A CYTOGENETIC STUDY OF NEW ZEALAND NUCLEAR TEST VETERANS: THE COMET ASSAY

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- ABSTRACT -

Between 1952 and 1958, forty thousand troops witnessed or assisted in the detonation of nuclear weapons in and around Australia and Christmas Island. Of these forty thousand troops there were 550 sailors from New Zealand; the remainder were mainly from Australia or Britain, together with a Fijian contingent. Since the end of this test series, the participants have maintained that they were exposed to radiation that has affected their health. The New Zealand test veterans say that their lifespan has been reduced by at least 10 years and there have been an unusually high number of genetic disorders among them and their children. The possible genetic effects of this radiation exposure have never been fully investigated.

One of the most popular techniques for detecting DNA damage is the single-cell gel electrophoresis assay (SCGE), also known as the COMET assay. The COMET assay was used throughout this study to determine if veterans of the Operation Grapple tests have long-term genetic effects as a result of their participation. The COMET assay measured three factors to determine the overall genetic damage in these veterans: the tail length; the tail moment; and the Olive tail moment. Only the tail length had a significant amount of difference after a comparison with a control group was conducted ($P = 0.046$). However, the mean genetic damage in these veterans was lower than that of the control group. It is unclear if this result is due to an anomaly in the data, or due to some other complex factor. An epidemiological analysis revealed a possible link between the mortality of these veterans and the number of weapons detonated.

The collection of these one hundred samples, not including re-collections, from several areas of New Zealand became a logistical nightmare. To minimise this problem a pilot study was also incorporated into this research to determine if blood samples could be cryopreserved for extended periods of time without an accumulation of genetic damage due to the freezing process. The COMET assay was also used to determine this damage. The cryopreservation of these samples induced extensive genetic damage. Only 7 from the total of 60 frozen samples were retrieved with a level of damage that was not significantly different from the original, unfrozen sample ($P = > 0.050$). It appears that the routine use of cryopreserved blood samples for cytogenetic testing is not possible at this time and further study is required.

- ACKNOWLEDGEMENTS -

An application was submitted to the human ethics committee for permission to use human subjects in this study. The application was considered and approved before research commenced. Copies of the letters of permission are included in Appendix One.

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- ABBREVIATIONS USED IN THIS REPORT -

α - Alpha

β – Beta

BrdU - 5-bromo-2-deoxy-uridine

Bq - Becquerel

C - Control group

Ca - Calcium

CA - Chromosome Aberration

CASP - COMET Assay Software Project

CDC - Centre for Disease Control

Ci - Curie

Cm - Centimetres

^{60}Co - Cobolt-60

^{137}Cs - Cesium-137

CT - Comet Threshold

DAPI - 4', 6-Diamidino-2-phenylindole

DMSO - Dimethylsulfoxide

DNA - Deoxyribose Nucleic Acid

DPS - Disintegrations Per Second

DSB - Double-Strand Breakages

E - Experimental (nuclear test veteran) group

EDTA - Ethylenediamine Tetraacetic Acid

EMF - Electromagnetic Field

EPA - U.S Environmental Protection Agency

Et al. - *Latin,* and others

FCS - Foetal Calf Serum

FISH - Fluorescence *In Situ* Hybridisation

γ - Gamma

G - Grams

GHz - Gigahertz

Gy - Gray

H^+ - Proton

^{2}H - Deuterium

^{3}H - Tritium

H_2O - Water

H_2O_2 - Hydrogen Peroxide

HCT - Head Centre Threshold

HT - Head Threshold

^{131}I - Iodine-131

IDDM - Insulin Dependent Diabetes Mellitus

^{192}Ir - Iridium-192

KT - Kilotons

L - Litres

LET - Low Linear Energy Transfer

Leuk - Leukocyte

LMAgarose - Low-Melting-point Agarose

MCi - Microcurie

µL - Microlitres

µm - Micrometers

µM - Molar concentration in micromoles/litre

M - Molar concentration in moles/litre

MA - Milliamperes

Mb - Megabytes

Mci - Millicurie

Mg - Milligrams

Ml - Millilitres

Mm - Millimetres

MN assay- Micronucleus assay

MqH_20 - Milli-Q water

Ms - Milliseconds

MSv - Millisieverts

MT - Megatons

NaCl – Sodium Chloride

NaOH - Sodium Hydroxide

Neutr - Neutrophil

NIDDM - Non-insulin Dependent Diabetes Mellitus

Nm - Nanometres

NMAgarose - Normal Melting-point Agarose

N or No. - Number

NTV - Nuclear Test Veterans

NZNTV - New Zealand Nuclear Test Veterans

NZNTVA - New Zealand Nuclear Test Veterans Association

OTM - Olive Tail Moment

P - HMNZS Pukaki

PBMC - Peripheral Blood Mononucleate Cells

PBS - Phosphate Buffered Saline

PHA - Phytohaemagglutinin

R - HMNZS Rotoiti

R.N.Z.N - Royal New Zealand Navy

ROS - Reactive Oxygen Species

RPMI - Roswell Park Memorial Institute

SCE - Sister Chromatid Exchange

SCGE - Single Cell Gel Electrophoresis

SD - Standard Deviation

SE - Standard Error

SSB - Single-Strand Breakage

Ssp. - Sub-species

^{85}Sr - Strontium-85

^{89}Sr - Strontium-89

^{90}Sr - Strontium-90

Std. Dev - Standard Deviation

TL - Tail Length

TM - Tail Moment

^{235}U - Uranium-235

V/v - Volume per volume

WU - U excitation (wide band) filter

x g - Gravities

^{90}Y - Yttrium-90

^{90}Z - Zirconium-90

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1.1 NUCLEAR WEAPONS AND NUCLEAR TEST VETERANS

Since 1945, when the first atomic bomb was detonated during the Manhattan Project in the United States of America (USA), nuclear weapons have proliferated across the globe. Other nations quickly joined the race to develop nuclear arms and at present, according to the Monterey Institute of International Studies, the former Soviet Union, Britain, France, the People's Republic of China, Pakistan, Israel, and India are also considered nuclear powers. More than 2,000 nuclear tests have been conducted at various locations around the world.

In 1946, the United States conducted Operation Crossroads, the first peacetime nuclear weapons test. The detonation was witnessed by an audience of worldwide press and visiting officials at the Bikini Atoll in the Pacific Marshall Islands. In 1949, the Soviet Union detonated its first atomic bomb.

In 1952, Britain became the third nation to detonate a nuclear device. During the 1950's and 1960's, Britain conducted a total of 21 atmospheric nuclear weapon tests at sites throughout the Pacific Ocean and Australia. These tests included approximately 40,000 participants from Britain, Australia, New Zealand, as well as a Fijian contingent, who were involved in several duties, ranging from technical to clerical and catering. However, according to Rabbitt Roff (1999b) all were ordered to witness the blasts in order to determine any possible troop effects in the event of a nuclear war. The operations conducted by the British were codenamed "Hurricane", "Totem", "Mosaic", "Buffalo", "Antler", and "Grapple".

1.1.1 OPERATION GRAPPLE

Operation Grapple consisted of 9 nuclear detonations between May 1957 and September 1958 at 2 sites: a series of 3 atomic (fission) detonations over ocean near Malden Island; and a further 4 detonations of atomic (fission) devices over ocean and 2 small thermonuclear (fusion) devices over land in the vicinity of Christmas Island (now known as Kiritimati Island). The locations of these islands are shown in Figure 1.1.

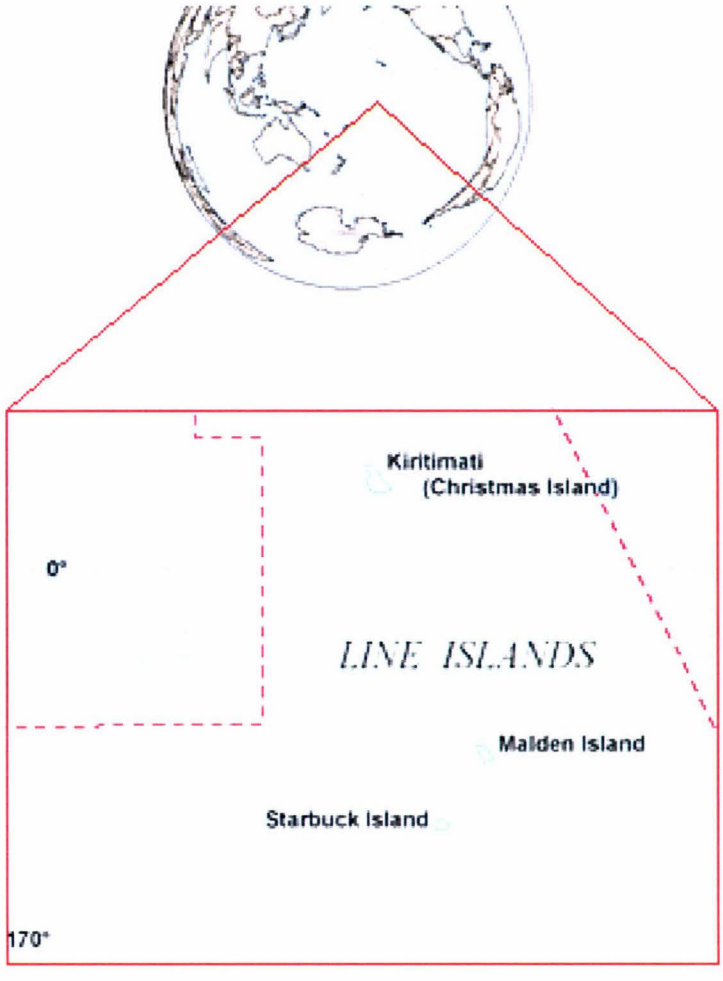

Figure 1.1. The geographic locations of Malden Island and Christmas Island.

All of the detonations conducted during these tests were airbursts from a plane or balloon, and were conducted at altitudes that were sufficient to ensure that no part of the fireball touched the surface to minimise any possible nuclear fallout. The Grapple series involved several naval vessels, and included 2 ships from New Zealand: The HMNZS Pukaki and the HMNZS Rotoiti. Over the course of these tests, a total of 550 New Zealand naval personnel manned these ships. Their duties consisted of witnessing the detonation of nuclear weapons, decontaminating the ships, and collecting weather data.

During the Operation Grapple tests, the New Zealand vessels were stationed at various distances of between 20 and 150 nautical miles upwind from ground zero; the point on the ocean surface above which the devices were detonated. These distances and positions were chosen to ensure that the ships crews would be well outside the range of all radioactive effects. The Pukaki was present in all of the 9 tests, while the Rotoiti was present only at the first 4 tests. Table 1.1 shows the detonation and distance information for each of these ships.

Table 1.1. The location and yields of each Operation Grapple weapon, and the position of each ship at the time of each weapon detonation (Crawford, 1989).

					Distance From Ground Zero (Nautical Miles)	
Round	**Date**	**Island**	**Height (m)**	**Yield**	**Pukaki**	**Rotoiti**
Grapple 1	15/05/1957	Malden	2400 m	Megaton	50	150
2	31/05/1957	Malden	2300 m	Megaton	50	150
3	19/06/1957	Malden	2300 m	Megaton	150	50
X	08/11/1957	Christmas	2250 m	Megaton	132	60
Y	28/04/1958	Christmas	2350 m	Megaton	80	-
Z1	22/08/1958	Christmas	450 m	Kiloton	28	-
Z2	02/09/1958	Christmas	2850 m	Megaton	35	-
Z3	11/09/1958	Christmas	2650 m	Megaton	35	-
Z4	23/09/1958	Christmas	450 m	Kiloton	20	-

After the tests, many of the participants removed any protective clothing that they had been issued with in order to swim in the ocean or to sit in the rains that often followed a detonation (nuclear weapons sometimes have a cloud-seeding effect). The sea and rainwater would most likely have been contaminated from the radioactive fallout. After the blasts, complaints were received from crewman regarding symptoms indicative of radiation sickness, such as blistering of the skin, vomiting, and diarrhoea (Roy Sefton, personal communication). This was a common occurrence during the British tests. When the participants were sent home, many felt that they had been affected, and there has been anecdotal evidence of rapid teeth and hair loss (Rabbitt Roff, 1999b). The unavailability of data from film badges worn by the participants during these tests makes it impossible to establish the levels of radiation these individuals were exposed to. The British government, however, claims that these men were not exposed to radiation levels outside of the safety parameters, despite the fact that many of the film badges used to monitor this exposure were not processed (Crawford, 1989).

For several years many veterans have claimed that their quality of life has been affected as a direct result of their participation in the British nuclear tests. They have also claimed that there is an increased prevalence of genetic disorders among them and their offspring. There have been reports of an increased frequency of multiple myelomas present in veterans of such

tests, based on the analysis of medical records for several thousand of the participants (Rabbitt Roff 1999a, b). Many veterans of the nuclear tests have had a history of afflictions such as cataracts (Phelps-Brown *et al*, 1997) and arthritis, or have died due to diseases that could be attributed to radiation exposure, such as gastrointestinal or respiratory disorders, and some types of cancer (Rabbitt Roff, 1997). Although several epidemiological studies have been conducted regarding the health of nuclear veterans from Britain, America, Australia, and New Zealand, all have yielded results that are inconclusive or insignificant (Pearce *et al,* 1990a; Rabbitt Roff, 1999a; Dalager *et al*, 2000; Muirhead *et al* 2003), as have studies involving the health of their offspring (Reeves *et al*, 1999; McLeod *et al*. 2001a, b).

Due to the small number of participants in the New Zealand group (550), epidemiological studies are difficult, as any radiation induced cancers that might result would not be detectable against background and expected range of variation of cancers that may arise spontaneously (McEwan, 1988). Nevertheless, some studies have found moderately significant increases in the incidences of haematological cancers, such as leukaemia, which may have arisen due to radiation exposure from the Operation Grapple tests (Pearce, 1990a). Pearce's study was based on findings from a much larger study involving 22,000 British veterans (Darby *et al*, 1988a). However, a comparison of the morbidity of the control group to the national cancer statistics showed that the group had abnormally low incidences of cancer, which may have skewed the results (McEwan, 1988). All of the claims made by the NZNTV thus far have been based on anecdotal evidence and yet to be supported experimentally.

It is for this reason that a genetic study, rather than an epidemiological study, was conducted to determine unequivocally whether the health of these veterans has been affected as a result of their participation in Operation Grapple. Cytogenetic testing has the unique ability to determine genetic damage that has not yet surfaced physically. No major genetic studies regarding the health of any group of nuclear test veterans have yet been reported.

1.1.2 THE CURRENT STUDY

My supervisor, Dr Al Rowland, began a study in early 2002 in order to determine for the first time, the possible effects that radiation from these nuclear weapons tests have had on the genetic material of New Zealand Nuclear Test Veterans (NZNTV) at the molecular level. All

of the studies that have been conducted to date have involved using mortality and morbidity statistical information in order to find differences through comparisons to the general public or a control group. This is the first major cytogenetic study of veterans that were involved in a nuclear weapon test series and has used 6 cytogenetic tests in tandem that are well known in biomonitoring studies. These tests are explained later in this report.

It should be noted that much of the genetic damage, if any, that was received at the time of the detonations would have been repaired by the cell's DNA repair mechanism and may not be measurable today. Nevertheless, recent studies have shown that radiation-induced stable chromosome translocations can be detected using current technology (Hande *et al*, 2003). The hypothesis of the current study does not only take this initial damage into consideration, but also considers the possible accumulation of genetic damage from normal internal and external sources due to a DNA repair mechanism that may have been disrupted as a result of exposure to a nuclear weapon detonation and subsequent fallout. There is also a possibility that long-lived radionuclides in nuclear fallout from these tests may have been ingested and retained by the participants, slowly releasing radiation into the surrounding tissues. These factors may have resulted in an increase in the levels of genetic damage in these individuals compared to what would be expected in a normal, unexposed person.

1.1.2.1 Previous Studies Of Nuclear Test Veterans

There have been a number of studies conducted that have observed the health of several groups of nuclear test veterans. Although the majority of these are epidemiological studies, the results that they have found are encouraging with regards to the current study.

In a study by Caldwell *et al* (1980), the American Centre for Disease Control (CDC) reported an apparent cluster of 9 incidences of leukaemia found among the 3,224 participants of Shot Smoky (part of the Plumbbob series) at the Nevada Test Site in 1957. A follow-up report (Caldwell *et al*, 1983) increased the count of leukaemia to 10 cases compared with 4.0 that were expected on the basis of U.S. cancer rates. However, no excess cancers were found at any other anatomical site (the total number of cancers observed was 112, compared with 117.5 expected). The Smoky test was the highest-yield tower shot ever conducted at the Nevada Test Site. However, the doses measured from the film badges of the Smoky

participants as a group were too low to explain the excess of leukaemia. Whether this cluster represents a random event, an underestimation of the doses for the small number of participants with leukaemia, or some other explanation remains unclear. This result may show that film badges, while a good measure of radiation exposure, cannot fully account for the effects of such an exposure on the individual. This is of great significance to the current study, as many of the film badges from the Operation Grapple participants were not actually examined, therefore, the full extent of their exposure to radiation is not known.

A follow-up to this study was conducted by the Medical Follow-up Council to study the health of the 13,685 veterans involved in the entire Plumbbob nuclear test series (Robinette *et al*, 1987). This again involved a comparison of the mortality and morbidity statistics of these personnel to that of the national statistics. Despite the fact that no significant difference was observed in this initial study, these results were reanalysed in a study by Bross and Bross (1987) who used a mathematical correction factor for a phenomenon known as the "healthy soldier bias". The military population consists of individuals who were deemed to be in good health upon entry into the armed forces, and those who were found to be in poor health were denied enlistment. Therefore, the morbidity and mortality statistics for the general military population are more favourable as a result of this selection criterion, and has appeared as the "healthy soldier effect". Because of this, false negatives may be seen during such a study, as the military population taking part may be, on average, healthier than the general public.

