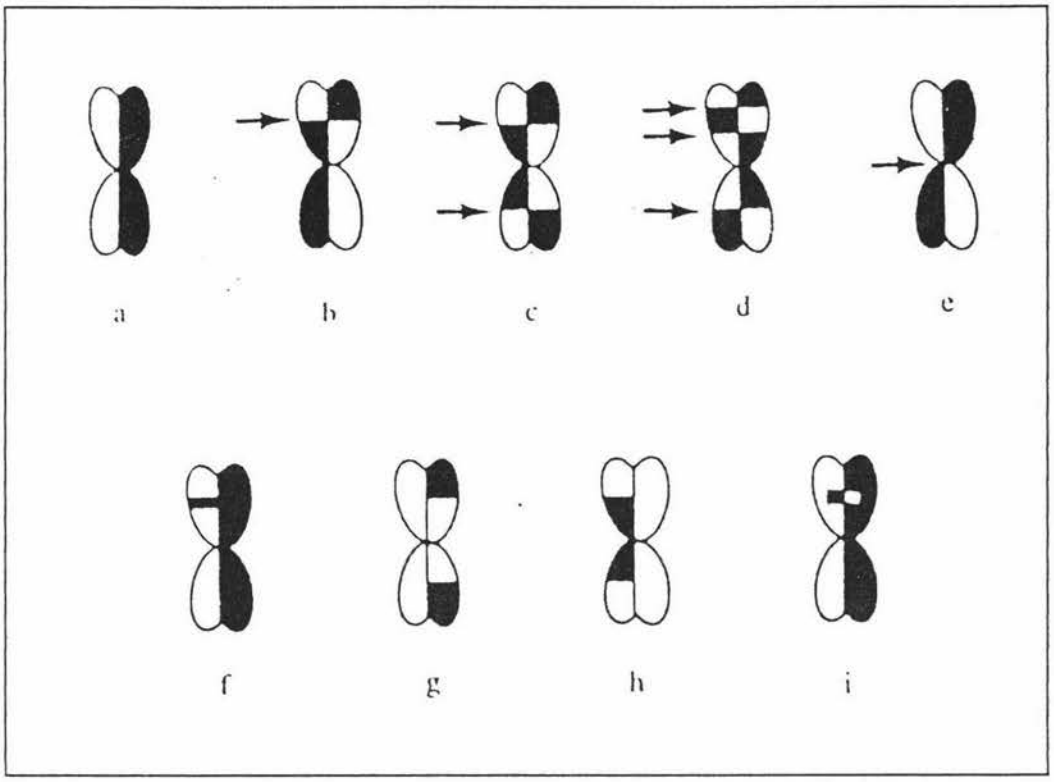


Figure 4.3. Schematic representation of various differential staining patterns observed after BrdU incorporation. (a) Incorporation of BrdU for two complete cycles or the first of two cycles of DNA synthesis. No SCEs are present. (b) A single SCE (arrow). (c) Two SCEs. (d) 3 SCEs. (e) An SCE occurring at the centromere. Unless this can be distinguished from a twist, this pattern should be scored as an SCE. (f) Possible staining artifact or incomplete incorporation of BrdU in part of the chromosome. Reciprocal light staining region is not found on the sister chromatid, therefore this is not scored as an SCE. (g and h) Incorporation of BrdU for more than two complete cycles of DNA synthesis. No reciprocal pattern, therefore not scored as second division SCEs. (i) Reciprocal pattern of staining less than one chromatid in width. These occur infrequently but should be scored as SCEs (Swierenga *et al* 1991).

Scoring Chromosome aberrations

Aberrations are scored by recording individual types of aberrations separately, but for statistical analyses, the aberrations must be grouped into classes based on the degree of chromosomal damage. The number of cells bearing multiple aberrations should also be taken into account when scoring.

Gaps should be recorded, but not included into the analysis of chromosome aberration incidence, since their cytogenetic significance is not known.

Aberrations were grouped into three groups:

a) Simple - terminal deletions, chromatid deletions and isochromatid deletions.

b) Complex - exchanges and rearrangements

c) Other - pulverised cells uncoiled (despiralized) chromosomes and heavily damaged cells containing ten or more aberrations.

Scoring Sister Chromatid Exchanges (SCE)

For the SCE assay, a minimum of 25 to 50 cells per culture and 50 cells per treatment were scored.

4.2 VANILLIN STUDY

4.2.1 Specimen Collection

Blood from one donor was collected into a heparanised tube and was used for all vanillin cultures. This reduced any variability in using different donors for culturing.

4.2.2 Lymphocyte Cultures

Fourteen culture tubes were set up, with two tubes per test. The test cultures were as follows:

- positive control - 0.01% Styrene Oxide
- negative control
- 0.1 mM vanillin
- 0.5 mM vanillin
- 1.0 mM vanillin

- 1.5 mM vanillin
- 2.0 mM vanillin

For chromosome aberration analysis each culture tube contained:

Medium 199	5.05 ml
Foetal calf serum	1.00 ml
PHA	0.10 ml

For SCE analysis each culture tube contained:

Medium 199	5.00 ml
Foetal calf serum	1.00 ml
PHA	0.10 ml
10^{-2}M BrdU	0.05 ml

Each tube was wrapped in foil to protect the cultures from light.

The tubes were inoculated with 0.3 ml of heparanised blood and then incubated at 37°C for 24 hours. 50 μl of each test solution was subsequently added to each culture and incubated for a further 48 hours and harvested as mentioned above in the smoking study.

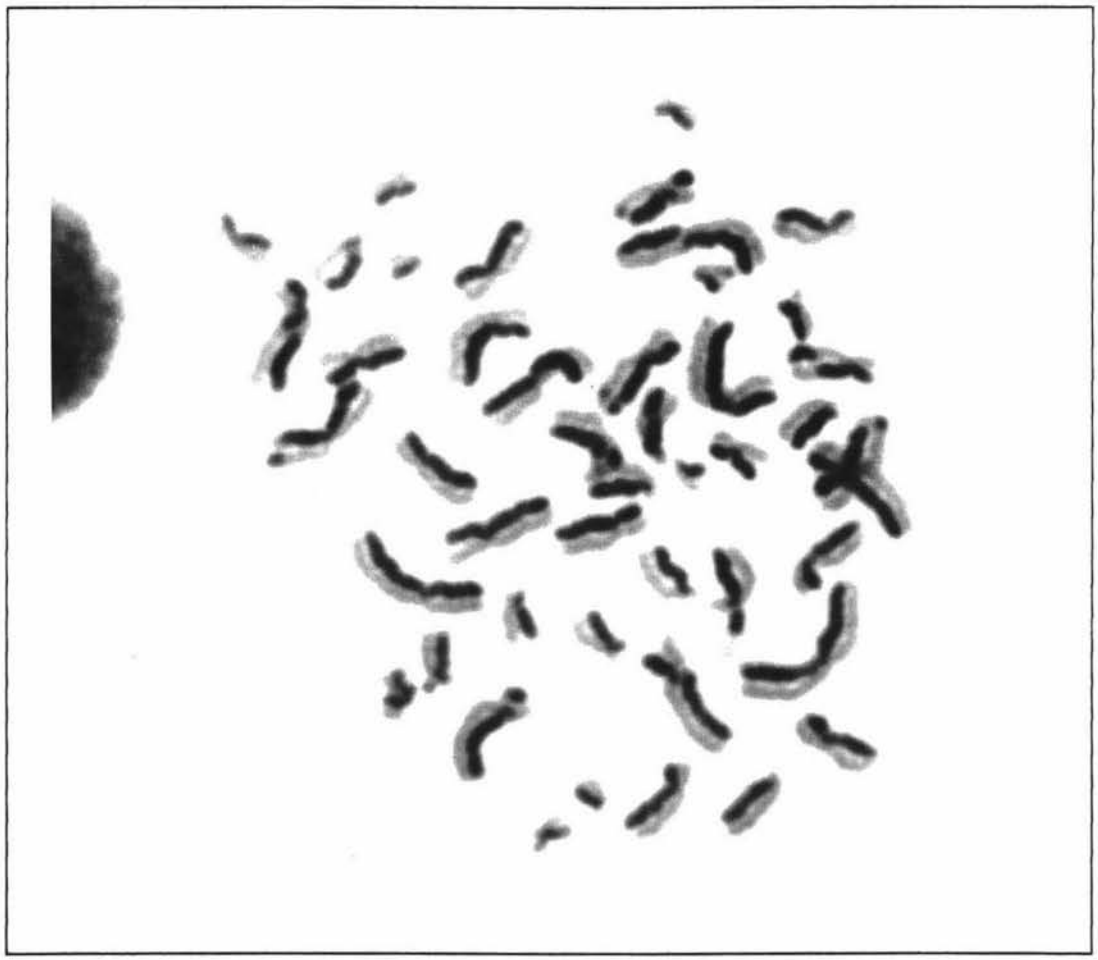
4.2.3 Scoring

Slides were coded and scored as in the smoking study.

5. RESULTS

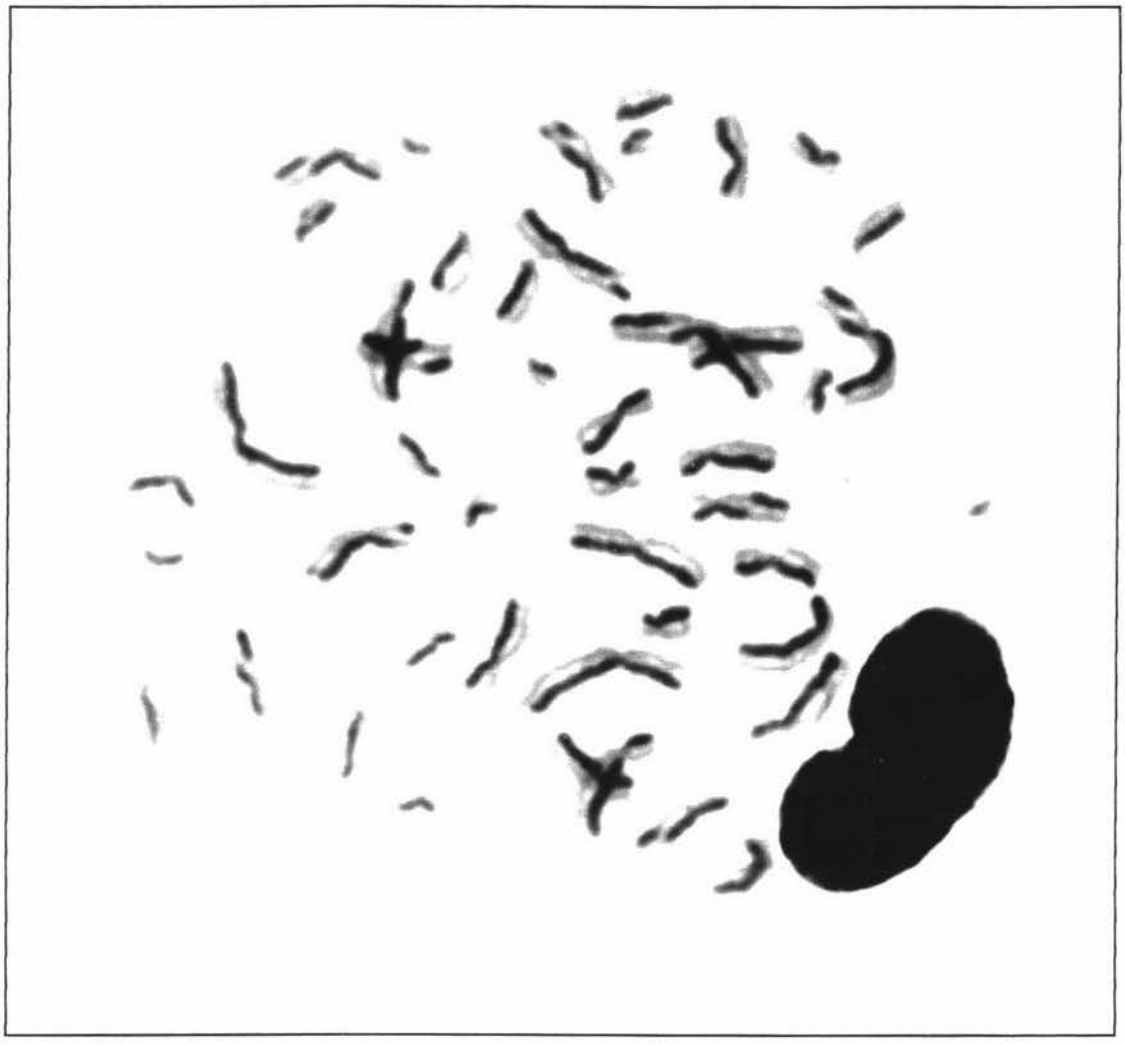
5.1 Effects of smoking: frequency of SCEs.