When this correction factor was applied, it was found that those veterans who were involved in the Plumbbob series had a cancer rate 61.6 % higher than the general public. This may signify that they had been exposed to harmful radiation during their participation. However, these results have been largely criticised by the scientific community (Jablon, 1987) as a "gerrymander" (manipulation) of the results to support the hypothesis advanced by Bross and Bross (1987). The healthy soldier effect was not an issue in the current study as both the experimental and the control groups were at some point members of the armed forces. As a result any bias was nullified.

In a morbidity study of 954 Canadian participants (Raman *et al*, 1987), no differences from matched controls were found. However, only very large effects would have been detectable in such a small sample size for an epidemiological study. In contrast, a larger study of 22,347 British participants of test programs conducted in Australia found higher rates of leukaemia

and multiple myelomas than in a matched control group (28 vs. 6) (Darby *et al*, 1988b). The cancer rates among the exposed veterans, however, were only slightly higher than those expected based on British cancer rates, whereas those in the control group were much lower than expected, and there was no dose-response relationship. No excess was found at any other cancer site. Although the difference between the exposed and unexposed groups was significant, the reason for the results gained has never been resolved.

Watanabe *et al* (1995) studied mortality and morbidity among 8,554 Navy veterans who had participated in Operation Hardtack 1 at the Pacific Proving Grounds in 1958. This was the first study of U.S. veterans to include a control group of unexposed military veterans. Overall, the participant group had a 10 % higher mortality rate, but the cancer excess was significant only for the combined category of digestive organs (a mean increase of 47 %). On average, the radiation doses were low, with a mean of 388 millirem (considering 600 – 700 rem over a short period is regarded a lethal dose). Nevertheless, there was a 42 % excess of all cancers among the 1,094 men who had received radiation doses that were greater than 1 rem. There were no categories of cancer sites that showed a significant excess or clear dose-response relationship. However, the number of deaths in any category was small.

Differences in the rates of mortality were evaluated for the approximately 40,000 U.S. Navy personnel who participated in Operation Crossroads, a 1946 atmospheric nuclear test series that took place in the Bikini Atoll in the Marshall Islands (Johnson *et al*, 1996). The epidemiology study looked for differences in 3 principal causes of mortality: all-causes, all-cancers, and only leukaemia, hypothesizing that increases in the latter 2 may result from radiation exposure. Among Navy personnel, the primary analysis group for this study, participants at the Crossroads nuclear test were found to have a higher mortality than a matched group of non-participating military controls. An increase in the incidences of all-causes mortality was 4.6 %, which was statistically significant. However, a comparison of mortality by cancer and leukaemia, although higher, was statistically insignificant.

An Institute of Medicine (IOM) study of nearly 70,000 nuclear test participants (Thaul *et al*, 2000), found that participants in the nuclear tests had a statistically insignificant 14 % higher death rate from leukaemia than those in a comparison control group. However, it was found that service members in land-based tests in the Nevada desert had a death rate from leukaemia that was 50 % higher than military personnel in similar units who did not take part in atomic

tests. Sea-based test participants in the South Pacific, however, did not differ from the leukaemia death rates in a comparison group. The results of this study clearly showed that those veterans exposed to atomic radiation whilst based on land were at an increased risk from leukaemia, nasal, and prostate cancer.

1.1.3 NUCLEAR RADIATION

Nuclear radiation is a well-known source of physical harm to organisms. The origin of this radiation is usually from nuclear power plant waste or from the aftermath of a nuclear weapon detonation. Normal background radiation that an organism receives on a daily basis is usually not sufficient to cause the visible affects that are often caused by these. Several studies have been conducted to determine the possible biological effects of an exposure to several different sources of radioactivity, and it has been found that the particles released directly interact and affect the DNA itself. If these particles contain enough energy they can physically break the DNA. The resulting genetic damage can be harmful if not correctly repaired.

Genetic damage can be the cause of the physical effects seen in an organism and may arise from a disruption in gene activity. This can lead to apoptosis, necrosis, or if the mechanism of cell death itself is affected, the control of a cell's proliferation can be affected and the cell can become cancerous. This can be a potentially fatal situation for a multicellular organism. The effect of radiation on an organism may be prompt, or may take several decades to present. It is therefore imperative for human health that the physical effects of possible radiation exposure be investigated to the fullest.

1.1.3.1 Studies Involving Individuals Exposed To Radiation

Extensive research has been conducted concerning the health of persons exposed to nuclear radiation for as long as its existence has been known. This research has focussed mainly on radiation sourced from nuclear power plants and atomic weapons, which is of high importance to those living in proximity to a nuclear power plant or a former nuclear testing ground. This section outlines radiation research that is of relevance to the current study.

The most famous incident concerning the release of dangerous materials from a nuclear power plant involved the meltdown of reactor number 4 at Chernobyl. A study by Lazutka and Dedonyte (1995) focused on genetic damage in 33 Chernobyl clean-up workers. Sister chromatid exchange (SCE) analysis of samples taken from participants 6 - 8 years after the incident showed a significantly increased number of exchanges in clean-up workers in comparison to a control group. However, a dose-related rate of genetic damage was not found.

In a second Chernobyl study, Sperling *et al* (1994) described an apparent cluster of Down's syndrome cases in Berlin, based on 2 cases that were found through prenatal diagnosis and 10 cases that were diagnosed in newborn infants born approximately 9 months after the accident at Chernobyl. This was thought to be due to increased atmospheric levels of radioactive iodine that were ejected from the reactor. Iodine is a rare, essential trace mineral. Any iodine that is present in the body is quickly scavenged and concentrated in the thyroid gland where it can become available to the growing foetus. Widespread deficiency of iodine in the diets of these people was thought to play a role in the increased prevalence in trisomy 21 cases.

India's first nuclear detonation took place in Western Rajasthan in 1974. An epidemiological study of malignancy was conducted during the 5 years between 1984 and 1988 (Sharma *et al*, 1992). In this time, 2662 new cancer cases were recorded with a high rate of skin cancer, urinary bladder cancer, bone malignancy, lymphoma, and leukaemia observed in this region. This was much higher than India's national average, indicating that radioactive materials produced as a result of nuclear detonations may have affected the nearby population. Similar increases were also observed during a study of Eastern Rajasthan (Sharma *et al*, 1994).

A survey of the genetic aberrations and birth defects in villages that were in close proximity to a power plant in Rajasthan were found to be much larger than those reported in distant villages that were out of range of the contamination (Gadekar, 1996). It was found that the deformity rate of the general public was 2.5 times higher in villages close to the plant than was seen in distant villages. When the deformity rates in children aged less than 18 years and less than 11 years were analysed, this increased to a factor of 3.45 and 5.7, respectively. Gadeker observed that the increase in deformity rates seems to correspond with the opening of the plant 15 years earlier. The numbers of spontaneous abortions or miscarriages, stillbirths, and deaths of one-day old babies had also increased significantly.

In the aftermath of the World War 2 atomic bombing of Hiroshima on the 6th of August 1945, many that were not killed by the initial blast succumbed to acute or chronic radiation exposure. In the following months and years a sharp increase in the incidence of cancers, foetal malformations, and genetic diseases arose due to the presence of radioactive isotopes in the soil. Even today, some 58 years later, significant levels of radiation can still be detected in the rebuilt city, which continues to affect the population (Hunter and Charles, 2002).

A study of 1,033 atomic bomb survivors with an average age of 59.5 years was undertaken in 1986 by the Investigative Committee of Atomic Bomb Victims of Hannan Chuo Hospital, Osaka, Japan, (Furitsu, 1996) in which comparisons were made to the expected level of disease reported for the same age group in the Japanese Ministry of Health's 1986 report. The most significant of the disorders that were investigated was leucopoenia. Leucopoenia is a condition involving a decrease in the numbers of white blood cells to fewer than 5,000 cells per cubic millimetre (mm^3), with leukocytes and neutrophils being the most affected cell type. This disorder can result from, among other causes, radiation poisoning. It was discovered that survivors of the atomic bombs had 13.4 times as many sufferers of leucopoenia than was found in an age matched control group. A similar comparison was conducted in the current study by using data of cell populations obtained from blood samples taken from the control group and the experimental group. Theoretically, if an individual was chronically exposed to high levels of radionuclides, such as those with long-term retention in bone, a decrease in the population of white blood cells may be observed.

1.1.3.2 Nuclear Fallout

The most important source of radiation to consider with regards to the current study is residual radiation produced by nuclear fallout. Fallout comprises 5 - 10 % of the total energy (Appendix VI) of a nuclear detonation and severe local fallout can extend far beyond the blast and thermal effects, particularly in the case of high yield surface detonations. In detonations near a water surface, the particles tend to be lighter and smaller and produce less local fallout, but will be distributed over a greater area. The particles contain mostly sea salts with some water; these can have a cloud seeding effect causing local rainout and areas of high local fallout. Indeed, it was noted from anecdotal accounts that it rained heavily after many of the Operation Grapple blasts, which could have exposed the participants to even more radioactive

material. However, it cannot be determined how much radiation each man was exposed to in his daily duties, as individual film badges were not labelled, and any estimate would be purely speculative (Rabbitt Roff, 1997).

Nuclear fallout contains radioactive isotopes that decay through the release of sub-atomic particles that can potentially cause harm to nearby organisms. An explanation of α-, β-, and γ-decay is given in Appendix VII.

1.1.4 DNA IONISATION, EXCITATION, AND OXIDATION

Radiation-induced genetic damage can occur via 2 mechanisms: 1) radiation can interact directly with DNA and may result in single- or double-stranded DNA breakages or a disruption of base pairing through an ionisation or excitation event, or 2) the radioactive particles can interact directly with other surrounding molecules within or outside of the cell, such as water, to produce free radicals and active oxygen species. Contact with an absorbing media, such as DNA, may result in ionisation and excitation of atoms or molecules. The transferred energy is usually dissipated as heat, which can cause more damage.

Ionisation involves the removal of an orbital electron from an atom or molecule, creating a positively charged ion. For an emitted particle to cause an ionisation event, the radiation must transfer enough energy to the electron to overcome the binding force exerted on the electron by the atomic nucleus. The ejection of an electron from a molecule can cause dissociation of that molecule. Excitation involves the addition of energy to an orbital electron, thereby transferring the atom or molecule from the ground state to an excited state. Oxidation involves highly reactive radicals and ions, such as those produced in normal metabolic processes. These can diffuse away from the site of interaction with radiation and interact with the DNA. This may also result in the breaking of molecular bonds.

If an emitted particle is able to penetrate the tissue of an organism, the ionisation, excitation or oxidation of a DNA molecule may occur. This can be a harmful event and may result in a single stranded or, less commonly, a double stranded DNA breakage. A single strand breakage (SSB) involves the breakage of 1 strand of the DNA helix, while a double strand breakage (DSB) involves the breakage of both strands of the helix at the same position.

Significant amounts of damage to DNA can lead to errors in gene coding of essential enzymes, proteins, and other molecules. DNA base-pair damage is the predominant type of DNA damage, followed by SSBs (which are 4 times less prevalent than base-pair lesions), DNA-protein cross-linkages, and double-strand breaks. At the molecular level, an important type of change to DNA that is frequently produced by radiation is the removal of a base, forming an apurinic or apyrimidinic site. The deletion or total destruction of DNA bases, destruction of deoxyribose residues, and the deamination of cytosine or adenine are only some of the many ways that radiation can alter the DNA at a molecular level. Minor damage left not repaired or damage that was not completely or correctly repaired can result in mutations.

If the cell survives the radiation-induced genetic damage and carries the mutations into future cell populations, 2 events can take place: 1) the cell may carry the DNA defect and express an adverse event, such as altered protein and enzyme synthesis, and defects in cellular metabolism. These defects can be numerous, depending largely on where in the genome the mutation takes place and how critical the normal gene is to normal cell function, or 2) the damage may result in a cell that is cancerous. The most important source of these radioactive particles is from long-lived nuclides that are produced during nuclear detonations.

1.1.5 RADIONUCLIDES PRODUCED BY NUCLEAR DETONATIONS

The fallout from nuclear weapons testing peaked in 1964 after 77 atmospheric detonations worldwide occurred in 1962 (Nuclear Regulatory Commission, 1985). Of the total worldwide fallout, 69 % was composed of carbon-14, 4 % was cesium-137, and 3 % was from strontium-90. The remaining 24 % was composed of radioactive isotopes of plutonium, rubidium, barium, iodine, iron, manganese, krypton, americium, hydrogen (tritium), and zinc. Biological hazards from nuclear fallout come in the form of radioisotopes with long half-lives that are formed by fission/fusion of fuels used in the detonation of nuclear weapons. These have the potential to be accumulated in an organism through ingestion from contaminated foods or by inhalation, and can case a variety of serious ailments, such as cancers.

The radioactive debris from a nuclear detonation is formed through a number of mechanisms. The main source is through the creation of fission products from larger radionuclides. The number of isotopes produced in such an explosion can be upwards of 200 from 35 elements.

Many are radioactive and have a short half-life; therefore, the initial radioactivity decreases quickly after the event. Many of these are potentially harmful to organisms. The yields of the principal nuclides per megaton of fission are shown in Table 1.2 (Eisenbud and Gesell, 1997).

Table 1.2. Approximate yields of the principal nuclides per megaton of fission.

Nuclide	Half-life	MCi [1]
^{89}Sr	53 Days	20.00
^{90}Sr	29 Years	0.10
^{103}Ru	40 Days	18.50
^{106}Ru	1 Year	0.29
^{131}I	8 Days	125.00
^{137}Cs	30 Years	0.16
^{131}Ce	1 Year	39.00
^{144}Ce	33 Days	3.70
^{95}Zr	65 Days	25.00

The most important radioisotopes produced in a nuclear detonation, with regard to their harmful effects on an organism, are outlined below.

Iodine-131 (^{131}I)

Iodine is a trace rare element, and the thyroid gland will scavenge and concentrate any that is available in the body. Because of this ^{131}I is perhaps the most important radioisotope with regard to its effect on human health. If the body is deficient in iodine, the thyroid gland will try to take as much from the body, including ^{131}I. ^{131}I is both a β- and γ-ray emitter and has a very short half-life of approximately 8 days. Therefore, any genetic damage that was caused as a result of exposure during the Grapple tests would be undetectable, or would have caused death due to lymphomas or similar cancers several years ago. Therefore, Iodine is not considered to be an important isotope with regards to the current study.

[1] MCi = millicuries. 1 curie (Ci) is equal to a radionuclide that decays at a rate of 37 billion disintegrations per second (DPS). This is the old unit for radioactivity and has been replaced by the Becquerel (Bq) (1 Bq = 3.7×10^{10} Ci).

Cesium-137 (^{137}Cs)

Cesium-137 (^{137}C) is produced during the fission of uranium and plutonium fuels and is an extremely reactive molecule. ^{137}C. Cesium is ingested by contaminated water of food, or inhalation of dust. This isotope has a relatively long half-life of approximately 30 years and undergoes β- and γ-decay to barium-137. Cesium is only retained by the body for a short time and is excreted through urine. ^{137}C is not considered to be an important isotope with regards to the current study.

Plutonium-239 and 240

Most of the radiation emitted by plutonium is in the form of α-particles, which have a short range and cannot penetrate the skin. External radiation from the passing cloud and from plutonium deposited on the ground can therefore be neglected. However, airborne particles of PuO_2 can be inhaled, and approximately 10 % of these would be retained in the deep lung with a retention half-life of about 20 - 50 years. A recent study by Hande *et al* (2003) found that by using M-band chromosome painting, it was possible to detect and quantify stable intrachromosomal translocations in the lymphocytes of workers who were exposed to plutonium isotopes from 1949 onwards as a result of their involvement in the production of nuclear weapons. This is of great relevance to the current study as several plutonium isotopes are present in fallout from the detonation of nuclear weapons. Plutonium may be important with regards to the current study.

Strontium-90 (^{90}Sr)

Strontium-90 (^{90}Sr) is one of 11 strontium isotopes produced by aboveground nuclear weapons testing in the 1950s, and as a result it is now widely spread in the environment. The radius of a strontium ion is chemically similar to a calcium ion (1.12 Angstroms), and so cellular proteins cannot distinguish between them easily. It is therefore absorbed by the body and stored in bone, as if it were calcium. The majority of the world's population has measurable amounts of ^{90}Sr present in their skeletons. This is due to the atmospheric testing of nuclear weapons and waste from nuclear power plants, which have resulted in the deposition of ^{90}Sr over the face of the Earth (Eisenbud and Gesell, 1997). This has lead to the

incorporation of strontium into the calcium pool of the biosphere. The daily intake of strontium by an individual is 1.9 mg, some of which may be ^{90}Sr.

A second isotope of strontium, strontium-89 (^{89}Sr), is also released in fallout. However, this is not considered a hazard due to its relatively short half-life (50.5 days), and is in fact injected routinely into patients in the treatment of metastatic bone diseases. ^{89}Sr, as well as other harmless strontium isotopes, is used in studies regarding the path of ^{90}Sr in the body due to their atomic similarities. All isotopes of strontium are cleared more efficiently than calcium from the blood and are excreted primarily in urine (Van Dilla *et al*, 1956). ^{90}Sr is the most important isotope with regard to the current study.

1.1.6 THE EFFECTS OF STRONTIUM-90

Theoretically, those who were in close proximity to the Operation Grapple blasts would have significantly greater levels of ^{90}Sr deposited in their skeletons compared to the general public. ^{90}Sr has a relatively long half-life of 29 years, and so a proportion of the ^{90}Sr produced by nuclear testing as fallout at Christmas Island that may have been absorbed by test veterans may still be present in their bones. Barring calcium turnover rates in bone, after 50 years of radioactive decay this would equate to approximately 30 % of the original mass, which is still radioactive.

^{90}Sr is a high-energy beta emitter that will decay to Yttrium-90 (^{90}Y), which is also a β-emitter, with a biological half-life of 64 hours. This in turn decays to the stable element Zirconium-90 (^{90}Zr), which is another β-emitter, with a biological half-life in the skeleton and the body of 22 years and 7 days, respectively. Both ^{90}Sr (Section 1.1.6.1) and ^{90}Y (Sigg *et al*, 1997; Lenarczyk *et al*, 2001) are considered to be hazardous materials, and can produce significant genetic damage. External sources of beta radiation are relatively harmless to organisms, as the emitted particles cannot penetrate tissues easily. Therefore, an organism exposed to an external source of ^{90}Sr will be unharmed for a short period. However, if the source is located in the organism's own skeleton, such as ^{90}Sr, the particles do not need to penetrate far to reach important tissues. As ^{90}Sr is deposited in bone it is very near to bone marrow, which is an important tissue for the production of white blood cells. Oral intake at high levels of activity has been found to result in irradiation of target organs and nearby tissues (NCRP, 1991).

The gastrointestinal tract absorption rate of ^{90}Sr is 30 %, while inhaled forms of the isotope is absorbed over a period of days, demonstrating minimal retention in the lung (EPA, 1994). Ingested and soluble inhaled forms of ^{90}Sr such as $^{90}SrCl_2$ are quickly absorbed into the bloodstream and are directed mainly to the skeleton. Retention of ^{90}Sr in the bone is long-term, with yearly turnover (replacement with calcium) of the existing levels in adults of 7.5 % from cortical bone, which makes up 80 % of the skeleton, and 30 % from trabecular bone, which makes up the remaining 20 %. This uptake and turnover in the skeleton is based on the concentrations of ^{90}Sr in human bone and human diet in the United Kingdom estimated by Papworth and Vennart (1984). Approximately 4.5 % of the dietary intake of ^{90}Sr reaches the skeleton, half going to cortical bone and half to trabecular bone. Strontium is known to decrease the normal rates of calcium turnover in the bones of estrogen deficient rats (Morohashi *et al*, 1995). Therefore, increased amounts of this isotope will prolong the normal exposure times.

Tolstykh *et al* (2003) used several radiological techniques to determine the ^{90}Sr content in teeth of residents living in settlements along the Techa River, Russia, who lived downstream from a nuclear power facility. All of the methods that were used found a spike corresponding to the presence of ^{90}Sr that had been deposited in the teeth of the participants due to radioactive waste material runoff. However, such a study is difficult to interpret due to the presence of background ^{90}Sr from atomic fallout. When this was taken into consideration, an increase in the internal levels of ^{90}Sr due to waste from the nuclear power plant was observed.

Strontium-90 concentration was measured in human bones and teeth collected in Greece between 1992 and 1996 (Stamoulis *et al*, 1999). ^{90}Sr concentration measurements in teeth demonstrated a pronounced structure, which clearly reflected contamination from the 1960s atmospheric nuclear weapons tests and the more recent Chernobyl accident. Through a fitting process, the model also yielded calcium turnover rates for compact bone, as a function of age, as well as an estimate of radiostrontium contamination of foodstuffs in Greece for the past four decades. The results obtained in this study indicate that radiostrontium environmental contamination in this area, and possibly the world, as a result of the atmospheric nuclear weapons tests in the 1960s exceed by far that caused by the Chernobyl accident. If these levels of ^{90}St were found in those who were clearly removed from the main atomic sources of this isotope, one can only imagine the levels that may have been present in those individuals who actually witnessed these detonations, such as the NTVs.

1.1.6.1 Cytogenetic Evidence For The Effects Of Strontium-90

The genetic effects of exposure to Sr^{90} are well documented. Brooks and McClellan (1968) injected small amounts of ^{90}Sr into Chinese hamsters and measured chromosomal aberrations at 2 and 14 days after injection of 0 - 5 microcuries (μCi) of Sr^{90}. They found that chromosomal breaks increased at greater concentrations after longer exposure periods. It was also noted upon autopsy of several mice that died during the trial, that bone marrow aplasia (failure) had been the cause of death. These mice had received between 3 and 5 μCi of Sr^{90}.

High exposure rates of ^{90}Sr have been found to result in radiation-induced hemorrhagic syndrome in test animals. This occurs through the uncontrolled activation of clotting factors and fibrinolytic enzymes throughout small blood vessels and results in tissue necrosis, internal and external bleeding, and death. At lower exposures, death results from destruction of the bone marrow, or from the formation of neoplasms (Pool *et al*, 1972; Hobbs and McClellan, 1986; NCRP, 1991). The most common cancers that arise from exposure to ^{90}Sr are leukaemia, which is an over-proliferation of immature white blood cells, and myelomas, which are tumours that arise from plasma cells in the bone marrow. The bone marrow is well within the range of any radioactive isotope that deposits in the skeleton. This is a common health effect observed during animal and human studies of Hiroshima and Nagasaki populations. The presence of strontium in bone may explain the increased frequency of leukaemia and multiple myelomas in the New Zealand veterans (Rabbitt Roff 1999a, b), as well as veterans of other weapon tests (Caldwell *et al*, 1980, 1983; Darby *et al*, 1988b).

Several studies of populations living in the vicinity of 4 nuclear reactors in New Jersey found high levels of radionuclides, especially ^{90}Sr, in the environment (Gould *et al*, 2001). Dosimetric tests of deciduous (baby) teeth from children in the area also showed high levels of ^{90}Sr (Gould *et al*, 2000, 2001; Mangano *et al*, 2003). The levels of ^{90}Sr detected in the environment were found to be similar to that seen in the late 1950s, when the U.S. and Soviet Union conducted large-scale atmospheric nuclear weapons tests. Cancer mortality rate for those aged 0 - 9 in Ocean and Monmouth Counties were found to be 74.1 % higher than the U.S averages and 54.0 % higher than the rest of New Jersey. In persons over the age 65 living in Salem and Gloucester Counties, cancer death rates soared between 1979-81 and 1996-98. These increases were mainly in leukaemia (130.4 %), Hodgkin's disease and non-Hodgkin's lymphoma (115.2 %), and multiple myelomas (74.5 %).

It is possible that veterans of the Operation Grapple tests may have been exposed to high levels of ^{90}Sr, as well as other sources of radiation, in the blast and fallout from the nuclear detonations. Analysis of the positions of the Pukaki and the Rotoiti during and after the weapon tests (Write, 2003) has shown that on several occasions the ships involved entered both the fallout zone and ground zero for extended periods soon after many of the detonations. Radionuclides may have been ingested through inhalation of fallout material or from contaminated food and water. These may have been absorbed by the body and incorporated and retained in the skeletons of those exposed where they remain, continually emitting damaging radiation into the surrounding tissues, especially the bone and bone marrow. This may explain the increased observed rates of cancers involving the bone marrow, such as myelomas and leukaemia, and little to no differences in the rates of other common types of cancers such as skin cancers. Boice and Land (1982) found that leukaemia and multiple myeloma are unquestionably radiogenic disorders.

It must be stressed, however, that data from film badges is not available, and dosimetric examinations have not yet been conducted on the participants of the current study. Therefore, any mention in this report regarding uptake and retention of radionuclides by participants involved in Operation Grapple is based purely on speculation.

1.2 ANALYSIS OF GENETIC DAMAGE

The maintenance of a genome's integrity and fidelity is essential for the proper function and survival of all organisms. This task is particularly intimidating due to constant assault on the DNA by genotoxic agents from both endogenous and exogenous sources, mistakes during DNA replication, and the inherent biochemical instability of DNA itself.

Failure to repair DNA lesions may result in the disruption of transcription and replication, mutagenesis, or cellular cytotoxicity. In humans, DNA damage has been shown to be involved in several genetically inherited disorders, in aging, and in carcinogenesis. It is therefore imperative for our understanding of cellular processes to study the effects of potential cytotoxic agents that pose a threat to the well being of humans, as well as determine the effects of the agent on those who have already had exposure. DNA strand breakage and genetic damage was outlined in Section 1.1.4.

1.2.1 CYTOGENETIC TESTS USED IN THE CURRENT STUDY

The study of genetic damage requires the use of specialised assays that have the ability to measure the levels of genetic damage in a single cell. There are many techniques that are at the disposal of researchers who wish to study genetic damage in an organism. These tests may range from classic karyotype analysis, to more specialised enzymatic assays. 6 tests were used by the researchers involved in the New Zealand Nuclear test veterans study to determine if the levels of genetic damage in nuclear test veterans were different to that of the general public. Only the COMET assay will feature in this current report. The 5 other tests were conducted by other investigators of our research team and will feature in a final published report at a later date. Outlines of the principles of these 5 remaining tests are found in Appendix V.

1.2.1.1 THE COMET ASSAY

One of the most widely used techniques for detecting DNA damage is the single-cell electrophoresis assay (SCGE), or COMET assay, as it is known. The COMET assay was the sixth test to be used in the current study. The assay's name is derived from the comet-like tails that are seen when cells are observed using a fluorescence microscope, as shown in Figure 1.2 below. The intensely stained head of the comet is made up of the remains of the cell's nucleus, while the tail is composed of broken DNA fragments that have been drawn out of the nucleus by an electric current.

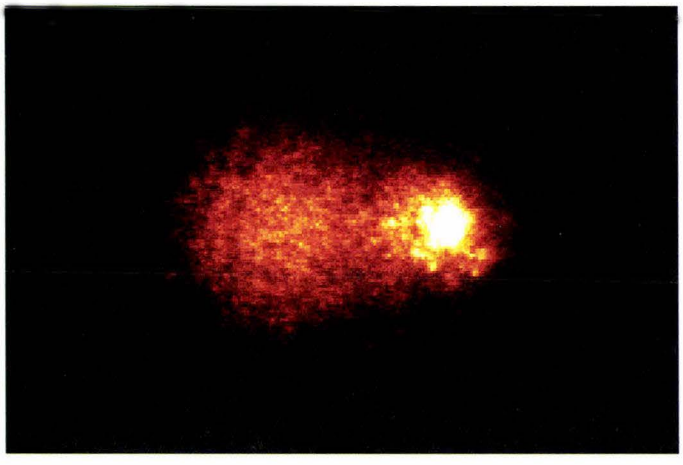

Figure 1.2. False colour, confocal microscopy image of a lymphocyte subjected to the COMET assay and stained with SYBR Green™.

The COMET assay first appeared in a paper by Östling and Johanson (1984), which described an assay that could be used to determine the level of DNA damage in a single cell by detecting DSBs that have occurred in the cell's DNA. This assay involved subjecting the cell's nucleus, which has been isolated from the cell through the use of ionic detergents, to an electric current. Free fragments of the negatively charged DNA could then migrate out of the nucleus towards the positive electrode. It was found that the length of the resulting "tail", which was composed of the fragments of DNA, was proportional to the amount of damage that the cell's DNA had received. Thus, by measuring the tail length the extent of the genetic damage could be ascertained. This method is described as the neutral single cell electrophoresis assay, and as it can only be used to detect DSBs, it is an effective test to use if a large amount of damage is expected.

Singh *et al* (1988) subsequently developed the single cell electrophoresis assay under alkaline conditions, which they found could be used to detect SSBs in DNA. High pH denatures the DNA and fragments arising from SSBs are released from the DNA helix so they can migrate through an agarose gel when exposed to an electric current, thus amplifying the ability of this assay to detect damage. This has made the assay much more sensitive than the neutral technique, which could only detect the less common DSBs, and it has become the assay of choice for DNA damage researchers. The assay is sensitive, inexpensive (when established), easy to perform, yields results that are simple to interpret, and only requires small amounts of tissue (< 100,000 cells) per assay, which can be obtained by a finger prick. Unless stated, any mention of the COMET assay hereafter refers to the alkaline version of the COMET assay.

This technique has been useful in studies of environmental toxins, cancer, and aging. Since the effects of these are often tissue specific it is important to see the DNA damage in an individual cell. Many different cell types have been assayed according to the large amount of literature that is available. This includes whole blood, bone marrow, or solid organs from most species of animal, as well as tissues taken from plants. Singh's group used this assay to study the effects of 2 of the most well known genotoxic agents, X-irradiation and hydrogen peroxide. They observed that the comet tails of cells exposed to either of these 2 agents were significantly longer than those of the control cells that had been sham exposed. It was concluded that they had developed a simple approach for the sensitive detection of DNA damage, as well as the assessment of the DNA repair capacity of individual cells.

Evidence of DNA damage is gained through the observation of the cell using fluorescence (or similar) microscopy. Microautoradiographs of tritium labelled cells have also been successfully used (Klaude *et al*, 1996). This information can be gathered using quantitative or qualitative methods. The comet tail can be analysed qualitatively according to its morphology and compared to healthy, control cells. Scoring can be made according to nominal, medium, or high intensity tail DNA content. Quantitative methods involve recording values such as tail migration distance, image length, nuclear size, and using these values to calculate the tail moment, which is a reliable and sensitive indicator of DNA damage. Several computer software packages are available to perform these actions, such as Komet™, and can be connected to microscopes via a digital camera for rapid analysis.

This assay has been the subject of hundreds of different studies and has enabled researchers to detect the effects of many known DNA damaging substances (Fairbairn *et al*, 1995; Olive, 1999; Albertini *et al*, 2000). Studies that have investigated the genotoxic effects of many chemicals and radiation sources have greatly benefited from the use of the COMET assay. It is due to these reasons that the COMET assay became a component of the current study, and will feature in this thesis. The COMET assay has been used successfully and consistently to detect genetic damage in affected cells.

1.2.2 CONFOUNDING FACTORS

As with any study, confounding factors can have a large impact on the results gained from the use of the COMET assay. Confounding factors are extraneous variables that result in observed effects that can obscure or exaggerate the true variable that is being measured. Due to the sensitivity of the assay, it may not only detect DNA damage caused by the factor that is being investigated, but also other factors as a result of the lifestyle of the participant involved.

When choosing subjects for the study of genetic damage several of these factors should be considered. Results gained from the COMET assay are severely influenced by such factors as age, gender, or lifestyle, such as if the individual is a smoker, drinker, is subject to high stress, or has an unhealthy diet. The occupation and health of the subject are also considered to be factors (Fairbairn *et al*, 1995). These factors were considered during the selection of subjects and controls for the current study.

1.2.2.1 Smoking

Smoking is one of the most active areas of study using the COMET assay and has been shown to be the most important confounding factor, markedly affecting the results gained from this test. It has been known for some time that the smoking of cigarettes is detrimental to one's health. Tobacco smoke contains many potential carcinogens such as the insecticide DDT, polycyclic aromatic hydrocarbons, aromatic amines, and aldehydes, among others.

Both the COMET assay and sister-chromatid exchange (SCE) assay were used by Betti *et al* (1995) to ascertain the genetic damage that may have occurred in the DNA of individuals as a result of cigarette smoking. Lymphocyte samples were obtained from 100 smokers and 100 non-smokers of similar ages every 3 months for 1 year. After applying the COMET assay to the samples it was found that the comet tails in lymphocytes taken from the smokers were significantly longer than those seen in samples taken from non-smokers. This showed that the DNA from the smokers had been damaged significantly more than that of the non-smoker. When the SCE was applied to the same samples they were unable to detect the effects of smoking in these samples. They concluded that the application of the COMET test to both *in vitro* and *in vivo* studies has shown its usefulness in evaluation of DNA damage and repair induced by physical and chemical agents in different cell systems.

Frenzilli *et al* (1997) used the COMET assay to observe the change in the levels of DNA damage in leukocytes taken from ex-smokers. 90 participants, who were heavy smokers, agreed to end their cigarette habit for 1 year. During this time, periodic blood sampling was conducted to determine the levels of genetic damage due to the decrease in tobacco smoking. During the trial, 28 participants abandoned the study, while 40 participants relapsed into their habit, albeit with a decreased number of cigarettes per day. 22 volunteers succeeded in stopping their habit. The levels of DNA fragmentation were then measured at 5 sampling periods and the results were compared. In this study, a statistically significant reduction in the levels of DNA damage in leukocytes was seen 1 year after cessation of smoking, compared to those who returned to their smoking habit. These were confirmed by a further sampling 6 months after the end of the initial trial. This sample showed a further decrease in the genetic damage in ex-smokers, most likely due to repair of DNA damage caused by their habit.

1.2.2.2 Aging

The age of a participant has been shown to be the main factor to severely affect the results of the COMET assay (Diem *et al*, 2002). When an individual ages, they will accumulate DNA damage due to normal exposure to factors (e.g. ultra violet light, hydrogen peroxide produced in normal metabolic reactions, background radiation) and their DNA repair mechanism may become impaired. Therefore, a cell sample that is taken from a healthy 60-year-old man will produce a longer COMET tail than a sample taken from a healthy 18-year-old man. Age matching is an important consideration when choosing subjects for a study.

Singh *et al* (1990) conducted a study involving 31 male and female subjects, aged between 25 and 91 years, to determine the rates of genetic damage and repair of donated lymphocyte cells exposed *in vitro* to 200 rads[2] of X-irradiation. Genetic damage was measured by the COMET assay. An analysis of the basal levels of genetic damage yielded an age-independent relationship, however, exposure to X-irradiation yielded an age-dependent relationship. An explanation for this result is unclear. It was not due to the levels of DNA repair, as the conditions used inhibited such repair. It was also unlikely to be due to a decrease of protective compounds that are reduced in older individuals. Further investigation is required to elucidate this response. Mendoza-Nunez *et al* (2001) have conducted similar research in this area.