Figure 5.1 and 5.2 shows an example of an SCE stained complement from a non smokers blood showing 11 SCEs. Figure 5.2 shows some unusual chromosome arrangements only due to the way the chromosomes lie, they do not show any abnormalities.



An SCE stained complement from a non smoking participant, with 11 SCEs.

Figure 5.1



An SCE complement from a smoking participant showing some unusual chromosome arrangements which are only due to the way the chromosomes lie across one another. There are no abnormalities. This complement shows 14 SCEs. **Figure 5.2**

Figure 5.3 shows a similar complement but from a smoking participant which shows 14 SCEs.

The results shown in Appendix 2 were obtained by examining 100 cells from each of 5 non smokers and 10 smokers. These were analysed using the statistical test, ANOVA (analysis of variance) with repeated measures. This tests for 'treatment' effect (in this case being smoking) using a special error term to adjust for replicate information. This is because a normal ANOVA looks at the difference between each value in the data. In this case we have repeated measures for several different people e.g. there are 5 non smokers

and 10 smokers. In each case 100 cells were examined and the number of SCEs determined. Therefore each person has 100 repeated measures. A special error term was used i.e. ID(TRT) which means the ID within the treatment, the ID in this case meaning each person.

SCE stained complement from a smoking participant showing 14 SCEs.

Figure 5.3

The analysis was carried out with results as in Table 5.1.

Dependent Variable: NUMSCE

Tests of Hypotheses using the Type III MS for ID(TRT) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr> F
	1	3118.79005	3118.79005	15.99	0.0015

Table 5.1

The circled value indicates that there is a significant difference between the two groups, non smokers and smokers, i.e. there is 0.15% probability of this occurring randomly.

The means were also calculated for the two groups. The non smokers had a mean of 9.712 SCEs per cell compared with the smokers who had a mean of 12.771 SCEs per cell.

5.2 Effects of smoking: frequency of chromosome aberrations

Figure 5.4 shows a smokers preparation showing a chromatid gap in one of the large chromosomes.

Appendix 3 shows the results obtained in examining 30 cells each of 5 non smokers and 10 smokers. These results were analysed statistically using an analysis of variance. Table 5.2 shows exchanges against treatment (i.e. smoking). There were no exchanges seen in any of the samples which would indicate that smoking did not significantly effect chromosome exchanges.

TABLE OF EXCHANGE AGAINST TREATMENT

EXCH Frequency Percent Row Pct Col Pct	TRT non-smoker	smoker	total
0	150	300	450
	33.3	66.67	100.00
	33.33	66.67	
	100.00	100.00	
Total	150	300	450
	33.33	66.67	100.00

Table 5.2

Chromosome complement showing a gap in the chromatid of one of the large chromosomes from a sample from a smoker.

Figure 5.4

Table 5.3 shows a table of breaks against treatment. The results in Table 5.4, show that there is 31.3% chance of randomly obtaining a positive result. This is not significant. Therefore cigarette smoking does not significantly affect the number of chromosome breaks per cell.

TABLE OF BREAK AGAINST TREATMENT

BREAK	TRT		
Frequency Percent Row Pct Col Pct	non-smoker	smoker	total
0	136	280	416
	30.22	62.22	92.44
	32.69	67.31	
	90.67	93.33	
Total	150	300	450
	33.33	66.67	100.00

BREAK	TRT		
Frequency Percent Row Pct Col Pct	non-smoker	smoker	total
1	14	20	34
	3.11	4.44	7.56
	41.18	58.82	
	9.33	6.67	
Total	150	300	450
	33.33	66.67	100.00

Table 5.3

STATISTICS FOR TABLE OF BREAK AGAINTS TREATMENT

Statistic	DF	Value	Prob
Chi-Square	1	1.018	0.313
Likelihood Ratio Chi-Square	1	0.987	0.320
Continuity Adj, Chi-Square	1	0.672	0.412
Mantel-Haenszel Chi-Square		1.016	0.314
Fisher's Exact Test (Left)			0.205
Fisher's Exact Test (Right)			0.883
Fisher's Exact Test (2-Tail)			0.346
Phi Coefficient		-0.048	
Contingency Coefficient		0.048	
Cramer's V		-0.048	
Sample Size = 450			

Table 5.4

Table 5.5 shows the number of chromosome abnormalities which include the whole chromosome against the treatment (i.e. smoking). '0' represents a negative result and '1' represents a positive result. The figures in table 5.6 show that there is 100% probability of getting a positive result. This is obviously not significant.

TABLE OF CHROMOSOME TYPE ABERRATION BY TREATMENT

CS Frequency Percent Row Pct Col Pct	TRT non-smoker	smoker	total
0	138	276	414
	30.67	61.33	92.00
	33.33	66.67	
	92.00	92.00	
Total	150	300	450
	33.33	66.67	100.00

(... continued overleaf)

CS	TRT		
Frequency Percent Row Pct Col Pct	non-smoker	smoker	total
1	12	24	36
	2.67	5.33	8.00
	33.33	66.67	
	8.00	8.00	
Total	150	300	450
	33.33	66.67	100.00

Table 5.5

STATISTICS FOR TABLE OF CHROMOSOME TYPE ABERRATIONS BY TREATMENT

Statistic	DF	Value	Prob
Chi-Square	1	0.000	1.000
Likelihood Ratio Chi-Square	1	0.000	1.000
Continuity Adj, Chi-Square	1	0.000	1.000
Mantel-Haenszel Chi-Square		0.000	1.000
Fisher's Exact Test (Left)			0.566
Fisher's Exact Test (Right)			0.580
Fisher's Exact Test (2-Tail)			1.000
Phi Coefficient		0.000	
Contingency Coefficient		0.000	
Cramer's V		0.000	
Sample Size = 450			

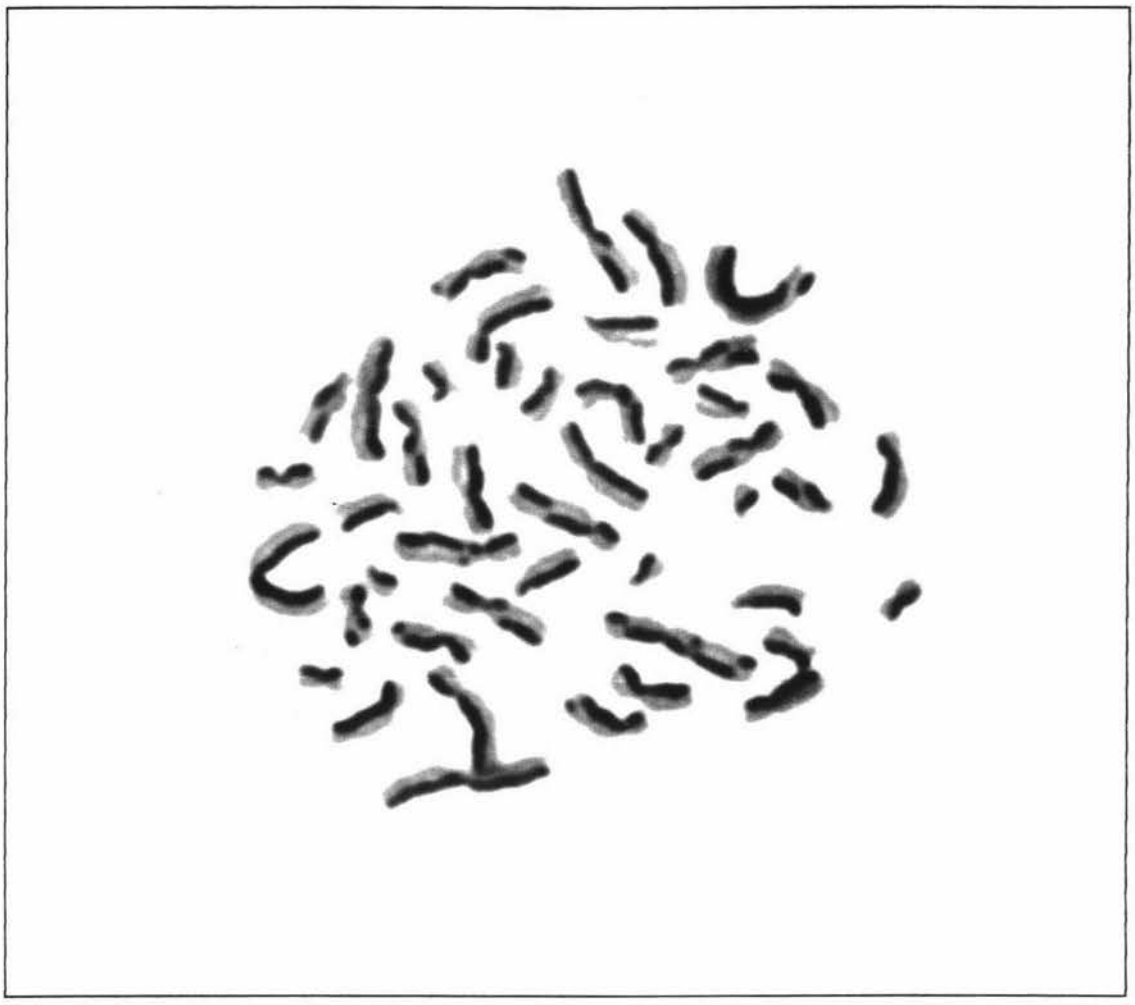
Table 5.6

5.3 Vanillin and SCEs

Figure 5.5 shows an SCE stained complement from the negative control showing 7 SCEs. Figure 5.6 shows an SCE stained complement from the positive control showing 23 SCEs.

This figure shows an SCE stained complement from the negative control. There are 7 SCEs.

Figure 5.5



This complement is from the positive control (styrene-7,8 oxide) and shows 23 SCEs.

Figure 5.6

Another analysis of variance test called the Dunnett's T test for variables, was used to look at the effects of vanillin concentration on SCEs. Table 5.7 shows the results of comparing each vanillin concentration against the negative control. The treatment comparison column in the table '3.0' represents the negative control and the other values are the vanillin concentration in mM.

Alpha=0.05 Confidence=0.95 df=693 MSE=8.627287
Critical Value of Dunnett's T=2.573
Minimum significant Difference=1.0689

Comparisons significant at the 0.05 level are indicated by '***'

TABLE OF VANILLIN CONCENTRATION AGAINST THE NEGATIVE CONTROL

TRT Comparison	Simultaneous Lower Confidence Limit	Difference Between Means	Simultaneous Upper Confidence Limit	
4.0 - 3.0	14.051	15.120	16.189	***
2.0 - 3.0	8.791	9.860	10.929	***
1.5 - 3.0	3.391	4.460	5.529	***
1.0 - 3.0	2.061	3.130	4.199	***
0.5 - 3.0	1.051	2.120	3.189	***
0.1 - 3.0	-0.089	0.980	2.049	

NOTE: This test controls the type I experimentwise error for comparisons of all treatments against a control.

Table 5.7

Table 5.8 shows the results of comparing the different concentrations of vanillin against the positive control, styrene oxide (in this case the positive control has been given the value of 4.0). Because the values of SCE per cell were greater with the positive control that the vanillin concentrations the values are negative but are still significant as shown by the '***".

TABLE OF VANILLIN CONCENTRATION AGAINST THE POSITIVE CONTROL

TRT Comparison	Simultaneous Lower Confidence Limit	Difference Between Means	Simultaneous Upper Confidence Limit	
2.0 - 4.0	-6.329	-5.260	-4.191	***
1.5 - 4.0	-11.729	-10.660	-9.591	***
1.0 - 4.0	-13.059	-11.990	-10.921	***
0.5 - 4.0	-14.069	-13.000	-11.931	***
0.1 - 4.0	-15.209	-14.140	-13.071	***
3.0 - 4.0	-16.189	-15.120	-14.051	***

Table 5.8

5.4 Vanillin and Chromosome aberrations

Table 5.9 shows a table of treatment by exchanges. As there were no exchanges this obviously shows no significant difference between each vanillin concentration level.

Table 5.10 shows a table of treatment by break. Table 5.11 shows that statistics which do not indicate any significant difference.

Table 5.12 shows a table of treatment by chromosome type aberrations. The statistics shown in table 5.13 also show no significance because the only group that showed any chromosome type aberrations was the positive control labelled '1' in this case.

TABLE OF TREATMENT AGAINST EXCHANGES

TRT Frequency Percent Row Pct Col Pct	EXCH 0	Total
1	30 14.29 100.00 14.29	30 14.29
2	30 14.29 100.00 14.29	30 14.29
3	30 14.29 100.00 14.29	30 14.29
4	30 14.29 100.00 14.29	30 14.29
5	30 14.29 100.00 14.29	30 14.29
6	30 14.29 100.00 14.29	30 14.29
7	30 14.29 100.00 14.29	30 14.29
Total	210 100.00	210 100.00

The numbers in the TRT (treatment column) represent each vanillin concentration. 1=the negative control, 2=0.1 mM vanillin, 3=0.5 mM vanillin, 4=1.0 mM vanillin, 5=1.5 mM vanillin, 6=2.0 mM vanillin and 7=styrene oxide (positive control).

Table 5.9

TABLE OF TREATMENT AGAINST BREAKS

TRT	BREAK		
Frequency Percent Row Pct Col Pct	0	1	Total
1	24	6	30
	11.43	2.86	14.29
	80.00	20.00	
	12.37	37.50	
2	28	2	30
	13.33	0.95	14.29
	93.33	6.67	
	14.43	12.50	
3	28	2	30
	13.81	0.48	14.29
	96.67	3.33	
	14.95	6.25	
4	28	2	30
	13.33	0.95	14.29
	93.33	6.67	
	14.43	12.50	
5	29	1	30
	13.81	0.48	14.29
	96.67	3.33	
	14.95	6.25	
6	28	2	30
	13.33	0.95	
	93.33	6.67	
	14.43	12.50	
7	28	2	30
	13.33	0.95	
	93.33	6.67	
	14.43	12.50	
Total	194	16	210
	92.38	7.62	100.00

The numbers in the TRT (treatment column) represent each vanillin concentration. 1=the negative control, 2=0.1 mM vanillin, 3=0.5 mM vanillin, 4=1.0 mM vanillin, 5=1.5 mM vanillin, 6=2.0 mM vanillin and 7=styrene oxide (positive control).

Table 5.10

STATISTICS FOR TABLE OF TREATMENT AGAINST BREAKS

Statistic	DF	Value	Prob
Chi-Square	6	8.254	0.220
Likelihood Ratio Chi-Square	6	6.789	0.341
Mantel-Haenszel Chi-Square	1	2.424	0.119
Phi Coefficient		0.198	
Contingency Coefficient		0.194	
Cramer's V		0.198	

Sample Size = 210
WARNING: 50% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Table 5.11

TABLE OF TREATMENT BY CHROMOSOME TYPE ABERRATIONS

TRT	CHROMOSOME TYPE ABERRATIONS		
Frequency Percent Row Pct Col Pct	0	1	Total
1	26 12.38 86.67 12.62	4 1.90 13.33 100.00	30 14.29
2	30 14.29 100.00 14.56	0 0.00 0.00 0.00	30 14.29
3	30 14.29 100.00 14.56	0 0.00 0.00 0.00	30 14.29
4	30 14.29 100.00 14.56	0 0.00 0.00 0.00	30 14.29
5	30 14.29 100.00 14.56	0 0.00 0.00 0.00	30 14.29
6	30 14.29 100.00 14.56	0 0.00 0.00 0.00	30 14.29
7	30 14.29 100.00 14.56	0 0.00 0.00 0.00	30 14.29
Total	206 98.10	4 1.90	210 100.00

The numbers in the TRT (treatment column) represent each vanillin concentration. 1=the negative control, 2=0.1 mM vanillin, 3=0.5 mM vanillin, 4=1.0 mM vanillin, 5=1.5 mM vanillin, 6=2.0 mM vanillin and 7=styrene oxide (positive control).

Table 5.12

STATISTICS FOR TABLE OF TREATMENT BY CHROMOSOME TYPE ABERRATIONS

Statistic	DF	Value	Prob
Chi-Square	6	24.466	0.000
Likelihood Ratio Chi-Square	6	16.049	0.013
Mantel-Haenszel Chi-Square	1	9.131	0.003
Phi Coefficient		0.341	
Contingency Coefficient		0.323	
Cramer's V		0.341	

Sample Size = 210
WARNING: 50% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Table 5.13

TABLE OF TREATMENT AGAINST GAPS

TRT	GAPS		
Frequency Percent Row Pct Col Pct	0	1	Total
1	25 11.90 83.33 13.02	5 2.38 16.67 27.78	30 14.29
2	28 13.33 93.33 14.58	2 0.95 6.67 11.11	30 14.29
3	28 13.33 93.33 14.58	2 0.95 6.67 11.11	30 14.29
4	28 13.33 93.33 14.58	2 0.95 6.67 11.11	30 14.29
5	28 13.33 93.33 14.58	2 0.95 6.67 11.11	30 14.29
6	28 13.33 93.33 14.58	2 0.95 6.67 11.11	30 14.29
7	27 12.86 90.00 14.06	3 1.43 10.00 16.67	30 14.29
Total	192 91.43	18 8.57	210 100.00

The numbers in the TRT (treatment column) represent each vanillin concentration. 1=the negative control, 2=0.1 mM vanillin, 3=0.5 mM vanillin, 4=1.0 mM vanillin, 5=1.5 mM vanillin, 6=2.0 mM vanillin and 7=styrene oxide (positive control).

Table 5.14

STATISTICS FOR TABLE OF TREATMENT AGAINST GAPS

Statistic	DF	Value	Prob
Chi-Square	6	3.281	0.773
Likelihood Ratio Chi-Square	6	2.836	0.829
Mantel-Haenszel Chi-Square	1	0.544	0.461
Phi Coefficient		0.125	
Contingency Coefficient		0.124	
Cramer's V		0.125	

Sample Size = 210

WARNING: 50% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Table 5.15

6. DISCUSSION

6.1 Smoking

The results from this study show that cigarette smoking has no effect on chromosome aberrations. These results are consistent with results of Nordenson *et al* (1978) and Hedner *et al* (1983) who found no correlation between the frequency of chromosome aberrations and smoking habits. This is in contrast with results found by Obe and Herha (1978), and Tawn and Cartmell (1989) who found an increase in total aberrations in smokers compared with non smokers. Kier *etal* (1989) found an increase of exchange type aberrations of the chromosome (dicentric and ring chromosomes) and chromatid (interchanges) types.

Many *in vitro* tests have also found that CSC increases the frequency of chromosome aberrations (Venema 1959; leuchtenberger *et al* 1973; Pandey *et al* l978). CSC, cigarette smoke and the urine of cigarette smokers have also been shown to be mutagenic in *Salmonella*. In this test, CSC seems to possess frameshift mutagens that require metabolic activation, and the majority of the mutagenic activity resides in the basic fraction. A small amount of activity is in the acidic fraction, and very little in the neutral fraction.

These conflicts could be due to the difficulty in chromosome aberration studies and population monitoring. Chromosome aberration studies are fraught with difficulties, especially in the present case, a shortage of scorers (i.e. only one) caused difficulties in reading enough cells for the test to be statistically significant, i.e., the number of cells to be scored in each group to five a 50% chance of asserting with 97.5% confidence that the mean of the exposed population is significantly greater than that of the control value, *e.g.*, Carrano and Natarajan (1988) suggest for mutagen to increase the control values by 50% (A control or background value in this case is 1 chromosome aberration per 100 cells), one would need to score 40 000 cells in each

population. If circumstances permit, one should initially consider employing an equal number of people in the control and exposed cohorts. For the analysis of aberrations, the minimum number in each group will be dictated by the expected control frequencies, the sensitivity desired, and economic factors. 10 people per group should be estimated as a minimum. Due to the extreme difficulty found in obtaining enough participants in the present study, greater than 10 people fro each group with similar lifestyles and medical histories would seem nearly impossible, although desirable to obtain more significant results. Chromosome aberration studies also require care and expertise on the part of the scorer.

It also appears infinitely valuable that if one wants to carry out cytogenetic studies that one gets in contact with other groups with similar objectives. Human specimens from exposed and control populations are a precious resource to the scientific community. Whenever possible, these specimens should be made available to interested callaborators for the concurrent or subsequent application of other genetic endpoints relevant to the suspected exposure. In the case of lymphocytes, this might be aided by cryopreserving them. The use of diverse methods of study on the same population can provide valuable information relative to the sensitivity of each method used as well as to the potential hazard for the population.

Results from this study conclude that cigarette smoking significantly increases the number of SCEs/cell compared to that of non smokers showing that smoking can induce genetic damage and therefore, carries with it a genetic risk. Similar studies have also shown similar results *e.g.* Lambert *et al* (1978), Husum *et al* (1982) and Husgafvel-Pursiainen *et al* (1980). Other previous studies have tried to understand unsuccessfully the way in which cigarette smoke might cause genetic damage and any diseases that may result from such damage. Other groups have found similar results for example Bender *et al* (1989) also found a significant effect of smoking on SCEs, equivalent to an absolute excess of about one SCE per cell, or 13%

There has also been conflicting reports on the effects of cigarette smoking on SCEs. For example Hedner *et al* (1983), Hollander *et al* (1978), Crossen

and Morgan (1980) have found no significant difference between the SCEs of smokers and that of non smokers. It must be noted that not all groups used a standardized white blood cell count in each culture. This is an important factor which must be included in further studies as Bender *et al* (1992b) found that the total lymphocyte inoculum in cultures contributed to the overall variance in SCEs. As the number of lymphocytes increased, the number of SCEs decreased.

6.2 Vanillin concentration

This study found no correlation between vanillin concentration and chromosome aberrations. This conflicts with results of Jansson and Zech (1987) who showed a slight increase in the number of chromosome aberrations with increasing concentrations of vanillin. However, only the highest tested concentration (4mM) showed a statistically significant effect with gaps included. The significance of counting and including gaps in aberration studies has been debated (Brögger 1982) and gaps are not included in the evaluation of chromosome aberration tests according to the OECD Guidelines for Testing of Chemicals (cited in Jansson and Zech 1987). However Jansson and Zech (1987) go on to say that the positive outcome of their study is valid as the dose-dependent effect can be seen both excluding and including gaps and especially on chromosom-type aberrations.

This study did show that as vanillin concentration increased so did the frequency of SCE. Jansson and Zech (1987), Jansson *et al* (1986) and Jansson *et al* (1988) also found that vanillin is a potent inducer of SCEs. For example Jansson and Zech (1987) found a control value of 14.3 SCEs per cell. A vanillin concentration of 1 mM increased values to 19.2 SCEs per cell and a concentration of 2 mM vanillin further increased values to an average of 24.2 SCEs per cell.

Jansson *et al* (1988) Found that all benzaldehydes tested except 2-hydroxybenzaldehyde induce SCE. It appears likely that benzaldehydes are direct-acting SCE inducers, since benzaldehyde itself showed an SCE-

inducing effect, while benzoic acid, a probable metabolite, was inactive. In conformity with this, 4-hydroxy-3-methoxyacetophenone, which differs from vanillin in having an acetoxy group instead of an aldehyde group, was also inactive. They therefore concluded that benzaldehydes in general are likely to induce SCEs. It is important to note that widely used food additives such as vanillin and ethylvanillin are found in this group of SCE inducers.

The majority of compounds discussed in this study having a carbon-carbon bond conjugated with the aromatic ring induce SCE i.e. styrene, 1-phenyl-1-propene, 2-(1-propenyl)-phenol, 2-methoxy-4-vinylphenol and 2-methoxy-4-(1-propenyl)-phenol. In contrast those having non-conjugated double bonds as well as the analogues having an alkyl instead of an alkenyl moiety are inactive. Since styrene is metabolised to the active metabolite styrene-7,8-oxide and both this compound and 1-(2-ethoxyphenyl)-1,2-epoxypropane induce SCE, it seems probably that the formation of an epoxide may be a prerequisite for activity of this type of compound.

Although many of the SCE-inducing compounds tested in the study by Jansson *et al* (1988) are ubiquitous in the human environment, genotoxicity data are lacking for most of them, including representatives used as flavour in foods, beverages and perfumes. It is therefore important that vanillin has been shown to increase SCE frequency in this study and indicates a great need for further studies to be carried out on this and other such compounds.