A DNA repair study was also conducted using these samples. Exposure to X-irradiation again showed age-dependent relationship, with cells from older participants showing higher initial levels of genetic damage. However, contrary to what was expected, the DNA damage from most cells, regardless of the age of their respective donor, was essentially repaired in the same length of time (approximately 2 hours). These results show that cells from older individuals have a similar, if not better, DNA repair mechanism than cells taken from younger individuals. However, it is usually standard practice to ensure that the participants of a study are matched for age, despite the results from Singh's research. In the current study the participants were age-matched (mean 66.95 years, P = 0.285), and so there should not be an age-related effect on the results gained. The P value was derived from a Student's T test and indicates that the difference between the mean ages of these 2 groups is not significant.

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[2] Rad: a unit of energy absorbed from ionising radiation equal to 100 ergs per gram, or 0.01 joules per kg of irradiated material. The gray (Gy) has replaced the rad as the standard unit.

1.2.2.3 Gender

The gender of an individual has also been shown to be important in a study of genetic damage (Baltaci *et al,* 1998). It is generally accepted that males have higher levels of genetic damage than age-matched females. Animal studies have shown that males can be more susceptible to DNA damage than females. Russell and Russell (1945) found that male mice exposed to radiation were more sensitive than females exposed to the same doses. In studies conducted using both males and females, the results gained from the COMET assay have differed significantly between genders.

Bajpayee *et al* (2002) conducted the most significant study thus far to determine differences in the levels of basal DNA damage in a normal, healthy group of 124 males and 106 females. All the individuals assayed belonged to a comparable socio-economic background and aged between 20 and 30 years. They were also matched for their smoking and dietary habits. This report has demonstrated for the first time significant differences in the basal level of DNA damage between males and females in a normal healthy Indian population. All of the variables measured using the COMET assay showed that males had a significantly higher level of basal DNA damage. Studies such as this have shown the importance of gender as a confounding factor. However, this was not an important factor for the current study, as all participants were male.

1.2.3 THE COMET ASSAY AND IONISING RADIATION

The current study was conducted to determine the levels of genetic damage in a group of individuals who may have been exposed to ionizing radiation. It is therefore appropriate to discuss other studies in which the COMET assay has been used to detect damage induced by radiation. As was outlined in Section 1.1, radiation is a potentially harmful source of genetic damage that has been rigorously studied for many years. The COMET assay has been used in many studies involving different types of radiation and is a valuable tool for determining the effects of radiation effect on living cells.

Both the alkaline and neutral versions of the COMET assay were used to measure the rate of rejoining of predominantly SSBs following *in vitro* exposure to 8 Gy and DSBs following *in*

vitro exposure to 75 Gy (Banath *et al*, 1998). All cells within a population responded in a similar fashion to the induction of SSBs and DSBs. However, a subset of the WBC appeared to rejoin SSBs more rapidly. 10 individual samples were examined, and it was found that the percentage of SSBs rejoined by the quickly repairing cells was 47 % and the rejoining half-time for the slow repairing cells was 1.3 %, as measured by the alkaline COMET assay. 24 h after an exposure of 8 Gy, 4.9 % of the initial SSBs remained. For DSB rejoining, 58 % of the initial damage was still present 4 hours after 75 Gy, and by 24 hours 32 % of the initial level of damage was still detected, as measured under normal conditions. This indicates the repair capacity of single cells in response to large amounts of genetic damage, as well as a possible difference in the repair capacity of individual samples.

The exposure of human beings to ionizing radiation is still of great concern in occupational and environmental medicine, and the widespread use of radiotherapy in the treatment of cancer has led to anxiety about the possible hazards to staff who are at risk of such occupational exposure. The COMET assay is becoming an important technique in biomonitoring studies due to its ability to determine small amounts of genetic damage. A study was conducted by Garaj-Vrhovac *et al* (2002) involving a worker that had been in contact with a radioactive cobalt source. The COMET assay was employed to determine the levels of genetic damage induced as a result of exposure to 221 millisieverts (mSv) from a Cobalt-60 (^{60}Co) source. Several blood samples were taken from the participant at different periods after the incident and extracted leukocytes were subjected to the COMET assay. A significantly increased level of genetic damage was recorded 1 day and 1 week after the incident. This genetic damage decreased further over the next year, however, after 1 year of recovery the levels were still higher than that seen in control participants.

Likewise, a study by Undeger *et al* (1999) used the COMET assay to measure the DNA damage in the peripheral lymphocytes of 30 technicians employed in radiation oncology departments for at least 1 year. The results were compared with those of 30 controls with comparable age, gender, and smoking habits who were not working in radiation oncology or chemotherapy services. The DNA damage observed in the lymphocytes of the technicians was significantly higher than that in the controls.

People are exposed to background radiation every day. However, some people, such as airline crew and frequent fliers, are exposed more than others. Cosmic radiation is made up of ions

travelling from outer space, and these individuals may receive larger doses than the general population because at high altitude the Earth's atmosphere is thinner and offers less protection. The exposure can rise from 100 to 300 times more than at sea level. It has been suggested that air personnel are exposed to higher than normal levels of genotoxic agents, the most hazardous of these being cosmic radiation (approximately 2-3 mSv per year, as well as combustion products from jet fuel, ozone, and electromagnetic fields (EMFs). Cavallo *et al* (2001) used the COMET assay to assess the differences in the levels of DNA damage between aircrew and unaffected controls. For this study, 40 male aircrew members, consisting of pilots and flight technicians, were matched with a control group of 40 ground personnel from the same air company. Personal information that represented possible confounding factors was also collected such as smoking, alcohol, and age. This study showed a difference between the controls and the experimental subjects, albeit statistically insignificantly. Nevertheless, the COMET assay was able to confirm results of previous work outlining the protective role of antioxidants, as well as a relationship between age and smoking on DNA integrity.

Most important for the current study, the effects of β-radiation from a ^{90}Sr source were studied using both the COMET assay and karyotype analysis (De Oliveira, 2001). Blood cells from 5 healthy donors were irradiated *in vitro* with doses of 0.2 - 5.0Gy from a ^{90}Sr source (0.2Gy/min). The cytogenetic results showed that the most frequently found aberration types were acentric fragments, double minutes and dicentrics. Statistical analysis showed that ^{90}Sr β-radiation was less efficient in inducing chromosome aberrations than other types of low linear energy transfer (LET) radiation such as ^{3}H β-particles, ^{60}Co γ-rays, ^{137}Cs and ^{192}Ir and X-rays, and had no effect on the modal chromosome number of irradiated cells. Concerning the COMET assay, there was an increase in DNA migration and tail moment or by visual (morphology) classification. In contrast to the cytogenetic data, ^{90}Sr β-radiation induced more DNA damage than ^{60}Co γ-radiation when the material was analyzed immediately after exposures. This study showed that the COMET assay could efficiently measure genetic damage induced in a cell by ^{90}Sr, which is part of the main rationale for the current study.

The COMET assay is an extremely sensitive test and has been used to detect the genetic effects of very low doses of irradiation, making it appropriate for the current study. Malyapa *et al* (1997) were able to detect significant differences between positive and negative controls after irradiation with gamma radiation doses as small as 1.0 centigray (the equivalent of 1 rad), indicated that the COMET assay was sensitive enough to detect very low levels of

induced DNA damage. Such studies show both the sensitivity of the COMET assay, and its ability to detect damage induced by radioactivity. Based on previous work the COMET assay appears to be an appropriate tool for the detection of any genetic damage that may be present in these veterans.

1.3 CRYOPRESERVATION OF HUMAN BLOOD CELLS

In nature, the survival of an organism below optimal temperatures in not an uncommon event and is sometimes required as part of their lifecycle. Vernalization is a phenomenon characteristic of some plants in which low temperature exposure is a prerequisite to flower bud initiation. Likewise, some species of frogs are able to become "solid blocks of ice" during winter, without a breath of air or a heartbeat for 4 to 5 months, before thawing and becoming active again. This natural form of cryopreservation is slowly becoming understood, and may one day be applied to the storage of whole animal tissues and organs.

"Freezing" in the context of cryopreservation, involves the storage of a cell or organism at a temperature well below the normal operating temperatures of that organism, while maintaining cellular integrity and viability. The storage temperatures used are usually below -70 °C and enables cells to be kept in a viable state of suspended animation for extended periods of time, while still able to return to a "live" state when recovered at higher temperatures. The storage of cellular tissues can be difficult, as the formation of ice crystals tends to cause irreversible damage to the cellular material. Therefore, a method is required that will protect these tissues from the harsh conditions involved with the freezing conditions.

Cryopreservation has been routinely used for the storage of cell samples since the early 1950's. In England, Polge, Smith, and Parkes were experimenting with spermatozoa from roosters to determine if they could be stored at freezing temperatures for later use. The normal protocol for handling sperm involved the use of saline solution, which would normally lead to an average motility of 5 % after cryopreservation. However, during one experiment they found that they were able to increase this mobility to > 50 %. It was later discovered that the labels for several containers used in making the saline had fallen off and been switched, and that glycerol, which was normally used to immobilise the sperm for microscopic analysis, had instead been used to make the solution. This was the first cryopreservant.

Glycerol is still used today for the storage of microbial cell lines at extremely low temperatures; however, other materials with cryoprotectant properties have become known since glycerol. According to Grout *et al* (1990), there are 2 categories of cryoprotectants: those that are able to permeate the cell's plasma membrane, such as glycerol, dimethyl sulfoxide (DMSO), sucrose, trehalose, methanol, glucose, 1.2 propanediol, proline, glycine betaine, fructose, galactose and lactose, and those cryoprotectants that do not permeate the cells, such as hydroxy ethyl starch, dextran and polyvinylpyrrolidone (PVP). The mechanism by which these cryoprotective agents operate is not understood.

Human and other animal cells are routinely frozen in the laboratory. Indeed, some animal cell samples and cell lines are stored for extended periods, as long as a decade, while still retaining their viability (Kleeberger *et al*, 1999). However, genetic integrity is often overlooked in favour of cellular integrity.

1.3.1 CRYOPRESERVATION PILOT STUDY

As well as the current study involving genetic damage in nuclear test veterans, the cryopreservation of human blood samples was conducted as a pilot study to determine if human lymphocytes could be stored for an extended period with minimum damage to genetic material. This study came about as a result of logistical problems found during the current study involving the collection and recollection of samples from 100 participants from several different locations. With a cryopreservation protocol in place, several samples from individuals exposed to a possible genotoxic agent could be taken at once and stored until such a time when they could be assayed. The collection of a large number of samples is not usually possible as only a small number of samples could be processed in a given week. This would increase the throughput of a study that may be halted due to delays in weekly sample collections. However, any protocol that is used must not cause genetic damage that significantly affects the results of any cytogenetic test used and must be refined so that the maximum genetic viability of the samples is achieved.

DMSO seems to be the most common and reliable cryoprotectant for human blood samples. DMSO is useful as it quickly penetrates cellular membranes and acts to protect cellular material from damage caused by extremely low temperature through an unknown mechanism.

DMSO was used in the current study for the cryopreservation of human lymphocyte cells. 20 samples were chosen from those obtained during the nuclear test veteran study, and were used to trial cryopreservation protocols.

1.3.2 CRYOPRESERVATION-INDUCED DNA DAMAGE

The inherent problem when storing blood samples using freezing conditions is the accumulation of genetic damage due to the cryopreservation process. Therefore, the process that is used to store these samples must ensure that this damage is kept at a minimum so as not to affect the results of any cytogenetic test they are used for. The first aspect of such a study that must be clarified is whether cryopreservation actually causes genetic damage.

It has been proposed that the injury is the result of several factors, including ice nucleation and dehydration (Mazur, 1970). However, recent studies have suggested oxidation due to the presence of oxygen free radicals is the main factor that contributes to freeze-thaw injury. This has been predicted through experiments using the snake *Thamnophis sirtalis* (Hermes-Lima *et al*, 1993) and the yeast *Saccharomyces cerevisiae* (Park *et al*, 1998) that have shown that an oxidative burst occurs upon thawing. Indeed, in a mutagenesis study by Park *et al* (1998) it was demonstrated that oxidative stress contributes to injury of yeast cells during the freeze-thaw process and that superoxide dismutase may be required for resistance to this injury. This was proven further in a study by Stead and Park (2000) that exploited the availability of mutations in 2 key components of the oxidative stress defence system of *Campylobacter coli*, superoxide dismutase and catalase, in order to determine the role of oxidative stress in the injury of these cells that occurs during freezing and thawing. They discovered that those bacteria with mutations in superoxide dismutase, but not catalase were far less viable than the parental, non-mutant strain. This indicates that $O^{\cdot}$, but not H_2O_2, was formed during freezing and thawing and is responsible for inducing DNA damage during cryopreservation.

Therefore, in order to successfully store blood samples, the levels of oxidation via reactive oxygen species must somehow be kept to a minimum. Antioxidant compounds that are able to scavenge these free radicals may solve these inherent problems, but this remains to be seen.

1.3.3 ANALYSIS OF CRYOPRESERVATION-INDUCED DAMAGE

Detection of genetic damage induced as a result of the cryopreservation procedure can be analysed using several cytogenetic techniques. Chromosome aberrations (Maurício Barbanti Duarte *et al*, 1999) and multicolour FISH (Karhu *et al*, 2002) have been used. However, the assay of choice for many studies that involve cryopreservation-induced genetic damage is the COMET assay. This is due to its ability to detect DNA fragmentation, such as that induced through cryopreservation (Men *et al*, 2003; Visvardis *et al*, 1995). The COMET assay was used in the current pilot study to detect any damage associated with the cryopreservation of the veterans' samples. There have been relatively few studies conducted regarding the genetic integrity of cells that have been cryopreserved. The majority of these studies have focussed on the transformation of these cells into immortalised cell lines, but not cytogenetic studies.

In perhaps the most important cytogenetic study involving cryopreservation, Visvardis *et al* (1995) used the COMET assay to collect important information concerning cryopreservation, and DNA damage and repair. Experiments were conducted using fresh and cryogenically preserved lymphocytes that had been exposed to gamma radiation or hydrogen peroxide in varying concentrations. They used the COMET assay to test the repair capacity of the cells. After exposing the samples to either gamma radiation or hydrogen peroxide, they allowed the cells to recover for 2 hours. In 2 hours of recovery time the cell was able to repair some of the DNA strand breakages that had been induced. They experimented with both fresh and cryopreserved lymphocytes and found that the cryogenically stored cells had a lower repair capacity than fresh cells. During their studies they found that there were different modes of action between the 2 agents, as well as the effect of cryopreservation on DNA repair.

Cryopreservation seems to be necessary only for long-term storage. Anderson *et al* (1997) conducted a joint study between 2 laboratories, one in England and one in Spain, to determine the effects of different storage temperatures on blood cells. Blood samples were taken from various donors and stored at room temperature. The samples were then examined 1, 2, 3, 4, 5, or 8 days after storage using the COMET assay. They found that for samples stored at 4 °C, after 4 days there was no significant increase in the levels of genetic damage, as measured by the COMET assay, and after 8 days there was only a moderate increase. This was only true for sample stored in RPMI (Roswell Park Memorial Institute) media. PBS seems to be only useful for fresh samples that are to be used immediately. On the other hand, samples

cryopreserved at -20 °C were found to have a significant increase in the levels of genetic damage, indicating that cryopreservation should not be used unless it is necessary for long-term storage.

The cryopreservation pilot study was conducted concurrently with the NTV study and involved determining whether human lymphocyte samples can be successfully stored and retrieved without the accumulation of genetic damage. The technique that was used to measure this damage was the alkaline COMET assay. If the results from this study show that the storage of human blood samples is possible, it may be feasible that this technique could be used for the bulk collection of samples with the intention of assaying them for genetic damage at a later date. This pilot study was based on research conducted by Visvardis *et al* (1995).

1.4 OBJECTIVES OF THIS THESIS

The main objective of this thesis was to test the hypothesis that as a result of witnessing the detonation of nuclear weapons during Operation Grapple, the servicemen have a level of genetic damage significantly greater than that of control military personnel who were not present during the weapons testing. The levels of genetic damage in these samples were measured using the alkaline COMET assay.

During the course of this project, a second aim was added. This involved determining whether human lymphocytes can be stored at extremely low temperatures and then recovered with minimal genetic damage as a result of the freeze/thaw process. This study would also involve the COMET assay as a test for genetic damage.

The following questions are addressed in this thesis:

- Can the COMET assay be used to detect genetic damage in lymphocyte samples exposed *in vitro* to a known genotoxic agent?
- Can the COMET assay be used to measure the abilities of the DNA repair mechanism of a lymphocyte sample after exposure to a known genotoxic agent?
- Are there any differences in the levels of genetic damage in the participants of Operation Grapple in comparison to matched controls?
- Can human lymphocytes be stored cryogenically without the accumulation of significant levels of genetic damage?

☢ - CHAPTER 2: MATERIALS AND METHODS - ☢

2.1 MATERIALS

The materials used during this research were stored at room temperature, unless stated. Sterilization of these materials was not required, unless stated, due to the nature of the assay. If sterilization was an issue, aseptic technique was used.

2.1.1 AGAROSE GELS

1 % Low Melting-point Agarose (LMA)

LMAgarose (Life Technologies Inc.)	1 g
1X PBS (Bio-Rad Laboratories)	100 ml

The agarose powder was mixed well with PBS and heated in a microwave with the cap loosened for ~30 seconds, or until solution turned clear. The agarose was prepared as stock and aliquots were melted using a hotplate and transferred into a smaller vial as required for experimental work.