6.3 Sources of variation

There are a number of possible causes for SCE variation:

- due to biological lifestyle factors such as smoking and diet.
- due to cell culture factors such as differential uptake of BrdU.
- or due to physiological factors such as age, gender, hormone levels and stress.

Because all participants were women one cannot overlook biological

rhythms and any hormone treatment especially for birth control. D'Souza *et al* (1988) report on the variaton in chromosomal damage as a function of biological rhythms in women. They found an enhanced frequency of SCEs (10.6/cell) during ovulatory stage, and a minimum level (7.0/cell) at progestonic stage, which also showed the lowest rate of chromosome aberrations (2.8%). Interestingly, the estrogenic phase revealed the highest frequency of chromosome aberrations (13%), though the frequency of SCEs (9.9/cell) was slightly lower than that of the ovulatory stage but was certainly higher than that of progestogenic stage. These results indicate that the hormonal variations during menstrual cycle play an important role in bringing about variation in the base line frequency of SCEs in women. The frequency of SCEs is found to be fairly constant if biologic rhythms are taken into account along with other potential variables (Das 1988 Cited in D'Souza 1988). Furthermore, it seems that female subjects are more prone to such rhythmic variation because of specific hormonal cycles and are susceptible to genetic damage during ovulatory and extrogenic stages of the menstural cycle. It would therefore be advantageous to take samples at the same stage in the menstrual cycle for each participant.

A possible explanation for the effects of hormones could be their direct influence on cells. It is well known that these steroid hormones retain their activity in target cells even when bound to receptors (Schulster *et al* 1976). Although they are known to function at the transcription level by binding to chromosomal nonhistone proteins, they may also induce DNA-polymerase and exonuclease activity. This may lead to initial lesions in the DNA strands which would favour exchanges such as SCEs. Nevertheless, it may simply be a natural phenomenon that these steroids may induce topoisomerase II which is an essential enzyme that operates during DNA replication, catalyzing a reversible and concerted DNA double strand, involved in breakage and rejoining. This may lead to a higher SCE formation due to more misrepair or errors in rejoining under hormonal influence (D'Souza *et al* 1988).

From a statistical point of view, there are several possible mechanisms for the observed variation of the mean SCE frequencies between and within

individuals:

- Most obvious possibility is sampling variation, i.e. the random statistical error that in inherent in any sampling process.
- Another possibility is that a shift in the number of SCEs in all the cells of the distribution has occurred, as might result from uniform exposure of lymphocytes or a systemic effect.
- Also the possibility is the presence of a small number of cells with exceptionally high or low SCE frequencies, due to exposure to a limited subset of the lymphocyte pool or a non-uniform response of the lymphocyte pool to a uniform insult.

In this study it was of utmost importance to minimise any such variables.

Cigarette smoke continues to represent a challenge to the biologists and chemists concerned with the effects on human health of complex mixtures such as cigarette smoke.

6.4 How is the damage caused and what does this mean?

The effect of age on chromosome aberrations in the general population is well documented (*e.g.* Tonomura *et al* 1983 and Galloway *et al* 1986) as mentioned in the literature review. Au *et al* (1991) also found that cigarette smoking enhances the age-dependent increase in chromosome aberrations. Age-dependent increase in development of disease (Jones *et al* 1975) and genetic outcome (Kram and Schneider 1978) have been documented. This information and data from studies by Au *et al* (1991) suggest that cigarette smoking may enhance these endogenous age-dependent deleterious processes. In addition, they found that both the amount and the duration of smoking contribute positively to the expression of chromosome aberrations.

Tobacco smoke condensate produces DNA lesions that result in SCE (Ghosh and Ghosh 1987) and there may be qualitative similarities between the response to tobacco tar of lymphocytes in virto and bronchial epithelium

in vivo. The level of carcinogenic ingredients that can react with cellular DNA may be particularly high in the respiratory airways of inhaling cigarette smokers. Epidemioligic studies indicate an association between cigarette smoking and lung cancer and there is increasing evidence to support the development of cancer as a result of somatic mutation (Hopkin and Evans 1980).

Vitamin intake has also been found to influence micronucleus frequencies in smokers. Au *et al* (1991) showed that the frequencies among female smokers who took vitamins were consistently reduced compared to those who did not take vitamins. Cigarette smoke is known to contain thousands of potentially hazardous chemicals including radioactive agents (Falk 1977). The formation of free radicals from radioactive and non-radioactive chemicals is probably one of the major pathways by which cigarette smoke causes genetic damage and cancer. Therefore, the supplementation of the diet with vitamins having free-radical-scavenging capability is a potentially useful approach to reduce genetic damage and to minimize adverse health outcomes from cigarette smoking.

It is also evident that cells from cigarette smokers may have DNA repair problems. A small increase in chromosome translocation frequencies has been detected in cells from smokers after exposure of lymphocytes to a single dose of X-rays (Au *et al* 1991). Data suggests that a major problem with cells from smokers is their delay in repairing damaged DNA compared with cells from nonsmokers. This may also explain the increase observed in this study of SCEs in smokers compared to non smokers as the proposed mechanisms for SCE formation suggests DNA repair and replication processes as being involved.

The significance of SCE induction in reflecting cancer proneness is still very speculative and evidence about the possible correlation is contradictory.

Husum *et al* (1981) examined SCEs in lymphocytes in peripheral blood in women with and without carcinoma of the breast and found no indication of an association of SCE frequency with malignancy/non-malignancy or the

tumor. Spontaneous SCE in lymphocytes is thus not an indicator of carcinoma of the breast.

Hopkin and Evans (1980) reported increased SCE rates in association with lung cancer. In vitro experiments showed a dose-related increase of SCE in human lymphocytes cultivated in three presence of cigarette smoke condensate, the increase being significantly greater in lymphocytes from lung cancer patients. Lung cancer has also been reported to be associated with normal SCE rates (Hollander *et al* 1978).

It is obvious that further studies need to be carried out on cigarette smoke exposure. Especially into the elusive mechanism of SCE formation which may in tern explain how chemicals cause genetic mutagenicity and whether this assists in cancer formation.

Another area where further research is required is into the use of the modified immunochemical assay for fast detection of DNA damage as described in the literature review earlier and by Timmerman *et al* (1995). This test appears to have several advantages over SCE and chromosome aberration assays in that it is simple, very quick (i.e. one hour) and also very sensitive. I would like to see a comparison study between this test, the SCE assay and chromosome aberrations and whether they compare with results found on effects of various mutagens among the population.

7. CONCLUSIONS

7.1 Vanillin

With respect to chromosome aberrations, there appears to be no effect of vanillin on chromosome aberrations. With this test you can only say that one variable is dependent on the other *i.e.* it does not show that chromosome aberrations are caused by vanillin, only that chromosome aberrations are dependent upon the vanillin concentration.

With respect to the affect of vanillin on SCE's, there is a significant effect of vanillin concentration of SCE's. As the vanillin concentration increased so did the number of SCEs per cell.

7.2 Smoking

Once again looking at chromosome aberrations there appears to be no difference between smokers and non smokers. With respect to SCE, there is a significant difference between smokers and non smokers in that there are more SCE in smokers compared with non smokers. Smokers were found to have a mean of 12.771 SCEs per cell compared to non smokers who had a mean of 9.712 per cell.

These results would suggest that there may be some effect of smoking and vanillin on the repair mechanisms of DNA as SCEs are thought to arise because of a fault in the DNA repair processes.

Appendix 1

PERSONAL HEALTH QUESTIONNAIRE

Please read the following questions carefully and answer them as thoroughly and accurately as possible. The information you give will not be associated with your name in any public document and will be known only to the principal investigator of this study. The answers you provide may have a direct bearing on the interpretation of our results. Therefore, we ask that you kindly cooperate fully in providing correct information. Thank you for your interest.

Name:.....

Address:.....

.....

.....

Contact phone no: .....

To be filled in by principal investigator:

Code Number:.....

Date:.....

This sheet is to be detached from the remainder of the questionnaire and filed by the principal investigator. Only the code number will be used as an identifier in subsequent pages. If additional space is needed for the completion of an answer, please write on the back of the page and identify the remaining part of the answer with the question number.

Code No. .....

Personal History

1. Date.....

2. What is your birth date? .....

3. What ethnic group do you consider yourself to belong to?

.....

Present and Past Occupational History

4. Are you currently employed? ❒ YES ❒ NO

5. What is the name of the company for which you now work or, if unemployed, last worked?

.....

6. For how long have you worked for this company?

.....

7. What type of work do/did you do?

.....

.....

.....

.....

.....

.....

.....

.....

Code No. .....

Exposure History (work and non-work related)

8. Have you ever been exposed to any of the following in your job?

		When were you first exposed? (month, year)	When were you last exposed? (month, year)	How long in terms of days, months, or years in total were you exposed?
Asbestos	❒ YES→			
	❒ NO			
Radiation	❒ YES→			
	❒ NO			
Coal products	❒ YES→			
	❒ NO			
Dust (such as wood, leather)	❒ YES→			
	❒ NO			
Pesticides Herbicides	❒ YES→			
	❒ NO			
Petroleum products	❒ YES→			
	❒ NO			
Dyes	❒ YES→			
	❒ NO			
Solvents	❒ YES→			
	❒ NO			
Other chemicals	❒ YES→ Specify in question No. 9.			
	❒ NO			

Appendix 1

Code No. .....

9. List the names of any specific substances to which you know you were exposed by either breathing or direct skin contact at work either in the last year or within the past 10 years.

In the last year (12 months)	How frequently exposed on a monthly average	Within the past 10 years	How frequently exposed on a monthly average

10. Please list any chemical or physical exposure you experienced in the last year while practising a hobby or other activities either at home or in other non-occupational settings. Refer back to the list in question No. 9. but do not limit your answers to only these substances.

In the last year (12 months)	How frequently exposed on a monthly average	Within the past 10 years	How frequently exposed on a monthly average

Code No. .....

Smoking History

11. Did you ever smoke? ❒ YES ❒ NO
If NO, go to Q 15.
If YES, go to Q 12.

12. Do you currently smoke? ❒ YES ❒ NO

If NO: How long did you smoke.....

When did you give up smoking?.....

If YES: go to Q 13.

13. How many packs do you smoke each day?

❒ less than half a pack
❒ half - 1 pack
❒ more than 1 pack:
If you smoke more than one pack a day please state how many:

.....

Do you smoke filtered cigarettes? ❒ YES ❒ NO

What is you usual brand?.....

14. Do you currently smoke cigars? ❒ YES ❒ NO

If YES: How many cigars do you smoke each day?
❒ 1 cigar
❒ 2-3 cigars
❒ 4 or more cigars

15. Do you currently smoke a pipe? ❒ YES ❒ NO

If YES: How many pipesful do you smoke each day?
❒ 1 pipeful
❒ 2-3 pipesful
❒ 4 or more pipesful

Code No. .....

16. What did you smoke in the past?

- ❒ cigarettes
- ❒ cigars
- ❒ pipe

17. Do you currently chew tobacco? ❒ YES ❒ NO

18. Do you smoke marijuana ❒ YES ❒ NO
If NO go to Q 19.

If YES, how many would you smoke?

- ❒ 1 or less per week
- ❒ 2-5 per week
- ❒ 6 or more per week

MEDICAL HISTORY

19. Have you taken any medication prescribed by a doctor in the past 1 year (or example, blood pressure pills, antibiotics, insulin, tranquillisers, muscle relaxants, etc.)?

❒ YES ❒ NO

If yes, please indicate below:

Type of medication	Dose	Frequency?	Time period: Began (month)	Ended (month)
.....				
.....				
.....				
.....				
.....				

Code No. .....

20. Have you taken any nonprescription medication *in the past 1 year* (for example, aspirin, antacids, antihistamines, sedatives, or other drugs?)

❒ YES ❒ NO

If yes, please indicate below:

Type of medication	Dose	Frequency	Time period Began (month)	Ended (month)
.....				
.....				
.....				
.....				
.....				

21. Do you take any vitamins currently or have you *in the past 6 months?*

❒ YES ❒ NO

If yes, please indicate:

What kind of vitamins	Dose	Frequency
.....		
.....		
.....		
.....		
.....		
.....		
.....		
.....		

Code No. .....

22. Have you ever had any of the following illnesses?

Cancer	❒ YES	❒ NO
Hepatitis	❒ YES	❒ NO
Mononucleosis	❒ YES	❒ NO
Herpes	❒ YES	❒ NO
AIDS	❒ YES	❒ NO
Meningitis	❒ YES	❒ NO
Bacterial or viral infections	❒ YES	❒ NO
Cardiovascular disease	❒ YES	❒ NO
Diabetes	❒ YES	❒ NO
Other major illness	❒ YES	❒ NO

If yes, please specify what illnesses, when you were ill, and indicate treatment.

Illness	Period of illness (month, year to month, year)	Treatment
.....	.....	.....
.....	.....	.....
.....	.....	.....
.....	.....	.....

23. List any other illness and their treatments you have experienced <u>in the past 12 months</u> (these should include colds, flu, etc)

Illness	Period of illness (month, year to month, year)	Treatment
.....	.....	.....
.....	.....	.....
.....	.....	.....
.....	.....	.....

Code No. .....

24. List any vaccinations which you have received in the past 12 months.

Type of vaccination Date administered

.....

.....

.....

.....

25. List any diagnostic or therapeutic X-rays other than dental you have received in the past 10 years.

Reason for X-ray Year received

.....

.....

.....

.....

26. Have you ever had any dental X-ray? ❒ YES ❒ NO

If yes within:

- ❒ the last month
- ❒ the last 6 months
- ❒ the last 6-12 months
- ❒ over one year ago

27. Have you had any surgery during the past year?

Date Reason

.....

.....

.....

Code No. .....

28. Give dates you have had any high fevers during the past year.

Date	Associated illness	Medications taken
.....	.....	.....
.....	.....	.....
.....	.....	.....

DIET HISTORY (Should only reflect current habits)

29. Do you eat vegetables? ❒ YES ❒ NO

30. Do you eat meat? ❒ YES ❒ NO

If yes, how often do you eat the following:

Days per week	1-2	3-4	5-6	Every day
Beef	❒	❒	❒	❒
Fish	❒	❒	❒	❒
Chicken	❒	❒	❒	❒
Pork	❒	❒	❒	❒
Other	❒	❒	❒	❒

How do you prefer your beef cooked?

❒ Rare ❒ Medium ❒ Well done

31. Do you use diet sweeteners? ❒ YES ❒ NO

How much per day or week? .....

32. Do you use diet drinks? ❒ YES ❒ NO

How many per day or week? .....

Code No. .....

33. Pertinent comments concerning diet not covered by above questions, e.g., special diet such as high protein, low carbohydrate, etc.

.....

.....

.....

.....

34. Do you drink coffee? ❒ YES ❒ NO

If yes, how much/day? .....

Decaffeinated? ❒ YES ❒ NO

35. Do you drink tea: ❒ YES ❒ NO

If yes, how much/day? .....

36. Do you drink beer? ❒ YES ❒ NO

If yes, please indicate your average weekly beer consumption:

- ❒ 1-6 cans (375 ml) a week or less
- ❒ 7-12 cans a week
- ❒ 13-24 cans a week
- ❒ more than 24 bottles a week, if this category is chosen, what is your average weekly beer consumption?
  ..... bottles or cans/week.

37. Do you drink wine? ❒ YES ❒ NO

If yes, please indicate your average weekly wine consumption:

- ❒ 1-4 glasses a week or less
- ❒ 5-8 glasses a week
- ❒ 9-16 glasses a week
- ❒ more than 16 glasses a week. If this category is chosen, what is your weekly wine consumption? ..... glasses/week.

Code No. .....

38. Do you drink other liquors (excluding beers and wines)?

❒ YES ❒ NO

If yes, please indicate your average weekly consumption for other liquors.

❒ 1-4 glasses (15 ml liquor) a week or less.

❒ 5-8 glasses a week

❒ 9-16 glasses a week

❒ more than 16 glasses a week. If this category is chosen, what is your average weekly consumption of other liquors?
..... glasses/week.

GENETIC HISTORY

39. Are you aware of any birth defects or other genetic disorders or inherited diseases which affect you parents, brothers, sisters, or their children?

❒ YES ❒ NO

If yes, please specify: .....

.....

40. Have you ever had difficulty conceiving (for a period of at least 12 months) or ever been diagnosed as infertile?

❒ YES ❒ NO

If yes, please specify (indicate when you experienced the difficulty or received the diagnosis):

.....

.....

37. Have you ever had children with birth defects or other genetic disorders or inherited disease? ❒ YES ❒ NO

If yes, please specify (indicate when the child was born and the nature of the disorder):

.....

Code No. .....

42. Have you ever had a still birth, a miscarriage, or a spontaneous abortion?

❒ YES ❒ NO

Thank you for your time

EFFECTS OF SMOKING ON SCE

	Number of SCEs														
	Non smokers					Smokers									
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	9	12	8	9	9	9	12	12	12	15	12	12	13	14	13
2	4	10	9	11	12	8	10	6	10	13	9	6	12	13	19
3	6	7	6	6	7	15	10	15	8	9	8	15	9	15	18
4	14	5	12	7	10	9	11	13	14	7	10	14	8	11	14
5	12	4	10	5	9	11	8	8	18	8	11	15	14	14	17
6	11	9	8	9	11	12	10	13	9	10	13	9	13	18	16
7	6	6	9	8	14	7	17	15	5	11	8	15	12	17	13
8	14	5	7	11	12	11	9	12	12	13	17	12	18	17	11
9	9	10	7	9	10	11	10	10	7	14	13	19	15	15	10
10	9	13	11	7	14	9	7	8	8	12	12	8	14	14	12
11	10	7	13	7	8	17	13	15	13	19	9	15	18	16	15
12	7	10	11	8	9	13	18	13	19	8	7	14	15	19	14
13	7	12	9	6	6	12	7	12	14	6	15	13	14	13	18
14	17	8	8	9	11	9	9	11	9	13	11	15	11	15	17
15	11	12	10	11	14	10	10	8	8	6	11	12	14	14	16
16	9	12	13	7	8	11	14	9	12	11	15	10	13	13	20
17	13	9	12	12	10	14	13	15	10	19	12	11	12	16	21
18	11	8	9	10	7	7	17	17	14	16	8	9	11	15	15
19	19	13	8	9	9	19	19	13	8	14	9	16	10	14	14
20	11	13	10	6	8	17	14	6	6	18	10	14	9	12	28

21	6	10	12	8	9	13	16	11	11	19	18	16	8	15	17
22	10	12	7	9	9	10	9	14	14	15	12	13	14	14	9
23	11	8	5	12	11	17	19	12	10	23	15	18	15	13	14
24	7	9	8	15	10	9	22	9	8	15	14	9	8	16	18
25	10	12	11	11	6	14	8	16	12	8	15	10	14	14	17
26	6	14	6	13	8	16	6	11	11	6	12	10	13	14	19
27	8	14	9	7	7	12	14	10	14	12	13	13	15	15	16
28	7	12	10	11	10	17	12	13	18	9	10	15	16	13	15
29	9	16	12	9	8	9	8	20	17	15	10	20	14	15	13
30	11	7	8	13	7	8	16	15	6	13	11	21	18	14	13
31	7	12	6	12	7	11	12	19	19	7	9	11	13	13	16
32	10	10	9	8	11	15	9	7	7	10	8	10	9	12	17
33	7	11	7	9	19	16	18	9	5	11	13	11	18	11	11
34	12	8	8	7	14	13	16	13	19	11	12	14	15	10	19
35	10	9	8	11	13	10	14	8	12	14	13	13	20	10	21
36	7	12	12	13	10	12	9	11	15	12	15	15	18	19	14
37	9	7	11	12	8	18	8	10	14	13	8	13	17	16	16
38	8	5	10	5	9	11	16	8	11	12	9	16	18	14	15
39	8	4	9	8	15	13	14	14	10	6	11	14	14	15	13
40	10	9	8	7	12	16	14	9	8	15	14	15	17	13	15
41	9	6	11	11	10	8	18	10	12	13	12	12	15	15	14
42	12	5	12	7	7	6	17	11	11	11	10	10	13	14	18
43	14	10	10	9	9	10	8	10	9	10	16	11	13	12	17
44	8	13	9	6	8	15	14	7	7	8	9	16	15	15	15
45	7	7	8	10	8	8	9	15	10	6	7	15	14	14	13
46	9	10	7	11	10	7	7	13	14	9	7	9	14	12	16

47	12	12	9	14	9	9	10	13	13	7	11	8	15	12	12
48	13	13	7	13	7	12	12	10	15	13	15	10	17	15	15
49	11	10	7	7	14	14	14	9	12	14	9	13	13	14	14
50	8	8	10	9	12	12	11	12	9	12	13	10	18	13	18
51	10	9	13	11	11	13	15	11	8	7	8	9	18	16	16
52	7	12	15	8	13	12	13	9	11	14	9	7	15	15	15
53	9	7	12	12	9	16	19	7	15	13	10	8	15	17	13
54	10	8	10	9	14	8	6	13	13	8	12	13	17	14	17
55	8	6	9	13	13	6	14	9	10	15	14	14	17	18	16
56	9	5	11	10	8	9	13	13	17	13	13	12	17	11	14
57	9	12	14	12	9	10	15	10	15	8	12	11	14	10	21
58	11	10	12	12	7	10	13	16	7	11	11	15	12	18	19
59	13	11	9	11	13	11	16	17	5	16	10	11	15	10	18
60	10	10	7	10	12	13	15	9	13	7	16	10	13	18	15
61	12	12	10	11	8	13	18	7	11	5	10	9	14	19	14
62	14	12	7	13	10	14	11	4	10	8	17	18	16	15	13
63	12	9	12	9	7	12	13	12	12	13	15	16	15	14	17
64	8	7	12	7	9	9	12	10	9	12	11	17	17	16	15
65	9	8	10	10	12	13	10	8	7	7	13	11	13	17	16
66	7	10	9	8	14	10	8	6	11	9	10	14	15	18	14
67	10	13	8	6	8	9	9	19	15	10	12	16	14	14	18
68	9	12	12	8	11	8	14	8	13	13	8	13	17	13	13
69	7	8	10	12	10	16	19	9	17	14	9	15	16	17	12
70	10	9	11	10	9	14	15	13	19	11	11	14	15	15	16
71	13	10	9	7	9	12	12	8	9	10	15	14	14	15	14
72	8	12	8	13	8	17	10	10	11	14	14	13	13	16	17

73	10	7	10	7	9	11	16	11	10	6	9	12	19	18	25
74	8	5	12	9	11	9	11	7	4	8	13	10	14	17	14
75	6	9	8	11	14	19	17	8	12	5	13	11	16	19	16
76	9	10	9	14	12	10	12	13	17	13	15	19	14	18	15
77	10	11	8	12	15	13	15	6	9	11	12	15	10	17	18
78	8	6	10	15	14	10	13	11	7	14	11	17	20	14	17
79	5	8	11	13	12	15		19	10	10	12	17	14	13	12
80	11	5	16	10	10	9	10	5	13	11	10	14	13	12	16
81	10	10	10	9	6	8	15	11	12	18	10	11	21	11	14
82	6	6	9	7	14	11	17	10	15	16	11	10	15	13	16
83	11	5	13	11	13	7	9	9	16	9	18	18	14	12	15
84	9	11	12	9	11	15	12	5	13	7	8	14	8	14	17
85	13	13	10	6	16	12	10	18	14	15	9	13	15	14	18
86	11	12	8	12	10	13	8	14	14	12	14	18	14	13	14
87	9	8	9	14	7	12	14	9	19	8	13	13	13	15	18
88	9	6	11	13	13	15	6	11	10	16	15	17	16	17	12
89	14	7	8	9	12	9	8	6	11	8	14	15	15	18	15
90	6	11	9	8	14	10	5	8	18	11	16	13	14	14	13
91	11	10	12	11	10	11	19	10	15	14	16	17	16	16	17
92	15	11	9	10	9	13	11	16	10	9	17	12	13	17	15
93	12	6	7	8	8	8	14	8	6	8	9	14	15	20	18
94	6	7	12	7	10	7	5	13	14	13	10	13	16	12	15
95	9	7	7	14	7	9	10	8	7	11	14	16	14	20	13
96	4	10	8	13	14	10	13	6	6	10	13	14	13	21	17
97	7	9	6	12	9	13	12	10	9	12	13	12	16	18	15
98	10	9	11	10	8	12	6	15	13	8	9	18	14	15	16