0.5 % Normal Melting Point Agarose (NMA)

NMA Powder (Life Technologies Inc.)	0.5 g
1X PBS (Bio-Rad Laboratories)	100 ml

The agarose powder was mixed well with PBS and heated in a microwave with the cap loosened for 60 seconds, or until solution turned clear. The agarose was prepared as stock and aliquots were melted using a hotplate and transferred into a smaller vial as required for experimental work.

2.1.2 CELL PREPARATION MEDIA

Ficoll-Paque media

Ficoll-Paque Plus media was obtained (Amersham Bioscience) and used undiluted. An equal volume of Ficoll was layered beneath a blood:PBS 1:1 mixture before centrifugation. Ficoll-Paque media was stored at 4 °C.

1x Phosphate Buffered Saline (PBS)

10X PBS (No Ca^{2+} or Mg^{2+}) (Bio-Rad Laboratories)	10 ml
Milli-Q Water (MqH_2O)	90 ml

10X concentrated PBS was mixed well with an appropriate volume of mqH_2O and refrigerated.

Trypan Blue Stain

Trypan blue stain (BDH Chemicals)	0.1 g
DH_2O	10 ml

The stain was mixed well until all powder had dissolved.

2.1.3 DNA STAINS

SYBR Green™ Stain

10,000X SYBR Green™ stain, (Trevigen)	1 µl
TE buffer, pH 7.5	10 ml

The stain was mixed well and stored at 4 °C in the dark. Temperature, light, and pH are important for the stain's viability.

4', 6-Diamidino-2-phenylindole (DAPI) Stain

Vectashield™ with DAPI was obtained (In Vitro Life Science Technologies Inc.) and used undiluted. DAPI was stored at 4 °C in the dark to maintain viability.

2.1.4 MOLECULAR BIOLOGY BUFFERS AND SOLUTIONS

Dimethyl Sulfoxide (DMSO)

DMSO was obtained (Life Technologies Inc.) and used undiluted. DMSO was stored at room temperature in the dark.

0.2M EDTA, pH 8

0.5 M EDTA (BDH Laboratories)	6 ml
MqH_2O	9 ml

A stock solution of 0.5 M EDTA was diluted using mqH_2O to reach the desired concentration.

0.5M EDTA, pH 8

EDTA (BDH Laboratories)	14.61 g
MqH_2O	50 ml
10 M NaOH (BDH Laboratories)	

The solution was mixed well and NaOH was added drop-wise until the desired pH was reached and all powder was dissolved.

70 % Ethanol Solution

Absolute ethanol (BDH Laboratories)	350 ml
DH_2O	150 ml

100 µM Hydrogen Peroxide (H_2O_2)

8.8 M Hydrogen peroxide (30 %) (BDH Laboratories)
MqH_20 to dilute

100 µM hydrogen peroxide solutions were made using 3 serial dilutions. 10 µl of an 8.8 M H_20_2 stock solution was added to 88 µl of mqH_20 and mixed well to make a 1 M. 1 µl of this 1 M solution was added to 1 ml of mqH_20 to make a 1 mM. 100 µl of this 100 mM solution was added to 900 µl of mqH_20 to make a 100 µM H_20_2 solution. This was stored on ice.

TE Buffer, pH 7.5

0.5 M EDTA (BDH Laboratories)	20 µl
1 M Tris, pH 7.5 (Invitrogen)	100 µl
MqH_2O	10 ml
10 M NaOH	

The solution was mixed well and NaOH was added drop-wise until the desired pH was reached and all powder was dissolved. The solution was stored at room temperature.

2.1.5 COMET ASSAY MATERIALS

All solutions for the COMET assay in this section were made fresh for each sampling day.

Alkaline Electrophoresis Buffer, ~pH 13

NaOH (BDH Laboratories)	12 g
0.5 M EDTA (BDH Laboratories)	2 ml
MqH_2O	1000 ml

The solution was mixed well until fully dissolved and left to chill at 4 °C for at least 60 minutes before use.

Alkaline Solution, pH 13

NaOH powder (BDH Laboratories)	0.6 g
0.2 M EDTA (BDH Laboratories)	250 µl
MqH_2O	50 ml

The solution was mixed well until fully dissolved and left to cool at room temperature for at least 60 minutes before use.

Lysis Solution

Per 10 slides:	
Lysis solution (Trevigen)	40 ml
DMSO (Life Technologies Inc.)	400 µl

The solution was mixed well by gentle agitation and stored at 4 °C for at least 30 minutes before use.

The lysis solution contains the following components (Singh *et al*, 1988).

NaCl	146.1 g
EDTA	37.2 g
NaOH	8.0 g
Trizma base	1.2 g

The components were added to approximately 700 ml of dH_2O, and mixed well. NaOH was added to allow powders to dissolve for about 20 minutes. The pH was adjusted to 10.0 using concentrated NaOH or HCl, before filling to 890 ml with mqH_2O. The solution was stored at room temperature. Before each assay, 35.6 ml of the above solution was measured and 400 µl of Triton X-100 and 4 ml of DMSO were added fresh to a final volume of 40 ml. The solution was then stored at 4 °C for at least 30 minutes before use. This protocol is included only for researchers who wish to make their own lysis solution. The lysis solution used in this thesis was obtained commercially.

2.1.6 CELL CULTURE MEDIA

Cell Culture Media

Wellcome media (media 99) (Gibco)	5 ml
AB serum (Gibco)	1 ml
Phytohaemagglutinin (PHA) (Gibco)	0.1 ml

The cell culture media was prepared fresh when required. Aseptic conditions were used during the use of this material to prevent bacterial contamination. The total volume of media prepared depended on what was required.

2.1.7 CRYOPRESERVATION MEDIA

Freezing Media

Dimethyl Sulfoxide (DMSO) (Sigma Chemicals Co.)	10 % (v/v)
Foetal calf serum (FCS) (Invitrogen Corp.)	50 % (v/v)
RPMI 1640 medium (Life Technologies Inc.)	40 % (v/v)

The freezing media was prepared fresh when required. FCS was heat-inactivated at 57 °C for 30 minutes before use and then added to chilled RPMI, under aseptic conditions. The total volume of media prepared depended on what was required.

Thawing Media

Foetal Calf Serum (FCS) (Life Technologies Inc.)	10 % (v/v)
RPMI 1640 medium (Invitrogen Corp.)	90 % (v/v)

The thawing media was prepared fresh when required. FCS was heat-inactivated at 57 °C for 30 minutes before use and then added to chilled RPMI, under aseptic conditions. The total volume of media prepared depended on what was required.

2.2 METHODS

2.2.1 EXPERIMENTAL DESIGN

In order to select participants for the current study, the current literature was consulted, as well as professional researchers, to determine the best possible approach. Prospective participants were asked to complete a questionnaire to establish any factors that could affect the results. Appendix VIII contains a copy of this questionnaire. Information such as lifestyle, employment, and medical history was collected and used to determine who amongst the individuals that completed the questionnaire would be suitable for the study. A face-to-face interview was also conducted.

For those who returned their questionnaires, several inclusion and exclusion criteria were set in order to select the participants of this study. For the experimental and control groups, the following criterions were set:

Experimental Group Selection Criteria

Inclusion Criteria

The participant was:

- Involved in Operation Grapple.
- Aged between 60 and 70 years at the beginning of the study.

Exclusion Criteria

The participant was not:

- Involved in another theatre of war/nuclear related area.
- Exposed to toxic substances for more than 1 year.
- Undergoing radiation or chemotherapy treatment.
- Enlisted in the air force.
- Too ill to be involved in the study.
- A resident of the South Island of New Zealand.

Control Group Selection Criteria

Inclusion Criteria

The participant was:

- Aged between 60 and 70 years at the beginning of the study.

Exclusion Criteria

As for the experimental group, plus

The participant was not:

- Involved in Operation Grapple.
- A recent immigrant.
- Educated to a high level (e.g. a surgeon).
- A participant of compulsory military training.

Of those who completed the questionnaire, 50 nuclear test veterans and 50 controls were chosen for the study using these criteria.

The study was conducted single blind. All samples were coded and recoded to ensure that no one involved in the study was aware which of the samples assayed were from an experimental (E) participant or from a control (C) participant. This method was used so that those involved in the testing could remain objective and the results gained could retain credibility. After a blood sample was taken from a participant by a trained phlebotomist, who was not associated with this research, the tubes containing the blood were given a code to identify that individual. These tubes were then re-coded by Massey University Student's Health Service, so the researcher could not determine the identities of the participants. The samples were decoded at the conclusion of the research for detailed analysis.

2.2.2 SAMPLE HANDLING

2.2.2.1 Lymphocyte Preparation

Peripheral blood lymphocytes (PBLs) were used in this study due to their relative ease of collection. They are also a good indicator of the body's overall health.

Lymphocytes were separated from donated whole blood using a method kindly supplied by Mr Chris Kendrick (personal communication, April 3, 2002). 10 ml of fresh, whole blood was collected from donors by venipuncture in heparinized tubes (Vacutainer™). The samples were stored in a custom-made incubator and stored overnight for transport at 25 °C. After the samples were received they were assayed for the percentage of different cell types present in each sample, and transported to the researchers for testing. Care was taken to ensure that damage due to exogenous sources was kept at a minimum. When blood samples were received in the laboratory for the COMET assay the tubes were stored on ice and in low light. All steps involving separation of lymphocytes were performed in a biohazard hood to prevent cross-contamination.

To isolate the lymphocytes, blood samples were mixed by a ratio of 1:1 in 8 ml Falcon™ tubes to a total volume of 5 ml with chilled phosphate buffered saline solution (PBS) and mixed by gentle agitation. The PBS contained no Ca^{2+} or Mg^{2+} to ensure minimal *in vivo* DNA repair during the assay procedure. 2.5 ml of the Blood:PBS mixture was carefully layered on to an equal volume of a Ficoll-Paque lymphocyte separation media (Amersham Bioscience) in a new 8 ml Falcon™ tube and centrifuged at 400 x g for 20 minutes using a Heraeus Megafuge 1.0 equipped with a 17 cm rotor. If the separation of lymphocytes from erythrocytes was incomplete, centrifugation was repeated at 800 x g for 20 minutes, or until the lymphocytes were completely separated from plasma and erythrocytes. After centrifugation the sample resembled Figure 2.1.

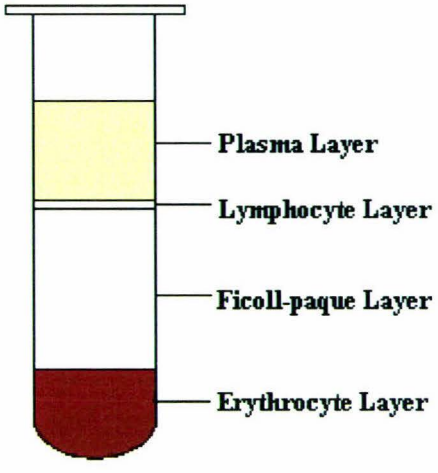

Figure 2.1. Diagram illustrating the separated layers after the centrifugation of whole blood using a Ficoll concentration gradient.

The top layer was a yellowish colour that consisted of plasma, platelets, and a small amount of erythrocytes and peripheral blood lymphocytes (PBLs). The large, clear layer contained the Ficoll gradient, and the dark red mass at the bottom of the tube consisted mainly of erythrocytes. A wispy, white "buffy" layer was observed between the plasma layer and the Ficoll layer that contained the majority of the lymphocytes. A Pasteur pipette was used to collect the lymphocyte layer in a swirling motion, with as little contamination from the upper and lower layers as possible. The lymphocytes were transferred to an 8 ml Falcon tube that contained 1 ml of chilled PBS buffer, mixed slowly by vortex, and stored on ice.

2.2.2.2 Lymphocyte Concentration And Cell Viability

To determine the lymphocyte concentration of the isolated sample, an "Improved Neubauer" haemocytometer was used (Weber Scientific International Ltd). A haemocytometer is a specialised slide with 2 chambers, each with grid patterns, that is used to determine the concentration of cells in a medium. Each grid is made up of 9 large squares with sides that are 1 mm in length. The layout of this grid is illustrated in Figure 2.2.

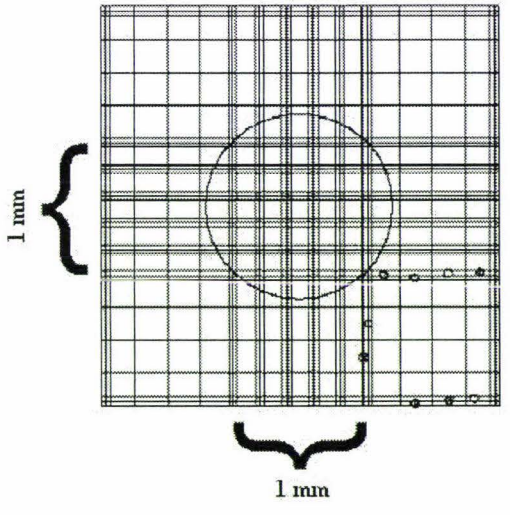

Figure 2.2. Diagram representing the dimensions of a haemocytometer counting chamber.

When the coverslip was placed on the clean haemocytometer there was a slight rainbow appearance over the edges of the coverslip. This indicated that the distance between the slide and the coverslip is 0.1 mm and therefore able to give an accurate cell count. The large square circled in Figure 2.2 was used for all counts. This square is made up of 25 smaller squares,

each with sides that are 0.2 mm in length. The volume contained in the large centre square is 0.1 mm^3 (1 mm x 1 mm x 0.1 mm). There is 1ml in $1cm^3$, and $1000mm^3$ in $1cm^3$, and 1 x 10^4 (1000 mm^3 x 1 x 10) volumes of the counting square in 1ml. Therefore, by counting the number of cells in the large centre square and multiplying this by 1 x 10^4, it will give the concentration of the cell sample in cells/ml.

For this study, the calculation of cell viability was required. Cell concentration and cell viability was determined using trypan blue exclusion and a haemocytometer. 1 drop of trypan blue stain was added to 1 drop of cell suspension in an Eppendorf tube, mixed well, and allowed to incubate for 1 minute. After 1 minute approximately 20 µl of the cell:stain solution was taken up using a Pasteur pipette and slowly added to the edge of the haemocytometer's coverslip, which is spread across the slide by capillary action. The sample was added until the chamber was filled, taking care not to overfill. The slide was observed under an Olympus® CHA light microscope using 400X magnification to determine the viability of the sample.

Non-viable cells are visible as dark blue structures due to the dye permeating the compromised cell membrane, while viable cells appear to be relatively clear against a darker background. Only cell samples with a cell viability of > 90 % were used for the COMET assay (Visvardis *et al*, 1994). A dilution factor of 2 was taken into consideration when determining lymphocyte concentrations due to addition of trypan blue stain. Lymphocytes were distinguished from erythrocytes by visual morphology only, although identification by other methods can be used, e.g. Turk's stain or using a haemolytic solution.

The total number of cells within the central counting grid was counted, as well as the number of viable cells. The total cell number was multiplied by 2 x 10^4 (1 x 10^4 multiplied by a dilution factor of 2) to give the approximate concentration of cells/ml. For example, if the number of cells in the large centre square was found to be 10, then the concentration of the cell solution is approximately 200,000 or 2 x 10^5 cells/ml. Cell viability was determined by the number of viable cells divided by the total number of cells counted, all multiplied by 100.

The cell concentration required for the COMET assay is approximately 1 x 10^5 cells/ml. Samples were diluted with PBS as required, or if the concentration was too low then they were concentrated by centrifugation and resuspended in a lower volume. Unless otherwise stated, a vortex mixer was used throughout this research for the resuspension of a pellet.

2.2.2.3 COMET Assay Positive Controls

A positive control was required in order to test the efficiency of the COMET assay for detecting DNA damage. This required a sample to be intentionally exposed to an agent that induced genetic damage. The most convenient genotoxin available was hydrogen peroxide (H_2O_2). A 30 % stock solution of H_2O_2 was prepared by serial dilution to produce the required concentration. 1 ml of this solution was added to 1 ml aliquots of a cell sample and incubated for 20 minutes on ice. The sample was then washed twice by centrifugation at 1000 x g for 5 minutes and the pellet resuspended with ice cold PBS. The samples were then processed as normal for the COMET assay (Visvardis *et al,* 1994).

2.2.3 SLIDE PREPARATION

2.2.3.1 CometSlide™ Method

CometSlides™ (Trevigen Ltd.) contain 2 sample wells that are coated with a layer that allows adherence of the gel without a pre-treatment step. An example of such a slide is illustrated in Figure 2.3.

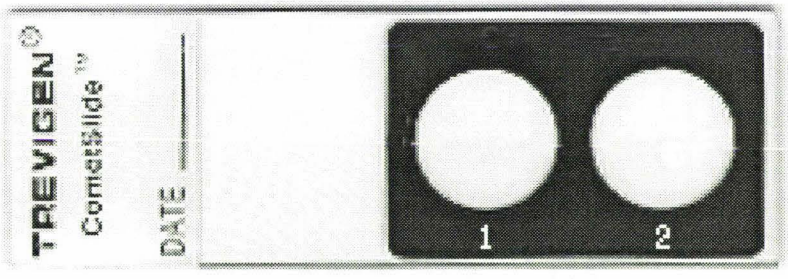

Figure 2.3. A Trevigen brand CometSlide™ that was used in the initial stages of this study. Each slide has 2 sample wells.

10 µl of the cell solution was mixed with 90 µl of Low Melting-point Agarose (LMA, Trevigen Inc, 1 % per volume) in an Eppendorf tube. This was mixed by gentle agitation and 75 µl of was applied to a well of the slide and spread evenly over the surface. The slides were then stored in the 4 °C room for 30 minutes to allow the agarose to solidify. These slides were used in preliminary experiments.