APPENDIX 2

99	12	6	12	11	14	14	18	7	12	9	11	15	13	19	18
100	7	11	8	8	9	16	7	5	19	15	7	14	11	14	14

EFFECTS OF SMOKING ON CA

Non smokers

	exch	break	c/s	exch	break	c/s	exch	break	c/s	exch	break	c/s	exch	break	c/s
1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
12	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
14	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
16	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
20	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

27	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Smokers														
	exch	break	c/s	exch	break	c/s	exch	break	c/s	exch	break	c/s	exch	break	c/s
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
9	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
12	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
13	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
15	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

24	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1
25	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
12	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
13	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
16	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
17	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
21	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0

22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

break = breaks

Exch = exchanges

c/s = chromosome type aberrations

APPENDIX 4

EFFECTS OF VANILLIN ON SCE

Number oF SCEs

	Negative vanillin	0.1 mM vanillin	0.5 mM vanillin	1.0 mM vanillin	1.5 mM vanillin	2.0 mM vanillin	positive control*
1	6	3	7	6	16	16	16
2	3	2	5	10	11	18	11
3	6	6	8	6	10	14	14
4	3	7	6	7	6	18	20
5	4	5	9	8	9	15	28
6	3	5	10	9	8	17	27
7	2	4	7	10	17	18	18
8	5	10	8	11	11	14	15
9	4	6	6	6	11	16	22
10	5	7	5	8	12	14	25
11	7	5	8	7	7	18	32
12	3	7	9	7	11	17	22
13	4	6	10	10	13	13	11
14	6	6	6	11	11	19	16
15	5	5	6	9	12	18	18
16	7	5	8	12	10	17	13
17	8	5	7	10	8	16	22
18	5	4	9	11	11	19	21
19	6	8	9	13	11	17	16
20	4	5	6	8	10	18	17
21	7	7	9	9	12	16	19
22	5	6	8	12	8	19	20
23	4	6	10	13	9	14	21
24	4	6	11	9	12	18	19
25	4	5	6	8	9	15	23
26	3	7	12	7	10	16	18
27	7	5	7	10	11	16	16
28	6	7	5	7	11	15	17
29	11	4	8	11	12	18	19
30	8	12	10	6	9	19	24
31	8	7	9	13	10	19	22
32	6	6	9	7	13	17	25
33	9	5	12	8	12	16	19
34	5	5	8	10	11	14	26
35	6	7	6	9	9	19	17
36	7	9	9	6	13	20	23
37	3	6	9	12	11	16	28

APPENDIX 4

38	4	4	11	8	12	18	32
39	5	6	7	7	10	21	13
40	5	6	6	9	9	17	16
41	4	11	9	8	13	19	17
42	6	9	12	10	15	19	22
43	6	8	14	11	12	16	19
44	7	8	8	6	15	9	15
45	7	4	7	7	13	12	26
46	8	9	6	11	12	15	31
47	6	8	7	10	10	17	27
48	5	10	6	9	8	14	28
49	4	7	4	7	6	12	21
50	4	9	3	11	7	10	26
51	3	10	5	8	11	16	18
52	6	13	7	10	14	14	17
53	5	7	8	6	10	17	20
54	5	8	6	7	8	18	20
55	4	6	10	9	7	14	19
56	3	5	7	11	14	15	16
57	2	6	8	8	12	16	23
58	7	12	5	7	10	17	32
59	7	5	10	7	13	18	30
60	5	7	8	10	11	14	26
61	6	7	5	9	10	15	29
62	7	5	7	12	9	13	27
63	6	14	6	10	7	12	22
64	6	7	12	13	6	17	24
65	5	6	9	10	8	20	20
66	7	8	9	9	9	14	16
67	4	9	6	7	9	12	19
68	8	11	8	8	10	11	18
69	4	7	12	10	12	16	20
70	3	13	5	6	8	18	29
71	4	8	8	12	9	14	26
72	5	6	6	16	12	17	25
73	7	8	8	7	11	19	24
74	6	6	7	8	9	20	14
75	5	5	10	7	13	14	11
76	4	4	7	7	9	13	16
77	6	7	9	5	10	17	22
78	6	5	6	6	8	16	13
79	7	6	8	9	8	18	32
80	9	10	5	8	6	19	15

81	11	4	7	12	9	19	22
82	7	6	5	11	12	12	11
83	6	8	6	10	11	13	31
84	5	7	6	10	9	11	25
85	5	5	8	9	12	15	24
86	4	5	11	11	8	19	27
87	6	8	12	5	9	16	28
88	3	6	9	6	12	15	20
89	5	11	7	7	12	10	25
90	8	7	6	9	10	11	19
91	4	6	7	12	8	13	13
92	9	8	5	11	7	15	21
93	9	4	12	13	5	18	22
94	6	7	6	9	11	17	16
95	7	5	5	6	12	19	15
96	4	5	8	7	10	16	18
97	7	6	7	11	15	12	27
98	7	4	13	7	9	15	19
99	6	8	10	9	8	13	17
100	8	10	12	8	7	16	23

* 0.01% styrene oxide

8. REFERENCES

Alberman, E., M. Creasy and M. Elliot (1976) Maternal factors associated with fetal chromosomal anomalies in spontaneous abortions, J. Obstet. Gynaecol. Br. Commonw., 83, 621-627.

Ammenheuser, M.M., A.B. Berenson, N.J. Stiglich, E.B. Whorton Jr., J.B. Ward Jr., (1994) Elevated frequencies of *hprt* mutant lymphocytes in cigarette-smoking mothers and their newborns, Mut. Res.320, 285-292.

Anderson R, AR Rabson, R Sher and HJ Koornhof (1974) The NBT test in cigarette smokers, Am. J. Clin. Pathol. 61, 878.

Andresen, BD., KJ Ng, JD Iams and JR Bianchine (1982) Cotinine in amniotic fluids from passive smokers, Lancet, I, 791-792.

Ardiot, G., L. Lamberti, E. Ansaldi, P. Ponzetto (1980) Sister chromatid exchanges in cigarette smoking human females and their newborns. Mut. Res., 78, 209-214.

Au, W., D.M. Walker, J.B. Ward Jr, E. Whorton, M.S. Legator and V. Singh (1991) Factors contributing to chromosome damage in lymphocytes of cigarette smokers. Mut. Res., 260, 137-144.

Bala Krishna Murphy and Prom (1979) SCEs in oral contraceptive users, Mut. Res., 68, 149-152.

Bender, M.A. R.J. Preston, R.C. Leonard, B.E. Pyatt, P.C. Gooch (1989) Chromosomal aberrations and SCE frequencies in peripheral lymphocytes of a large human population sample: II extension of age range, Mut. Res., 212, 149-154.

Bender, M.A., R.J. Preston, R.C. Leonard, B.E. Pyatt and P.C. Gooch, (1992a) On the distribution of spontaneous SCE in human peripheral blood lymphocytes, Mut. Res., 281, 227-232.

Bender, M.A., R.J. Preston, R.C. Leonard, B.E. Pyatt and P.C. Gooch, (1992b) Influence of white blood cell count on SCE frequency in peripheral lymphocytes, Mut. Res., 283, 87-89.

Benedict, W.F., N. Rucker, J. Faust and R.E. Kouri (1975) Malignant transformation of mouse cells by cigarette smoke condensate, Cancer Res., 35,857-860.

Betti, C., (1994) Microgel electrophoresis assay (comet test) and SCE analysis in human lymphocytes from 100 normal subjects, Mut. Res., 307, 323-333.

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

Bock, F.G., A.P. Swain and R.L. Stedman (1971) Composition studies on tobacco, XLIV, Tumor-promoting activity of cubfractions of the weak acid fraction of cigarette smoke condensate, J. Natl. Cancer Inst., 47, 429-436.

Bostock CJ, Christie S (1976) Analysis of the frequency of sister chromatid exchange in different regions of chromosomes of the kangaroo rat (*Dipodomys ordii)* . Chromosoma 56, 275-278.

Bottoms, S.F., B.R. Kuhnert, P.M. Kuhnert and A.L. Reese (1982) Maternal passive smoking and feotal serum thiocyanate levels, Am J Obstet. Gynecol., 144,787-791.

Brandom, W.F., G. Saccomanno, V.E. Archer, P.G. Archer and A.D. Bloom (1978) Chromosome aberrations as a biological dose-response indicator of radiation exposure in uranium miners, Radiat. Res., 79, 159-171.

Bridges, B.A., J. Clemmesen and T. Sugimura (1979) Cigarette smoking - Does it carry a genetic risk? Mut. Res., 65, 71-81.

Brögger, A. (1982) The chromatid gap - a useful parameter in genotoxicology?, cytogenet. Cell Genet., 33, 14-19.

Carrano A.V., J.L. Minkler,D.G. Stetka and D.H. Moore II (1980) Variation in the baseline SCE frequency in human lymphocytes, Environ. Mutagen., 2, 325-337.

Carrano, A.V. (1982) SCE as an indicator of human exposure, in: B.A. Bridges, B.E. Butterworth and I.B. Weinstein (Eds), Branbury Report 13, Indicators of Genotoxic Exposure, Cold Spring Harbour Laboratory, New York, pp307-318.

Carrano, A.V. and A.T. Natarajan (1988) IPEMC: Considerations for population monitering using cytogenetic techniques, Mut. Res., 204, 379-406.

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

Changanti R.S.K., S. Schonberg, and J. German (1974) A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. Proc. Natl. Acad. Sci (USA) 74,4508-4512.

Cleaver, J.E. (1981) Correlations between sister chromatid exchange frequencies and replicon sizes. A model for the mechanism of SCE production, Exp. Cell Res., 136, 27-39.

Cnattingius, S.,B. Haglund, and O. Meirik (1988) Cigarette smoking as risk factor for late foetal and early neonatal death, Br. Med. J., 197, 258-261.

Cole, P.V., L.H. Hawkins and D. Roberts (1972) Smoking during pregnancy and its effects on the foetus, J. Obstet, Gynaecol, Br, Commonw., 79, 782-787.

Cone F., J. Lellouch and D. Schwarts (1971) Smoking and leucocyte counts: results of an epidemiological study, Lancet, 2, 632-634.

Crossen, P.E., and W.F. Morgan (1980) Sister chromatid exchange in cigarette smokers, Hum. Genet., 53, 425-426.

Csillag, C., P. Aldhous (1992) Signs of Damage by Radicals, Science, 258, 1875-1876.

Davidson R.L., E.R. Kaufman, C.P. Dougherty, A. Ouellette, C.M. DiFolco , S.A. Latt (1980) Induction of sister chromatid exchanges by BUdR is largely indepepdnet of the BUdR content of DNA. Nature 284,74-77.

Davies, P., G.S. Kistler, C. Leuchtenberger and R. Leuchtenberger (1975) Ultrastructural studies on cells of hamster lung cultures after chronic exposure to whole smoke or the gas vapour phase of cigarettes, Beitr. Path. Bd., 155, 168-180.

De Raat, W.K., (1977) The induction of sister chromatid exchanges by cyclophosphamide in the presence of differently induced microsomal fractions of rat liver, Chem. Biol. Interactions 19, 125-131.

De Weerd-Kastelein, E.A., W. Keijzer, G. Rainaldi, D. Bootsma (1977) Induction of sister chromatid exchanges in xeroderma pigmentosum cells after exposure to ultraviolet light, Mut. Res., 45, 253-261.

De Marini, D.M. (1981a) Mutagenicity of fractions of cigarette smoke cindensate in *Neurospora crassa* and *Salmonella typhimurium,* Mut. Res., 88, 363-374.

De Marini, D.M. (1981b) Mutagenicity of cigarette smoke condensate in *Neurospora crassa,* Mut. Res., 66,253-259.

De Marini, D M. (1983) Genotoxicity of tobacco smoke and tobacco smoke condensate, Mut. Res., 114, 59-89.

Dilehay, L.E., S.C. Denstman and J.R. Williams (1987) Cell cycle dependence of sister-chromatid exchange induction by DNA topoisomerase II inhibitors, Cancer Res., 47, 106-110.

Dilehay, L.E., D. Jacobson-Kram and J.R. Williams (1989) DNA topoisomerases and models of sister-chromatid exchange, Mut. Res., 215, 15-23.

Department of Health and Department of Statistics (1991) Tobacco statistics 1991: Trends in Tobacco consumption and smoking prevalence in NZ.