2.2.3.2 Pre-treatment Method

In the actual veterans study, Trevigen CometSlides™ were not used due to the costs involved. Instead, COMET assay slides were prepared from conventional microscope slides using a modified version of the pre-treatment method outlined by Singh *et al* (1988).

26 mm x 76 mm microscope slides (Lomb Scientific Ltd) were first marked at the centre with an electronic etcher to identify the sample number that was to be added to each slide. Vivid or similar markers could not be used as the ink is removed by the COMET assay solutions. The slides were then cleaned using a lint-free tissue to remove grease, moisture, and dust, all of which can cause the gel to detach during the assay. An aliquot of electrophoresis-grade, normal melting-temperature agarose (NMA, 0.5 % per volume, Life Technologies Inc.) was melted using a microwave, and 2 aliquots of 75 µl of the molten agarose was pipetted onto each of the slides, 1 at each end. A 22 mm x 22 mm glass microscope coverslip (Biolab Scientific) was placed on top of each aliquot to act as a type of mould. The slides were stored at 4 °C for at least 60 minutes to allow this base layer of agarose to solidify. This layer served as an attachment layer for later agarose gel layers. During seasons of low humidity, such as winter, these gels are prone to drying too fast before a coverslip can be added. To overcome this, slides were placed on top of a slide warmer that had been covered with a damp tissue to create artificial humidity during application of the base gel layer. This change would not affect results, as no cells were present in this layer.

An aliquot of electrophoresis-grade, low melting-temperature Agarose (LMA, 1 % per volume, Life Technologies Inc.) was melted using a boiling water bath. After 10 minutes the LMA was transferred to a 42 °C water bath to cool for at least 10 minutes. Cells were not added until the gel had cooled in order to prevent temperature-associated damage (Speit *et al,* 1999). The coverslip was carefully removed from the base layer by sliding, taking care not to tear or disturb the base agarose layer. 10 µl of the cell sample from a participant (Section 2.2.2) was mixed well with 90 µl of the molten LMA by gentle agitation in an Eppendorf tube. 70 µl of this cell/agarose mixture was added to each of the base gel layers and a fresh coverslip was again placed on top of each of the new layers. The slide was stored at 4 °C for 20 minutes to allow the agarose to solidify. The coverslip was again removed and another 70 µl of LMA 42 °C layer was added. This layer contained no cells and functioned as a

protective layer to prevent detachment of the cells from the gel while in solution. A fresh coverslip was then placed on top of this new layer and the slide was stored at 4 °C for 30 minutes to allow the agarose to solidify. A diagrammatic representation of the gel layers is seen in Figure 2.4.

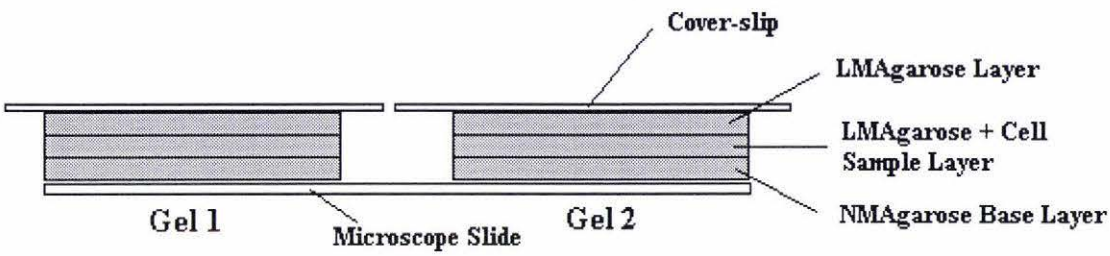

Figure 2.4. Image illustrating how the gel layers were formed using the pre-treatment method. Image is not drawn to scale.

2 replicate slides were prepared for each sample, each with 2 gels (designated Gel 1 and Gel 2 as illustrated in Figure 2.4) at either end. Each slide was processed 1 step apart from its counterpart to take into account any variability that may occur between sample runs, i.e. while the first slide was immersed in alkaline solution (the second step of the COMET assay) the second slide was immersed in the lysis solution (the first step of the COMET assay). 2 gels per slide were prepared as a precaution as the gels would sometimes become detached from the slide while in solution, and hence will become unusable.

After the gels had solidified the coverslips were carefully removed so that the slides could be subjected to the COMET assay (Section 2.2.4).

2.2.4 THE COMET ASSAY

The COMET assay was performed using a modified version of the methodology described by Singh *et al.* (1988). All solutions for the COMET assay were prepared at least 1 hour before the assay was commenced. Unless stated, solutions were prepared using mqH_2O. The lysis solution and electrophoresis solution were prepared and stored in a 4 °C refrigerated room, while the alkaline solution was cooled and stored at room temperature. Samples were kept in the dark during all stages of the assay. Figure 2.5 shows the basic steps of the alkaline COMET assay.

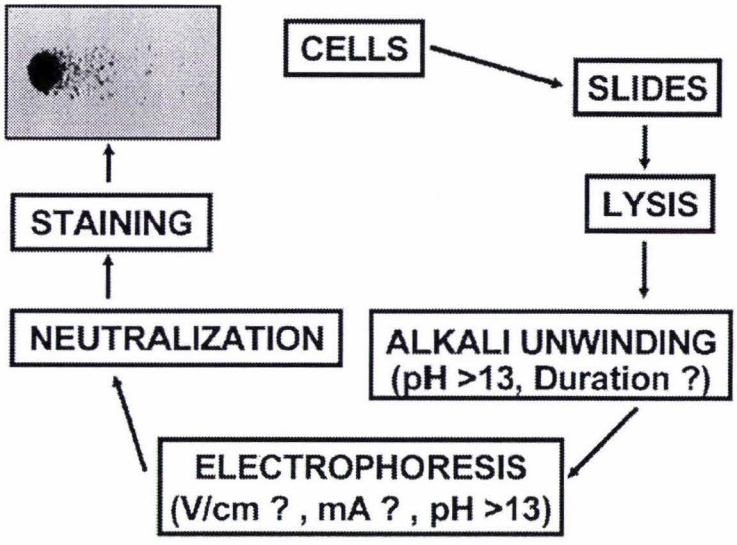

Figure 2.5. Figure taken from Tice *et al* (2000) outlining the basic steps of the COMET assay.

2.2.4.1 Lysis Solution

The lysis solution contains non-ionic detergents and high salts that will remove the lipid membranes and most of the cellular proteins. The detergents will lyse the cells including the nuclear membrane and, to a certain extent, dissociate the non-histone proteins from the DNA. The high levels of NaCl in the lysis buffer remove the histones from the nuclear complex. The remaining nucleoids contain DNA in the form of long, supercoiled loops that are anchored in the residual nuclear matrix (Gasiorowski *et al*, 2001). The DNA and its matrix remain embedded in the agarose. Without encapsulation by the nucleus, any fragments are able to freely migrate through the agarose gel under electrophoretic conditions.

The lysis solution is also supplemented with Dimethyl Sulfoxide (DMSO). DMSO is a chelator of Fe^{2+} and Fe^{3+} ions that are released from haemoglobin when red blood cells are lysed. Research has shown that these ferrous ions can indirectly cause DNA damage by way of a Fenton reaction; a process that causes the accumulation of hydroxyl radicals through an iron mediated reaction. Therefore, by using DMSO to chelate any free Fe ions, oxidative DNA damage induced by the lysis of blood cells will be reduced (Tice *et al*, 1991).

After the coverslips were removed, the slides were immediately immersed in 40 ml of lysis solution (Trevigen Inc.), which had been chilled at 4 °C or on ice for at least 60 minutes

before use. The slides were immersed until levels just covered the gels. Immersion was conducted at 4 °C for 40 minutes in the dark. The slides were then carefully removed from the solution, drained, and then immediately transferred to the alkaline solution (Section 2.2.4.2).

2.2.4.2 Alkaline Solution

Hydrogen bonding is very sensitive to H^+ concentration. At pH greater than 11.3 (alkaline conditions) all hydrogen bonds in DNA are eliminated and the DNA helix is completely denatured into its single stranded form. This is caused by deprotonation of the species that are involved in creating the hydrogen bonds between nucleotide pairs. Acid conditions will also denature DNA, however this has been found to cause damage to the phosphodiester linkages and will affect the results of the COMET assay. It is therefore not appropriate to use acidic conditions for the COMET assay. Phosphodiester bonds are resistant to alkaline pH, and so a pH > 11 is the method of choice for denaturing DNA in the laboratory.

When DNA receives a DSB, it is broken into 2 fragments. When an electric current is applied to the nucleus the fragments, assuming that they are small enough, can migrate to the positive electrode. This is the basis of the neutral COMET assay. However, if the DNA receives a SSB, the DNA helix will remain intact due to the hydrogen bonds between base pairs that hold the 2 strands together. This is the reason that alkaline conditions are used. By separating the strands, this effectively releases any fragments that have been induced by SSBs. The fragments are then free to migrate when an electric current is applied. This makes the alkaline COMET assay much more sensitive for the detection of DNA damage than that of the neutral assay, due to the fact that SSBs occur more frequently than DSBs.

Yendle *et al* (1997) found that the alkaline step was the most significant step of the COMET assay, as any deviation from the amount of time a cell is exposed to the alkaline solution will affect the results significantly. Using several exposure times from 30 minutes to 18 hours it was observed that the amount of damage measured by the COMET assay increased significantly. This was due to more fragments being released from the nucleus as a result of increased denaturation of the DNA helix. Therefore, the time used for the alkaline step should be strictly adhered to. A similar study involving the lysis solution showed no measurable effect.

The slides were immersed in 50 ml of alkaline solution and incubated for 40 minutes at room temperature, in the dark. At no point during this stage was the container of alkaline solution disturbed or moved, as gels tend to detach from the slides at this point due to the conditions. The slides were then carefully removed and any excess solution was drained off. Slides were immediately transferred to the electrophoresis apparatus (Section 2.2.4.3).

2.2.4.3 Alkaline Electrophoresis

DNA is negatively charged. Therefore, it will migrate to the positive electrode when subjected to gel electrophoresis. The rate of migration will depend on the size of the fragments; a smaller fragment will migrate further than a larger fragment. This is important when using the COMET assay because the length of the tail will rely on the amount and size of fragmentation that has been induced by the genotoxic agent that is being investigated. Therefore, smaller fragments will create a longer tail than the same number of larger fragments. This is the basic principle behind the COMET assay. When the electric current is applied the fragments that have broken free from the main helix will leach out of the nucleus, to be drawn along the gel.

An electrophoresis apparatus was filled with 1 Litre (L) of chilled, alkaline electrophoresis buffer at least 1 hour before use. The volume used will depend upon the apparatus used. Work involving the apparatus was conducted at 4 °C in the dark to reduce any temperature-related damage due to heating of the solution by the electric current. Heat may affect the results of the assay, or cause the gel to detach from the slide. A maximum of 5 slides were placed in the centre of the apparatus in each run. All slides were placed in the apparatus so that all comet tails were directed from left to right in order to accommodate the COMET assay analysis software used in later experiments. The settings for electrophoresis were applied at 1 volt per cm in length of the electrophoresis apparatus at 300 milliamperes (mA). The apparatus was approximately 30 centimetres (cm) in length and so the voltage applied was 30 volts (V). A current of 300 mA was applied to normalise the volume of electrophoresis buffer used between gel runs. This current is applied regardless of the size of the apparatus. The amount of buffer will affect the electrophoresis current, and so buffer was added or removed until this current was achieved. The voltage was applied for a time of 30 minutes. The placement of slides in the apparatus is illustrated in Figure 2.6.

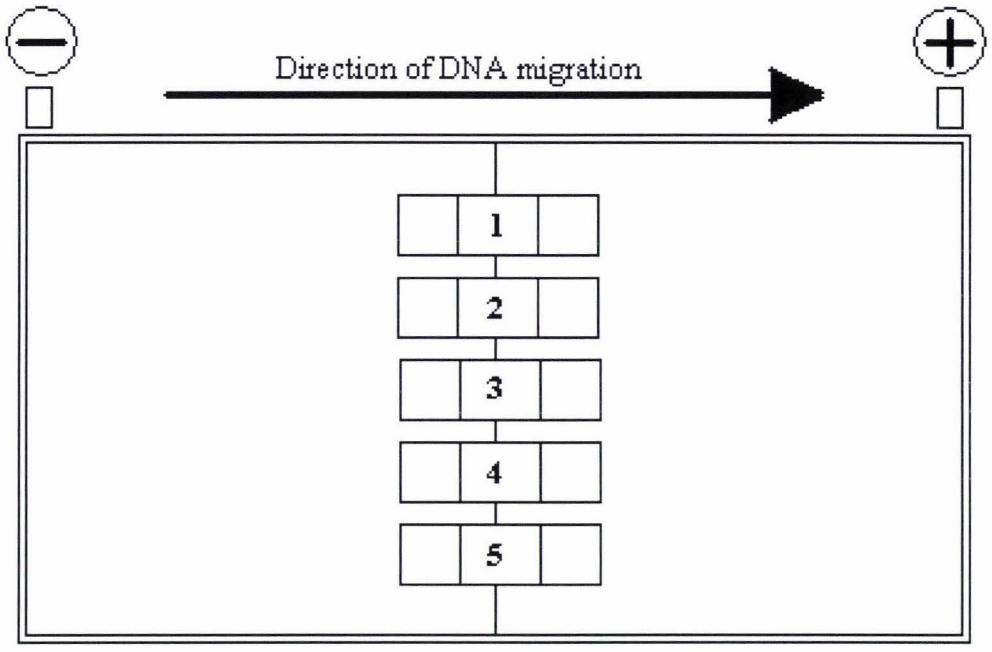

Figure 2.6. Image indicating the placement of microscope slides in electrophoresis tank.

The slides were then removed from the apparatus and immersed in 70 % ethanol for 5 minutes. The slides were removed from the ethanol and allowed to dehydrate overnight in a slide box that contained desiccant (silica, AnalaR). The drying step is important as it brings the cells into the same field of view to allow observation by fluorescent microscopy.

2.2.5 SLIDE ANALYSIS

There are several methods for scoring cells that have been subjected to the COMET assay. The earliest method to be used was to measure the length of the tails of cells through the use of an eyepiece micrometer (Östling *et al*, 1984; Singh *et al*, 1988). This was later paired with a qualitative system involving the manual allocation of a grade to each cell based on the morphology of the comet cell. Several methods for scoring have emerged in the literature as COMET assay techniques have been developed, such as the head and tail DNA content and comet length. However, the most useful has been the advent of the tail moment and the Olive tail moment (Olive *et al*, 1990). These 2 measurements are similar and are derived from an equation involving the percentage of the cell's DNA present in the tail and the length of the cell's tail. These measurements appear to be the most reliable in terms of assessing the levels of DNA damage present in a sample.

Several variations of these measurements have been reported. Instead of relying on the morphology, Gasiorowshi *et al* (2001) used the head and tail DNA content to assign a grade to damaged cells during a DNA repair study. A study by Heaton *et al* (2001) used the tail length multiplied by the assigned morphology grade to produce a value know as the damage grade to determine the levels of damage in cats and dogs in a nutritional study. There is no consensus protocol for the analysis of samples subjected to the COMET assay.

The inherent problem with using a qualitative measurement, such as assigning a grade based on morphology, is the objectivity of the scorer. If possible, it is recommended to use commercially available software, which takes the subjectivity out of the analysis of the COMET assay. Nevertheless, research conducted by Kobayashi *et al* (1995) involving the comparison of manual scoring systems to software-oriented automatic scoring found that either method was able to give an accurate indication of the levels of damage. A software package known as the CASP™ was used in the current study for the analysis of genetic damage, as measured by the COMET assay. However, the use of either a quantitative or qualitative method for scoring the COMET assay is generally accepted.

The number of cells that should be assayed for each sample varies from paper to paper. As little as 50 (Valverde *et al*, 1999) and as many as 1000 cells per sample have been scored (Ivancsits *et al*, 2002). However, Tice *et al* (2000) recommends that a minimum 100 cells should be scored for each sample. This was the number of cells that were analysed during the current study.

For the visualisation of cells several fluorescent stains have been used. These include: Acridine orange, Ethidium bromide, DAPI, Hoechst 33258 and 33342, Propidium iodide, SYBR™ Gold, SYBR™ Green, and Yo-Yo Dye. Several other lesser-known DNA stains have also been used.

2.2.5.1 Qualitative Analysis

The following method of analysis was trialed successfully before the software package subsequently used in the NTV study became available. It is included as a reference for any future researcher who wishes to apply it. To score the CometSlides™, the gels were stained

using SYBR™ Green stain (Trevigen Ltd), however, other stains have been successfully used for the COMET assay such as ethidium bromide, DAPI, YO-YO1 or acridine orange. 50 µl of SYBR™ Green stain was applied to each gel, which was then allowed to rehydrate for approximately 5 minutes. A coverslip was not required for observation. Analysis was conducted using a fluorescence microscope (Zeiss) with an excitation/emission filter of 494 nm and 521 nm respectively.

For each cell scored, the length of the tail from the centre of the nucleus to the most distant DNA fragments in the tail was recorded using arbitrary eyepiece micrometer units under the 16X objective lens. The centre was determined by eye as the brightest and widest area of the comet's "head". An example of this is illustrated in Figure 2.7.