D'Souza, D., I.M. Thomas, and B.C. Das (1988) Variation in spontaneous chromosomal damage as a function of biologic rhythms in women, Human Genet. 79, 83-85.

Evans, H.J. and M.L. O'riordan (1975) Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests, Mut, Res. 31, 135-148.

Evans, J.J., J. Fletcher, M. Torrance and T.B. Hargreave (1981) Sperm abnormalities and cigarette smoking, Lancet, 1, 627-629.

Falk, H.L. (1977) Chemical agents in cigarette smoke, in:D.H.K. Lee (Ed.), Handbook of Physiology: Reaction to Environmental Agents, Waverly, Baltimore, MD, pp. 199-211.

Funes-Cravioto, F., C. Zapate-Gayon, B. Kilmodin-Hedman, B. Lambert, J. Lindsten, E. Norberg, M Nordenskjold, R. Olin, A. Swensson, (1977) Chromosome aberrations and sister chromatid exchange in workers in chemical laboratories and a rotoprinting factory and in children of women laboratory workers, Lancet II, 322.

Galloway, S.M., and H.J. Evans (1975) Sister chromatid exchange in human chromosomes from normal individuals and patients with atazia telangiectasis. Cytogenet. Cell. Genet, 15, 17-29

Galloway, S.M., H.J. Evans (1981) Sister chromatid exchange in human chromosomes form normal human lymphocytes. Mut. Res., 81, 395.

Galloway, S.M., P.K. Berry, W.W. Nichols, S.R. Wolman, K.A. Soper, P.D. Stolley and P. Archer (1986) Chromosome aberrations in individuals occupationally exposed to ethylene oxide, and in a larger control population, Mutation Res., 170, 55-74.

Gairola, C. (1982) Genetic effects of fresh cigarette smoke in *Saccharomyces cereviciae* , Mut. Res., 102, 123-136.

Gerbhart, E. (1981) Sister chromatid exchange (SCE) and structural chromosome aberrations in mutagenicity testing. Hum. Genet. 58:235-254

Godfrey, B., (1981) Sperm morphology in smokers, Lancet, 1, 894-896.

Ghosh, R., and P.K. Ghosh (1987) The effect fo tobacco smoking on the frequency of SCE in Human Lymphocyte chromosomes, Canc. Genet. and Cytogenet., 27, 15-19.

Gosh, P.K., Nand R., (1979) Reduced frequencies of sister chromatid exchanges in human lymphocytes cultured in autologous serum, Hum Genet 51,167-170.

Guerrero, R.E., D.E. Rounds and T.C. hall (1979) Bioassay procedure for the detection of mutagenic metabolites in human urine with the use of sister chromatid exchange analysis, J. Natl. Cancer Inst., 62, 805-809.

Hedner, K., B. Högstedt, A. Kolnig, E. Mark-Vendel, B. Strömbeck and R. Mitelman (1982) Sister chromatid exchange and structural chromosome aberrations in relation to age and sex, Hum. Genet., 62, 305-309.

Hedner, K., B. Högstedt, A.-M. Kolnig, E. Mark-Vendel, B. Strömbeck and F. Mitelman (1983) Sister chromatid exchanges and structural chromosome aberrations in relation to smoking in 91 individuals, Hereditas 98, 77-81.

Heim, S., B. Johansson, F. Mertens (1989) Constitutional chromosome instability and cancer risk, Mut. Res., 221, 39-51.

Heim,S., and F. Mitelman (1987) Cancer Cytogenetics, Alan R. Liss, New York, pp 309.

Hirsch, S., K.K. Sentz, M. McGue (1992) Genetic and environmental influences on base-line SCE, Environ. Mol. Mutagen 20, 2-11.

Hoffmann, D., and E.L. Wynder (1976) Environmental respiratory carcinogenesis, in: C.E. Searle (Ed.), Chemical Carcinogens, American Chemical Socitey, Washington, DC, pp324-365.

Holden, H.E., J.F. Barett, C.M. Huntingtion, P.A. Muehlbauer and M.G. Wahrenburg (1989) Genetic profile of nalidixic acid analog: a model for the mechanism of sister-chromatid exchange induction, Environ. Mol. Mutagen., 13, 238-252.

Hollander, D.H., M.S. Tockman, Y.W. Liang, D.S. Borgaonkar and J.K. Frost (1978) Sister chromatid exchanges in the peripheral blood of cigarette smokers and in lung cancer patients; and the effect of chemotherapy, Hum. Genet., 44,165-171.

Hollinshead, W.H. (1979) The consequences of smoking in pregnancy, Rhode Island Med. J., 62, 207-212.

Hopkin, J.M. and H.J. Evans (1979) Cigarette smoke condensates damage DNA in human lymphocytes, Nautre 279, 241-242.

Hopkin, J.M. and H.J. Evans (1980) Cigarette smoke induced DNA damage and lung cancer risk. Nature 283, 388-390.

Hsu T.C., S. Pathak (1976) Differential rates of sister chromatid exhanges between euchromatin and heterochromatin. Chromosome 58, 269-273, 1976.

Husgafvel-Pursiainen, K., J. Mäki-Paakkanen, H. Norppa, M. Sorja (1980) Smoking and sister chromatid exchange, Hereditas 92, 247-250.

Husgafvel-Pursianinen, K., K. Koskimies and M. Sorsa (1988) Tobacco smoke exposure during pregnancy, Hereditas 108, 118-124.

Husum, B., H.C. Wulf, E. Niebuhr (1981) Sister-chromatid exchanges in lymphocytes in women with cancer of the breast, Mut. Res., 85, 357-362.

Husum, B., H.C. Wulf, E. Niebuhr (1982) Increased SCE frequency in lymphocytes in healthy cigarette smokers, Hereditas, 96, 85-88.

Husum, B., H.C. Wulf, E. Niebuhr (1986) SCE frequency correlates with age, sex and cigarette smoking in a 5-year material of 553 healthy adults, Hereditas, 105, 17-21.

Hutton, J.J., and C. Hackney (1975) Metabolism of cigarette smoke condensates by human and rat homogenates to form mutagens detectable by *Salmonella typhimurium* TA1538, Cancer Res., 35, 2461-2468.

IARC (1987a) Monographs on the Evaluation of Carcinogenic Risks to Humans, Overall Evaluations of Carconogenicity: An Updating of IARC Monographs Volumes 1 to 42, International Agency for Research on Cancer, Llyon, Supplement 7.

IARC (1987b) Monographs on the Evaluation of Carcinogenic Risks to Humans, Genetic and Related Effects: An Updating of IARC Monographs Volumes 1 to 42, International Agency for Research on Cancer, Llyon, Supplement 7.

Jones, K.L., D.W. Smith, M.A.S. Harvey, B.D. Hall and L. Quan (1975) Older paternal age and fresh gene mutation, data on additional disorders, J. Pediat., 86, 84-88.

Jansson, T., M. Curvall, A. Hedin and C.R. Enzell (1986) In vitro studies of biological effects of cigarette smoke condensate: II. Induction of sister-chromatid exchanges in human lymphocytes by weakly acidic, semivolatile constituents, Mut. Res., 169, 129-139.

Jansson, T., and L. Zech (1987) Effects of vanillin on SCE and chromosome aberrations in human lymphocytes, Mut. Res., 190, 221-224.

Jansson, T., M. Curvall, A. Hedin and C.R. Enzell (1988) In vitro studies of the biological effects of cigarette smoke condensate. III. Induction of SCE by some phenolic and related constituents derived from cigarette smoke: A study of structure-activity relationships, Mut. Res., 206, 17-24.

Joseph-Lerner, N.M. Fejgin, I. Ben-Nun (1993) The correlation between the frequency of SCE and human reproductive hormones, Mut. Res. 300, 247-252.

Kao-Shan C., R.L. Fine, J. Whang-Peng, E.C.Lee, and B.A. Chabner (1987) Increased fragile sites and SCE in bone marrow and peripheral blood of young cigarette smokers, Cancer Res., 47, 6278-6282.

Kato, H., N.F. Stich (1976) Sister chromatid exchanges and repair deficient human fibroblasts. Nature 260, 447-448.

Kelsey, J.L., T. Dwyer, T.R. Holford and M.B. Bracken (1978) Maternal smoking and congenital malformations: An epidemiological study, J. Epidemiol. Commun. Health, 32, 102-107.

Kier L.D., E. Yamasaki, B.N. Ames (1974) Detection of mutagenic activity in cigarette smoke condensates. Proc. Natl. Acad. Sci (USA), 71, 4159-4163.

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

Kram, D., and E.L. Schneider (1978) An effect of reproductive ageing: increased risk of genetically abnormal offspring, in: E.L. Schneider (Ed.), The Ageing Reproductive System, Rowen, New York, pp. 237-270.

Knuutila, S., A. Harkki, K. Ellimaki, R. Salunen (1979) Decreased sister chromatid exchange in Down's syndrome after measles vaccination, Hereditas 90, 147-150.

Kurvink K., C.D. Bloomfield, J. Cervenka (1978) Sister chromatid exchange in patients with viral disease, Exp. Cell Res. 113,450-454.

Lambert, B.O., A. Lindblad, M. Nordenskjolk, B. Werelius (1978) Increased frequency of SCE in cigarette smokers, Hereditas, 88, 147-149.

Lambert B., A. Ehrnst, K. Hansson, A. Linbald, M. Morad, B. Werelius (1979) Sister chromatid exchanges in peripheral lymphocytes of subjects vaccinated against measles. Hum Genet 50, 29-32.

Lambert, B, I Berntsson, J Lindsten, M Nordenskjöld, S Söderhäll, B Holmstedt, L Palmér, B Jernström and L Marsk (1982) Smoking and sister chromatid exchange, in M Sorsa and H Vianio (Eds) Mutagens in Our Environment. Progress in Clinical and Biological Research, Liss, New York, pp 401-414

Lasnitzki, I. (1958) Observations on the effects of condensates from cigarette smoke on human foetal lung in vitro, Br. J. Cancer, 12,547-552.

Lasnitzki, I. (1968) The effect of hydrocarbon-enriched fraction of cigarette smoke condensate on human foetal lung grown in vitro, Cancer Res., 28, 510-516.

Lähdetie, j., K. Engström, K. Husgafvel-Pursiainen, L. Nylund, H. Vainio and M. Sorsa (1993) Mut. Res., 37-43.

Latt, S.A. (1973) Microfluorometric detection of deoxyribonucleic acid replication in human metaphase chromosomes, Proc. Natl. Acad. Sci. (USA), 70, 3395-3399.

Latt S.A., G. Stetten, L.A. Juergens, G.R. Buchanan and P.S. Gerald (1975) Induction by alkylating agents of sister chromatid exchanges and chromatid breaks in Fanconi's anemia. Proc. Natl. Acad. Sci. (USA) 72: 4066-4070

Latt, S., J.W. Allen, S.E. Bloom, A.V. Carrano, E. Falke, D. Kram, E. Schneider (1981) Sister Chromatid Exchanges, Mut. Res., 87, 17-62.

Lehman, A.R., S. Kirk-Bell, C.F. Arlett, M.C. Paterson, P.M.M.Lohman, E.A. de Weerd-Kastelein, Bootsma S., (1975) Xeroderma pigmentosum cells with normal levels of excision repair have a defect in DNA synthesis after UV-irradiation, Proc. Natl. Acad. Sci. (USA), 72, 219-223.

Leuchtenberger, C., R. Leuchtenberger and A. Schneider (1973) Effects of marijuana and tobacco smoke on human lung physiology, Nature (London), 241, 137-139.

Liu, L.F., C.C. Liu and B.M. Alberts (1980) Type II DNA topoisomerases: enzymes that can unknot a topologically knotted DNA molecule via a reversible double-strand break, Cell, 19, 697-707.

Lundgren, K., W.L. Lucier (1985) Differential enhancement of sister-chromatid exchange frequencies by a-napthoglavone in cultured lymphocytes from smokers and non-smokers, Mut. Res., 143,131-135.

Mau, G., and P. Netter (1974) Die Auswirkungen des vaterlichen Zigarettenkonsums auf die perinatale Sterblichkeit und die Missbildungshäufigkeit, Deut. Med. Wochschr., 99, 1113-1118.

Mazrimas J.A., D.G. Stetka (1978) Direct evidence for the role of incorporated BUdr in the induction of Sister chromatid exchanges. Exp Cell Res 117,23-25.