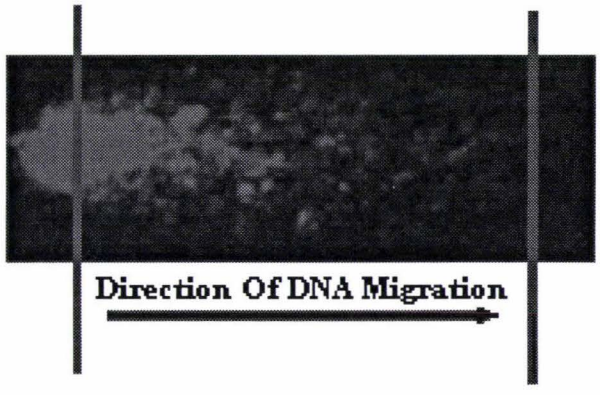

Figure 2.7. Image illustrating the points where the tail length of each comet was taken. Image obtained from www.cometassay.com.

A stage micrometer was used to calibrate the eyepiece micrometer, and it was found that 20 arbitrary eyepiece units = 120 µm. However, for simplicity these units were not converted.

A visual grade was assigned based on the morphologies illustrated in Figure 2.8. These ranged from grade 0, which is a normal cell with little to no tail, to grade 5, the "exclamation point" morphology in which only the nucleolus is present at the head of the comet. Figure 2.8 was used as an example of the 6 different morphologies when assigning such a grade.

These 2 factors (length and morphology) were studied because they represent the 2 most important factors. Other factors may be studied such as DNA signal intensity (which indicates DNA content), or tail moment but these were not calculated in this section due to time

constraints. Specialised software (subsequently used) is required to determine these values and was not available to the researcher at the time these qualitative experiments were conducted. It has been found in several studies that qualitative results are as valid as quantitative results (Kobayashi *et al,* 1995). The tail length and the visual grade were multiplied together to give the overall damage unit (Heaton *et al*, 2001).

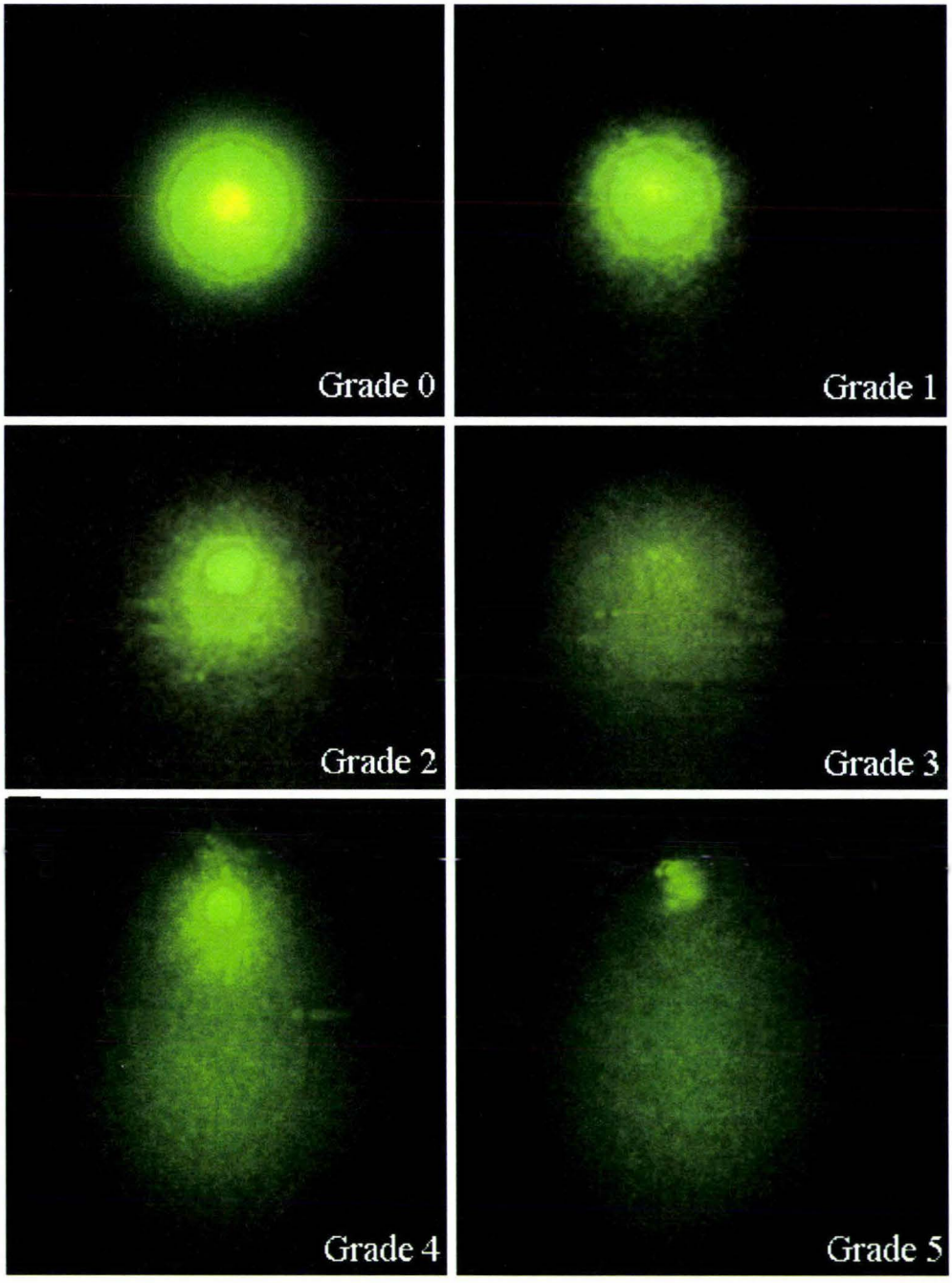

Figure 2.8. Chart illustrating the different morphologies that were used when assigning a grade to each lymphocyte. The cells have been stained with SYBR™ Green stain. This image is a based on a similar image found at www.cometassay.com.

2.2.5.2 Quantitative Analysis

Fluorescent microscopy was utilised to visualise the slides after applying the COMET assay. Various fluorescent DNA stains were trialled to detect comets in this study, such as SYBR Green™, and propidium iodide. Although these stains give clear images, they were very photosensitive and faded quickly, making them unreliable for digital imaging. 4', 6-Diamidino-2-phenylindole (DAPI), however, is a semi-permanent stain and during trials for a suitable stain was found to be very effective in regards to both clarity of image and stability.

15 µl of DAPI was added to the centre of each gel and a coverslip was place on top to allow the stain to spread. The slides were then refrigerated overnight at 4 °C and observed the following day. All microscope work and digital imaging was conducted using an Olympus® BX51 fluorescent microscope, under 400X magnification. The wavelengths for excitation and emission of DAPI occur at 358 nm and 461 nm, respectively, therefore the stain was detected using a U excitation (wide band) filter (abbreviated WU). Digital imaging was conducted using an Optronics MagnaFire S99802 digital camera with MagnaFire™ frame-grabbing software on a 2 GHz Pentium 4 ™ computer with 256 MB of RAM and a 128 MB video card. Cell images were captured using an exposure time of 200 ms throughout the sampling period. Figure 2.9 shows the hardware used during the current study.

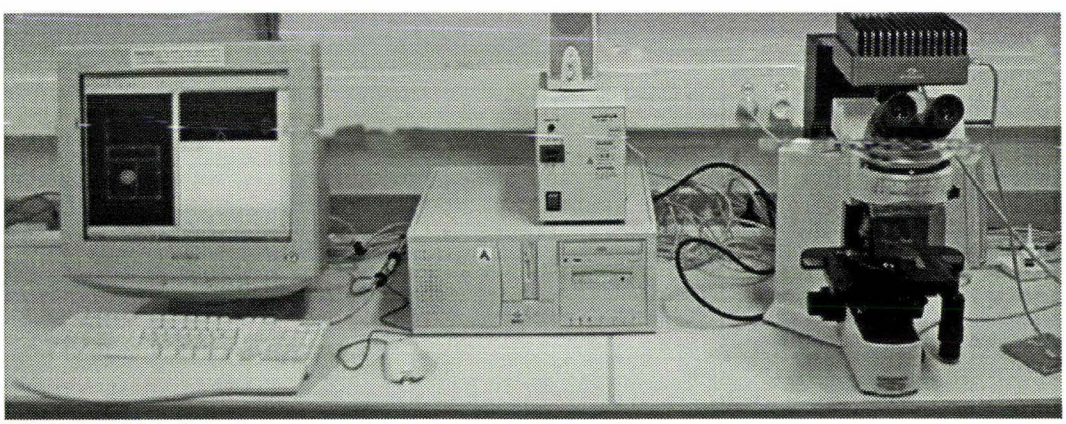

Figure 2.9. Image capturing hardware used during the current study.

Several papers indicate that a minimum of 100 cells for each sample should be assayed. Therefore for each sample approximately 100 cells were photographed, with 50 cell images captured from each of the 2 replicate slides. Depending on how many of the 2 gels per slide were still attached, the 50 cells were divided between them; if 2 gels were still attached to a

slide then 25 cells were captured from each, whereas if only 1 gel was still attached to the slide then all 50 cells were captured from this single gel.

Cell images were captured so that they would accommodate the COMET assay analysis software (The CASP, © Krzysztof Konca). The main requirement is that COMET tails were to be directed from left to right as illustrated in Figure 2.10.

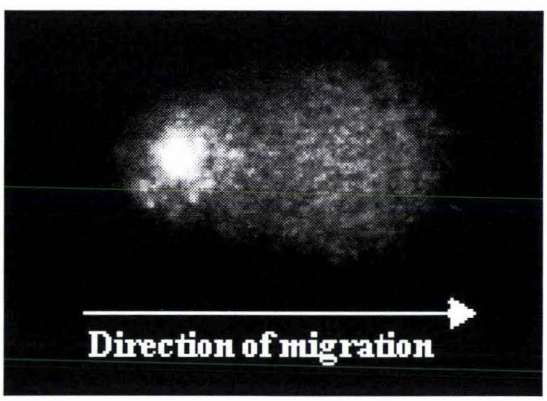

Figure 2.10. Orientation of cell (directed left to right) for analysis by the CASP software.

Each cell that was to be captured was required to be free of debris and other cellular material, and be completely separated from other comets (both head and tails) around the perimeter of the cell of interest. A set of 50 random microscope coordinates (X and Y positions) for each of the 2 gels per slide were generated using Microsoft Excel™ (Appendix II) by determining the perimeter of the 2 gels per slide and the corresponding area of the microscope stage. These coordinates were used in all microscope work throughout this study. Once the microscope had been directed to the coordinate, separate images were taken of all comets that could be seen in the field of view that matched the prerequisites outlined in this paragraph.

Approximately 100 black and white cell images were taken from each sample and stored on a recordable CD for analysis at a later date.

Images of comet cells were analysed using The CASP version 1.01 (The COMET Assay Software Project, Krzysztof Konca ©) software package with a 902 MHz Intel Celeron ™ computer, with 256 MB of RAM and a 64 MB video card. The CASP software captures 13 variables from each comet cell, which can be used to determine the extent of the DNA damage. An explanation of these 13 variables is outlined below:

HeadArea - Area of the comet head in pixels (the sum of the pixels in the head).

TailArea - Area of the comet tail in pixels (the sum of the pixels in the tail).

HeadDNA - Amount of DNA in the comet head (derived from the sum of intensities of pixels in the head).

TailDNA - Amount of DNA in the comet tail (derived from the sum of intensities of pixels in the tail).

HeadDNA% - The Percentage of the cell's total DNA that is located in the comet head.

TailDNA% - The Percentage of the cell's total DNA that is located in the comet tail.

HeadRadius – The radius of the comet head (in pixels).

TailLength – The length of the comet tail measured from right border of head area to end of tail (in pixels).

CometLength – The length of the entire comet from the left border of head area to the end of tail (in pixels).

HeadMeanX – The centre of gravity of DNA in the head (X coordinate).

TailMeanX – The centre of gravity of DNA in the tail (X coordinate).

TailMoment - TailDNA% x TailLength = ([the percent of DNA in the tail] x [tail length]).

OliveTailMoment (OTM) - TailDNA% x (TailMeanX-HeadMeanX) = ([the percent of DNA in the tail] x [the distance between the centre of gravity of DNA in the tail and the centre of gravity of DNA in the head in x-direction]).

A pictorial explanation of some of these variables is illustrated in Figure 2.11.

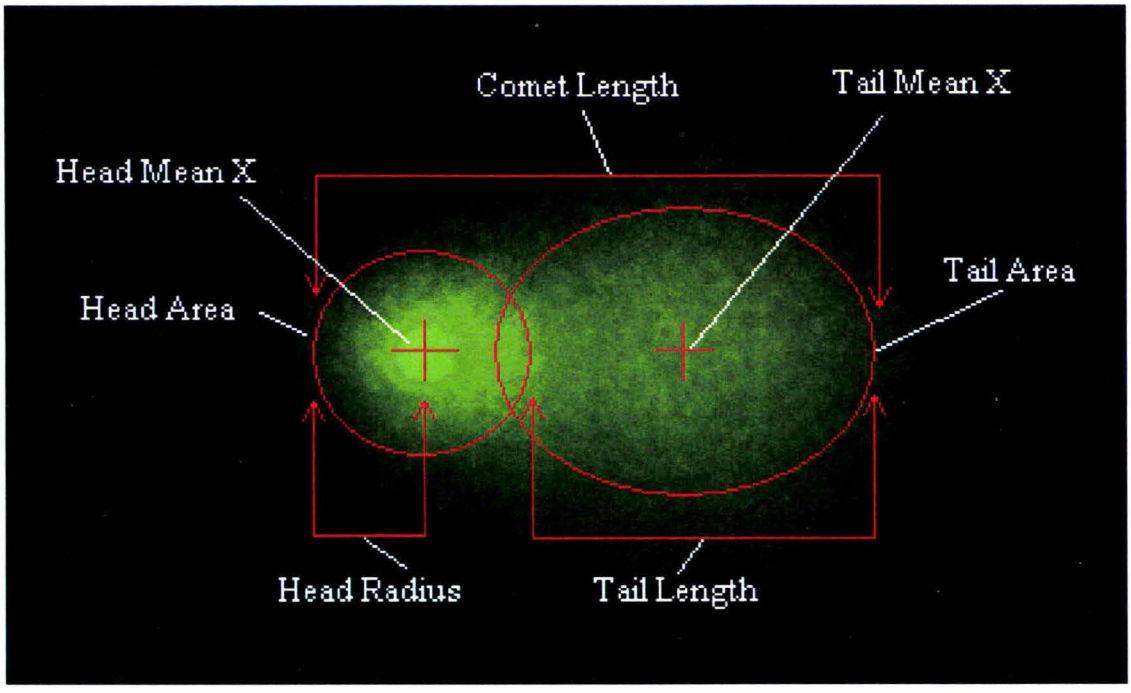

Figure 2.11. Image outlining 7 of the 13 different variables that are measured by the CASP™ software.

The steps for the analysis of comet cells using the CASP software are outlined in Appendix III.

Of the 13 variables, only 3 have been found to be the most indicative of genetic damage. The comet's Tail Length (TL) was used for many years due to its ease of measurement via an eyepiece micrometer. Tail moment (TM) and Olive Tail Moment (OTM) (Olive *et al*, 1990) have become more prominent in recent literature. These 3 variables were used as a measure of genetic damage in cells throughout this research.

2.2.6 DETECTION OF DNA REPAIR

Human lymphocytes were separated from whole blood and resuspended in PBS (Section 2.2.2). 1 ml aliquots were exposed to an equal volume of 100 µM H_2O_2 in an 8 ml Falcon™ tube for 20 minutes on ice. The lymphocytes were washed twice with PBS at 1000 x g for 10 minutes to remove any H_2O_2. The cells were resuspended in a cell culture medium and incubated at 37 °C for 24 hours. 10 µl aliquots of the sample were taken at time periods of 0, 6, and 24 hours, added to a CometSlide™ (Section 2.2.3.1) and subjected to the COMET assay (Section 2.2.4).

2.2.7 CRYOPRESERVATION

2.2.7.1 Cell freezing

PBLs were cryopreserved using a modified version of the protocol outlined in Daid's Virology Manual for HIV Laboratories. 7.5 ml of whole blood was mixed 1:1 with chilled PBS and divided between 2 Falcon™ tubes (8 ml). The lymphocytes were separated (Section 2.2.2) and the lymphocytes from the 3 tubes were pooled into a single 8 ml Falcon tube that contained 1 ml of fresh, chilled PBS buffer.

The cells were washed once by centrifugation at 1000 x g for 10 minutes and the supernatant was removed. The pellet was resuspended in an 8 ml Falcon tube with 3 ml of cold freezing media added drop wise down the inside of the tube so that a concentration of approximately 1 x 10^6 lymphocytes per ml was achieved, as measured by a haemocytometer. 1 ml aliquots of the sample were carefully transferred to 1.5 ml cryopreservation tubes (Sigma). The tubes in this study were numbered from 1 - 60 based on their donor and which sampling period they were required for. 3 cryopreservation tubes were prepared for each sample.

The viability of cryopreserved lymphocytes can be maintained by controlling the cell's freezing rate. For best results a rate of -1 °C per minute is recommended. However, this requires the purchase of expensive cryopreservation vials. Nevertheless, it has been found that by wrapping the cryopreservation tubes in several layers of tissue paper and storing the tubes in a polystyrene container a similar freezing rate can be achieved when these tubes are placed in a - 80 °C freezer (K. M. Stowell, personal communication, February 17, 2003). This freezing rate was not checked and is taken only as an approximation. These "bundles" contained 3 tubes from the same donor and were placed upright, initially in a -20 °C freezer for 1 hour, and then stored in a -80 °C freezer overnight. The following day the tubes were quickly and carefully removed from the bundles and stored at the bottom of a -80 °C freezer for several months until required. These frozen tubes were handled carefully at all times to reduce any mechanical damage to the cells.