Mizusaki, S., H. Okamoto, A. Akiyama and Y. Fukuhara (1977a) Relation between chemical constituents of tobacco and mutagenic activity of cigarette smoke condensate, Mut. Res., 48, 319-326.

Mizusaki, S., T. Takashima and K. Tomaru (1977b) Factors affecting mutagenic activity of cigarette smoke condensate in *Salmonella typhimurium* TA1538, Mut. Res., 48, 29-36.

Morgan W.F., and P.E. Crossen (1977) The incidence of sister chromatid exchanges in cultured human lymphocytes. Matat Res 42, 305-312.

Morgan W.F., P.E. Crossen (1981) Factors influencing sister chromatid exchange rate in cultured human lymphocytes, Mut. Res., 81, 395-401.

Murthy, P.B.K. (1979) Frequency of sister chromatid exchanges in cigarette smokers, Hum. Genet., 52, 343-345.

Nakayama, T., M. Kaneko, M. Kodama, C. Nagata (1985) Cigarette smoke induces DNA single stranded breaks in human cells, Nautre 314, 462-464.

National Research Council (1986) Environmental Tobacco Smoke. Measuring Exposures and Assessing Health Effects, National Academy Press, Washington, DC, pp 1-337.

Nordenson, I., G. Beckman, L. Beckman and S. Nordstrom (1978) Occupational and environmental risks in and around a smelter in northern Sweden, II. Chromosomal aberrations in workers exposed to arsenic, Hereditas, 88, 47-50.

Obe, G., B. Beek, G. Dudin (1975) the human leucocyte test system. V. DNA synthesis and mitosis in PHA stimulated 3-day cultures, Human genetik 28, 295-301.

Obe, G., and J. Herha (1978) Chromosomal aberrations in heavy smokers, Hum. Genet., 41,259-263.

Ozkul, Y., A. Erenmemisogly, N. Cucer, A. Menevse, C. Saatci (1995) Sister-chromatid exchange inducing effect of smokeless tobacco using on T-lymphocyte chromosomes.

Pandey, K.N., J.F. Benner and P.S. Sabharwal (1978) Cytogenetic effects of the gaseous phase of cigarette smoke on root-tip cells of *Allium sativum* L., Environ. Exptl. Bot., 18, 67-75.

Painter, R.B. (1980) A Replication Model for Sister-Chromatid Exchange, Mut. Res., 70, 337-341.

Perry P., and H.J. Evans (1975) Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature 258,121-125.

Perry, P., and S. Wolff (1974) New Giemsa method for the differential staining of sister chromatids, Nature (London), 258, 121-125.

Pescitelli, A.R. (1979) Mutagenicity of cigarette smoke condensate and cigarette smoke in *Drosophila melanogaster*, Environ. Mutagen., 1,118.

Pommier, Y., L.A. Zwelling, C.-S. Kao-Shan, J. Whang-Peng and M.O. Bradley (1985) Correlations between intercalator-induced DNA strand breaks and sister-chromatid exchanges, mutations, and cytoxicity in Chines hamster cells, Cancer Res., 45, 3143-3149.

Rasmussen, R.E., C.H. Boyd, D.R. Dansie, R.E. Kouri and C.J. Henry (1981) DNA replication and unscheduled DNA synthesis in lungs of mice exposed to cigarette smoke, Cancer Res., 41, 1583-1588.

Rees, E.D., A. Shuck, J. Lowry, H. Lipscomb and R. Wolfe (1973) Chromosomal aberrations and neoplasms - slight effect of two smoke fractions, Tobacco and Health Workshop Conference Proceedings, 4th, pp. 188-197.

Reif, A.E. (1958) International Cancer Congress, Science, 128, 1512-1522.

van Rensburg, C.E.J., A. Theron, G.A. Richards, C.A. van der Merwe, R. Anderson (1989) Investigation of the relationships between plasma levels of ascorbate, vitamin E and ß-carotene and the frequency of SCEs and release of reactive oxidants by blood leucocytes from cigarette smokers, Mut. Res., 215, 167-172.

Reuterwall, C. (1990) A Nordic data base on somatic chromosome damage in humans: Nordic Study group on the Health Risk of Chromosome Damage, Mut. Res., 241, 325-337.

Rhim, J.S., and R.J. Huebner (1973) In vitro transformation assay of major fractions of cigarette smoke condensate (CSC) in mammalian cell lines (37162), Proc. Soc. Expt. Biol. Med., 142, 1003-1007.

Royal college of Physicians (1977), Smoking or Health, Pitmans, London.

San Sebastian J.R., J.P. O'Neil, A.W. Hsie (1980) Induction of chromosome aberrations, sister chromatid exchanges, and specific locus mutations in Chinese hamster ovary cells by 5-bromodeoxyuridine. Cytogenet Cell Genet 28,47-50.

Sasaki, M.S. (1982) in A.A. Sandberg (Ed) Progress and Topics in Cytogenetics Volume 2; Sister Chromatid Exchange, Alan R. Liss, Inc., New York, pp 135-161.

Sato, S., Y. Seino, T. Ohaka, T. Yahagi, M, Nagao, T. Matsushima and T. Sugimura (1977) Mutagenicity of smoke condensates from cigarettes, cigars and pipe tobacco, Cancer Lett., 3, 1-8.

Sato, S., T. Ohka, M.Nagao, K. Tsuji and T. Kosuge (1979) Reduction in mutagenicity of cigarette smoke condensate by added sugars, Mut. Res., 60, 155-161.

Schnedl W., W. Pumberger, R. Czaker, P. Wagenbichler, H.G. Schwarzacher (1976) Increased sister chromatid exchange events in the human late replicating X. Hum Genet 32, 199-203.

Schubert I., R. Reiger (1981) Sister chromatid exchanges and heterochromatin. Hum Genet 57, 119-123.

Schuler C.R., S.A. Latt (1978) Sister chromatid exchange test in Chinese hamster cheek pouch mucosa. J. Dent Res 578, 211-213.

Schulster, D., S. Burstein, B.A. Cooke (1976) Molecular endocrinology of steroid hormones. Wiley, New York.

Schvartzman J.B., F. Cortes, A. Ganzales-Fernandes, C. Gutierres, J.F. Lopes-Saez (1979) On the nature of sister-chromatid exchanges in 5-bromodeoxyuridine-substituted chromosomes. Genetics 92, 1251-1252.

Schvartzman J.B. and R.R. Tice (1982) in A.A. Sandberg (Ed) Progress and Topics in Cytogenetics Volume 2; Sister Chromatid Exchange, Alan R. Liss, Inc., New York, pp 123-134.

Shafer, D.A. (1982) Alternative replication bypass mechanisms for sister-chromatid exchange formation, in A.A. Sandberg (Ed), Progress in Topics in Cytogenetics: sister Chromatid exchange, Liss, New York, pp. 67-98.

Sharma T., and B.C. Das (1986) Higher incidence of spontaneous SCEs and X-ray induced chromosome aberrations in peripheral blood lymphocytes during pregnancy. Mut. Res., 174,27-33.

Sheshardri, R., E. Baker and G.R. Sutherland (1982) Sister-chromatid exchange analysis in mothers exposed to DNA-damaging agents and their newborn infants, Mut. Res. 97, 139-146.

Surgeon General (1986) The Health Consequences of Involuntary Smoking, U.S. Department of Health and Human Services, Government Printing Office, Rockville, MD, pp 1-359.

Swain, A.P., J.E. Cooper and R.L. Stedman (1969) Large-scale fractionation of cigarette smoke condensate for chemical and biological investigations, Cancer Res., 29, 579-583.

Swierenga, S.H.H., J.A. Heddle, E.A. Signal, J.P.W. Gilman, R.C. Brillinger, G.R. Douglas and E.R. Nestmann (1991) Recommended protocols based on survey on current practice in genetoxicity testing labs. IV Chromosome aberration and sister-chromatid exchange in Chinese hamster ovary, V79 Chinese hamster lung and human lymphocyte cultures, Mut. Res. 246,301-322.

Taubes, G. (1993) Clam of higher risk for women smokers attacked, Science, 262, 1375.

Tawn. J.E. and C.L. Cartmell (1989) The effect of smoking on the frequencies of asymmetrical and symmetrical chromosome exchanges in human lymphocytes, Mut. Res. 224, 151-156.

Taylor, J.H., P.S. Woods and W.L. Hughes (1957) Types and frequencies of chromosome aberrations in peripheral lymphocytes of general populations, in AA Sandberg (Ed) Radiation-Induced Chromosome Damage in Man, Liss, New York, pp 605-616.

The Dominion (Tuesday March 2 1993) page 13

Tice, R., J. Chaillet and E.L. Schneider (1976) Demonstration of spontaneous sister-chromatid exchanges in vivo, Exp. Cell Res., 102, 426-429.

Timmerman, A.J., R.H. Mars-Groenendijk, G.P van der Schans, R.A. Baan (1995) A modified immunochemical assay for the fast detection of DNA damage in human white blood cells, Mut. Res. 334, 347-356.

Tonomura, A., K. Kishi and F. Saito (1983) Types and frequencies of chromosome aberrations in peripheral lymphocytes of general populations, in: T. Ishihara and M.S. Sasaki (Eds), Radiation-Induced chromosome Damage in Man, Progr. Mut. Res., Vol 4, Liss, New York, pp. 605-616.

Trichopoulos, D., A. Kalandidi, L. Sparros, and B. MacMahon (1981) Lung cancer and passive smoking, Int. J. Cancer, 27, 1-4.

Tucker, J.D., M.L. Christensen, C.L. Strout and A.V. Carrano (1986) Determination of the baseline sister-chromatid exchange frequency in human and mouse peripheral lymphocytes using monoclonal antibodies and very low doses of bromodeoxyuridine, Cytogenet. Cell Genet., 43, 38-42

Tucker, J.D., L.K. Ashworth, G.R. Johnston, N.A. Allen and V. Carrano (1987) Variation in the human lymphocyte SCE frequency as a function of time: Results of a daily and twice-weekly sampling, Environ Mol Mutagen., 10, 69-78.

Tucker, D., L.K. Ashworth, G.R. Johnston, N.A. Allen, A.V. Carrans (1988) Variation in the human lymphocyte SCE frequency: Results of a long term longitudinal study, Mut. Res., 204, 435-444.

Underwood, P.B., K.F. Kesler, J.M. O'Lane and D.A. Callagan (1967) Parental smoking empirically related to pregnancy outcome, Obstet. Gynecol., 29, 1-8.

Van Duuren, B.L. and B.M. Goldschmidt (1976) Catechol shows potent co-carcinogenic action in benzo (*a*) pyrene carcinogesis, J. Natn. Cancer Inst. 56, 1237-1242.

VanVunakis, H., JJ Langone and A milunsky (1974) Nicotine and cotinine in the amniotic fluid of smokers in the second trimester of pregnancy, Am. J. Pbstet. Gynecol., 120, 64-66

Venema, G. (1959) The influence of substances extracted from cigarette-smoke on mitotic proesses in *Allium cepa,* Chromosoma, 10, 679-685.

Wolff, S., J. Bodycote, G.H. Thomas, J.E. Cleaver (1975) Sister chromatid exchanges in xeroderma pigmentosum cells that are defective in DNA exisision repair or post-replication repair, Genetics, 81, 349-355.

Wulf H.C., A.S. Iversen, B. Husum, and Niebuhr (1985) Decreased sister chromatid exchange frequency in Seventh-day Adventists, Mut. Res 200, 145-156.

Wynder, E.L., and D. Hoffmann (1967) Tobacco and tobacco smoke, Studies in Experimental Carcinogenesis, Academic Press, New York, pp. 225-231.

Wynder, E.L., and D. Hoffmann (1979) Tobacco and health: A societal challenge, N. Engl. J. Med., 300, 894-903.

Wynne Griffith, G., (1976) in Scientific Foundation of Oncology, Heinmann, London, pp245-255.

Yamasaki, E., and B.N. Ames (1977) Concentration of mutagens from urine by adsorption with the nonpo;ar resin XAD-2: Cigarette smokers have mutagenic urine, Proc. Natl. Acad. Sci. (USA), 74, 3555-3559.

Yoshida, Y., and T. Matsumoto (1980) Amino-α-carbolines as mutagenic agents in cigarette smoke condensate, Cancer Lett., 10, 141-149.

Zajicek G. (1985) Inflammation initiates cancer by depleting stem cells, Med. Hypotheses, 18, 207-219.