Care was taken to ensure that cells were not exposed to DMSO at temperatures above 37 °C as it has been found that DMSO can be a toxic to cells at these temperatures.

2.2.7.2 Cell Retrieval

The 3 tubes for each individual studied were thawed at periods of either 2, 4, or 7 months after freezing in order to determine whether genetic damage had occurred to the cells during prolonged cryopreservation. The procedure used in the thawing process was a modified version of the protocol outlined in Daid's Virology Manual for HIV Laboratories.

Each tube was carefully removed from the freezer and readied for centrifugation **ONE TUBE AT A TIME.** Tubes were only centrifuged when all of the samples had been prepared using the thawing media. This is to reduce any damage that may occur due to exogenous factors as a result of waiting for other tubes to defrost. The tubes were carefully removed from the freezer to prevent any mechanical injury to the cells and flash-thawed in a water bath at 37 °C for approximately 1 - 2 minutes until a small amount of ice crystals remained. The cells were then carefully transferred from the cryopreservation tubes by slowly added drop-wise down the inside of a 15 ml Falcon™ conical centrifuge tube that contained 10 ml of thawing media making a total of 11 ml.

Lymphocytes were retrieved by centrifugation at 1000 x g for 10 minutes at room temperature (Heraeus Megafuge 1.0). 10 ml of supernatant was removed and the pellet resuspended in the remaining 1 ml of supernatant. The lymphocytes were transferred to an Eppendorf tube and centrifuged again at 1000 x g for 10 minutes at 20 °C using a temperature-controlled microcentrifuge (Eppendorf 4417R). After centrifugation, 800 µl of supernatant was removed and the pellet was resuspended in 300 µl of chilled PBS buffer. The pellet was washed once more by centrifugation and the pellet was resuspended in 300 µl of chilled PBS buffer. The lymphocyte cell concentration and viability of the sample was determined using a haemocytometer and the concentration was adjusted with PBS (Section 2.2.2). The sample was then mounted in an agarose gel slide (Section 2.2.3.2) and subjected to the COMET assay (Section 2.2.4).

☢ - CHAPTER 3: RESULTS - ☢

3.1 GENETIC DAMAGE MEASURED BY THE COMET ASSAY

The single-cell electrophoresis assay, or COMET assay, is a reliable and sensitive test for the detection of DNA damage in eukaryote cells. It has been used extensively to test genetic damage in cells as a result of exposure to a genotoxic agent. Before the COMET assay could be applied to the current study involving the New Zealand Nuclear Test Veterans (NZNTV), a preliminary pilot study was conducted to determine the reliability of the assay to detect genetic damage, and to determine if the assay could be consistently replicated in the laboratory. For this to be accomplished, a cell sample was treated artificially *in vitro* with a known genotoxic agent and subjected to the COMET assay. The agent that was used to induce this damage was hydrogen peroxide (H_2O_2), which is a powerful oxidant. Once the abilities of the COMET assay were successfully established, the NTV study could proceed.

3.1.1 MEASUREMENT OF *IN VITRO* OXIDATIVE DAMAGE.

Lymphocyte cells were separated from whole blood taken from a single, healthy donor (Section 2.2.2) and challenged using a 50, 100, or 200 μM H_2O_2 solution for 20 minutes (Section 2.2.2.3), before being subjected to the COMET assay (Section 2.2.4). These were then compared to non-challenged (0 μM H_2O_2 sham exposed), healthy lymphocytes using qualitative methods (Section 2.2.5.1) to determine if the COMET assay was capable of detecting the change in the levels of genetic damage in response to challenges with low and high H_2O_2 concentrations. Concentrations of 500 μM and 1000 μM were also used during these experiments, however, these consistently resulted in cells that were much too damaged to be reliably scored. Therefore, the results for the cells exposed to these concentrations are not shown. Results for these experiments are shown in Table 3.1 and Figure 3.1.

Table 3.1. Oxidative DNA damage induced by a 20-minute exposure to different concentrations of H_2O_2, as measured by the COMET assay (qualitatively).

	0 µM H_2O_2	50 µM H_2O_2	100 µM H_2O_2	200 µM H_2O_2
Number Of Cells	75	75	75	75
A) Tail Length (Arbitrary Unit)	2.08	13.79	13.96	17.80
B) Morphology Grade (Out Of 5)	0.27	3.00	3.72	3.89
Damage Grade (A x B)	0.56	41.36	51.93	69.30

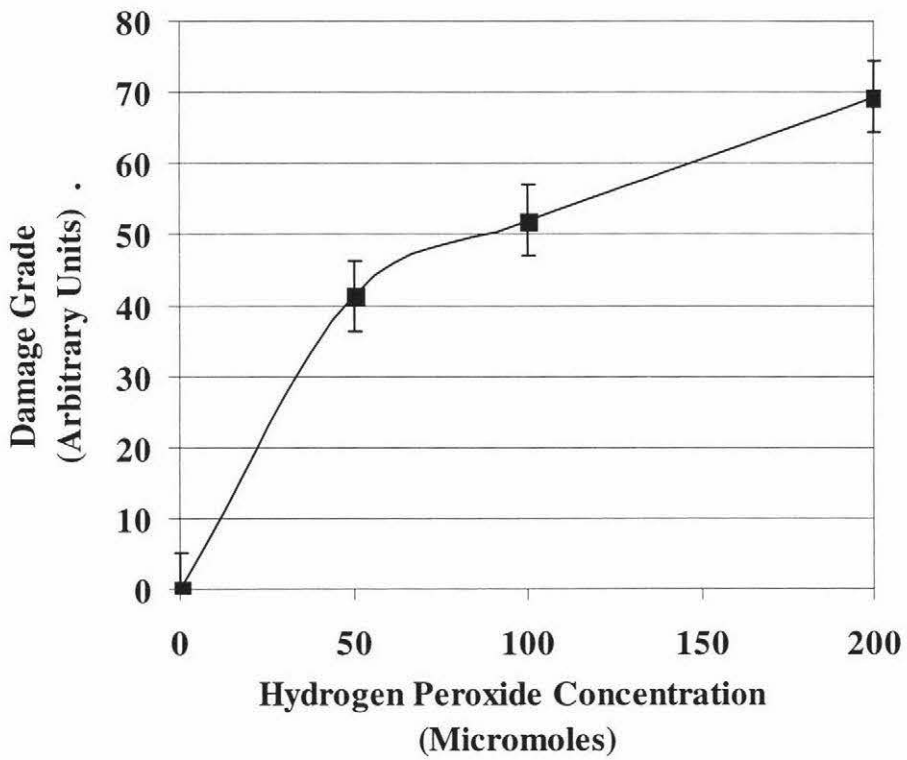

Figure 3.1. Graph of oxidative DNA damage induced by 4 concentrations of H_2O_2, as measured by the COMET assay. Error bars represent the standard error.

The purpose of this experiment was to determine whether different amounts of genetic damage caused by exposing cell samples to different concentrations of H_2O_2 could be distinguished by the COMET assay. Figure 3.1 clearly shows a dose related pattern of damage

with a higher concentration causing a greater amount of damage than a lower concentration. A similar pattern was seen in each replication of this experiment. However, the results for the replications were not always consistent, i.e. the differences between replications were statistically significant ($P = < 0.050$). This may have been due to the inconsistencies in the exposure times as a result of the washing steps from replication to replication, but was most likely due to inconsistencies in the qualitative scoring system.

The analysis of damaged cells was performed "by eye", as an automated scoring system was not available at the time of this experiment, and so the inconsistencies may have resulted from the subjectivity of the scorer. An automated analysis system is often recommended as it takes this subjectivity out of scoring the COMET assay. The experiment was repeated when the CASP™ software package was acquired, with the following revision: instead of using a qualitative scoring strategy (Section 2.2.5.1), a quantitative scoring strategy using the CASP™ software was performed (Section 2.2.5.2). The results for this experiment are shown in Table 3.2.

Table 3.2. Oxidative DNA damage induced by a 20-minute exposure to different concentrations of H_2O_2, as measured by the COMET assay (quantitatively).

	0 µM H_2O_2	**50 µM H_2O_2**	**100 µM H_2O_2**	**200 µM H_2O_2**
Number Of Cells	93	87	63	78
TL[3] (Arbitrary Units)	11.73	22.67	44.14	45.79
TM (Arbitrary Units)	5.87	17.22	23.33	19.67
OTM (Arbitrary Units)	4.41	8.58	11.51	10.01

[3] The tail length (TL) is measured in pixels. However, it is labelled as an arbitrary unit from this point on for simplicity. The tail moment (TM) and Olive tail moment (OTM) are measured as arbitrary units.

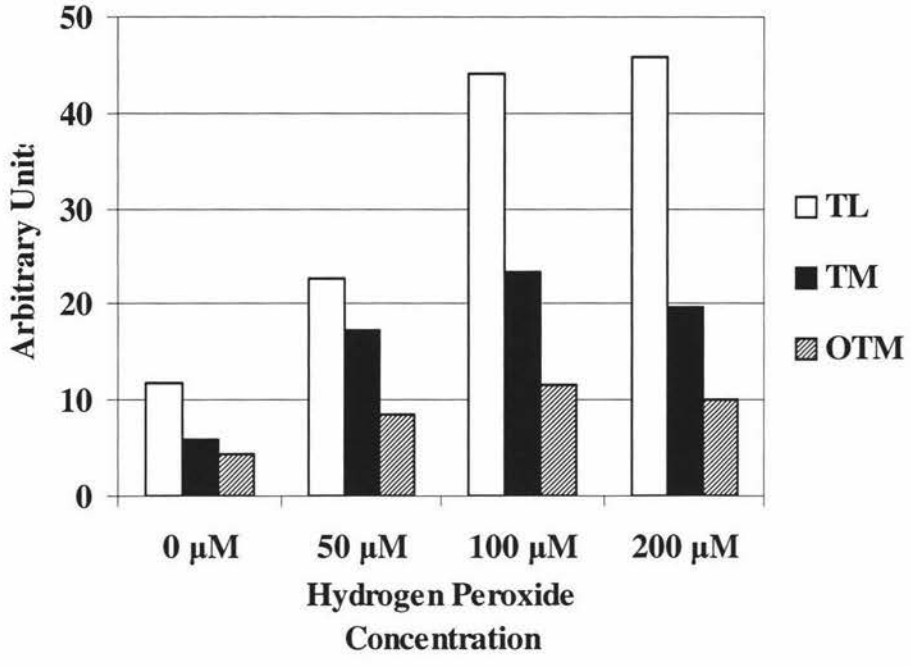

Figure 3.2. Graph of oxidative DNA damage induced by 4 concentrations of H_2O_2 (20 minute exposure), as measured by the COMET assay.

The quantitative experiment yielded results that were similar to the previous qualitative trial: a dose-related increase in the levels of genetic damage was seen as the concentration of H_2O_2 increased. An exact replication of this experiment on a separate occasion showed no statistically significant differences, indicating that the COMET assay can be conducted with reproducible results ($P = > 0.050$). However, only a comparison between 0 µM and 50 µM, and 0 µM and 100 µM yielded a statistically significant difference. A comparison between the results gained from cells exposed to 50 µM and 100 µM, and 100 µM and 200 µM were not found to be statistically different. This was due to the CASP software's inability to distinguish the cell's comet tail from the background in several of the images taken. Because of the extremely fragmented DNA signal in these images, the software could not detect the high levels of genetic damage without modification of the sensitivity setting. Therefore, several highly damaged cells could not be included in the final analysis and the results were lower than expected.

As a result of the above findings, the experiment was again repeated with the following revision: the 20-minute H_2O_2 exposure time was decreased to a 5 minutes exposure to minimise the induced genetic damage to a level that could be detected by the software. This yielded the results shown in Table 3.3 and Figure 3.3.

Table 3.3. Oxidative damage induced in cells by a 5-minute exposure to different concentrations of H_2O_2, as measured by the COMET assay (quantitatively).

	0 μM H_2O_2	50 μM H_2O_2	100 μM H_2O_2	200 μM H_2O_2
TL (Arbitrary Units)	11.73	36.83	42.10	31.45
TM (Arbitrary Units)	5.87	14.14	18.44	11.90
OTM (Arbitrary Units)	4.41	8.06	9.75	6.21

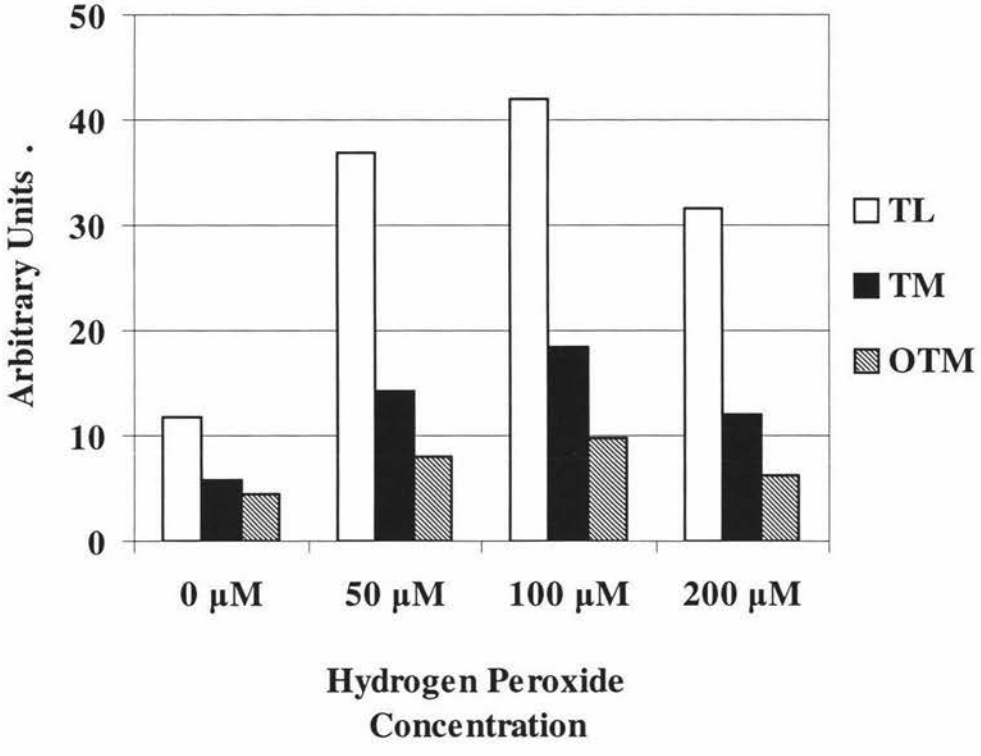

Figure 3.3. Graph of oxidative DNA damage induced by 4 concentrations of H_2O_2 (5 minute exposure), as measured by the COMET assay.

This yielded results that were similar to those gained from the 20-minute exposure. While the amount of damage induced was slightly decreased, the larger concentrations again resulted in cells that were difficult to analyse by the software. The only concentration to show a significant difference from the control was 100 μM. This was significant for all 3 factors

(P = 0.147, 0.0040, and 0.0160, respectively) when compared to the negative control. The results gained from the 50 μM-exposed cells were not found to be statistically different from the control.

The same effect that was seen with a 20-minute exposure to 200 μM exposures was also seen in the 5-minute exposures. The cell's DNA was much too damaged for the software to distinguish it from the background. It appears that this software can only be used if a moderate amount of damage is expected. Large amounts of damage cannot be analysed without modifying the sensitivity settings of the software, which would affect the validity of the results. These settings must remain unchanged (Appendix III). The results gained in this section show that while the COMET assay can distinguish between the genetic damage induced by increasing concentrations of a genotoxin, there is an upper and a lower limitation to its ability. The assay can detect genetic damage when compared to a negative control. If the damage is too high, however, then the software has difficulty distinguishing the cell from the background.

These experiments were also conducted using low frequency electromagnetic fields (EMF) and a dose related pattern was also observed (Mohammed Abdul Wahab, personal communication, 2003). The results for this are not shown.

3.1.2 REPAIR OF OXIDATIVE DNA DAMAGE

When it was established that the COMET assay was able to detect genetic damage induced by an external factor, it was then prudent to determine if the assay could detect the repair of this damage. The protocol outlined by Visvardis *et al* (1994) was modified for these experiments. Lymphocytes were separated from whole blood (Section 2.2.2) taken from a single, healthy male donor and the resulting 2 ml sample was divided between twin 8 ml Falcon™ tubes. The first tube was challenged using a 100 μM Hydrogen peroxide (H_2O_2) solution for 20 minutes on ice (Section 2.2.2.4), while the remaining tube was sham exposed and kept under the same conditions. The samples were then washed twice and resuspended in a cell culture medium and allowed to recover over 24 hours (Section 2.2.6). During this time several samples were taken at 0, 6, and 24 hours after exposure from both the exposed and the sham-exposed sample and processed using the COMET assay (Section 2.2.4). The results from the

challenged sample (100 μM H_2O_2) were then compared to the results from the non-challenged (0 μM H_2O_2 sham exposed) healthy lymphocytes using qualitative methods (Section 2.2.5.1) to determine any possible changes in the levels of genetic damage in these samples over time. This experiment was not replicated and was only conducted as a trial experiment to determine the ability of the COMET assay to follow the repair of oxidative damage. A time course of the DNA repair of oxidative damage is shown in Table 3.4 and Figure 3.4. The sham-exposed sample is represented as the basal measurement.

Table 3.4. Repair of DNA damage over 24 hours, as measured by the COMET assay.

	Basal	0 hours	6 hours	24 hour
Number Of Cells	75	75	75	75
A) Tail Length (Arbitrary Units)	4.85	9.04	8.72	8.19
B) Morphology Grade (Out Of 5)	0.80	2.95	1.71	1.77
Damage Grade (A x B)	3.883	26.638	14.911	14.496

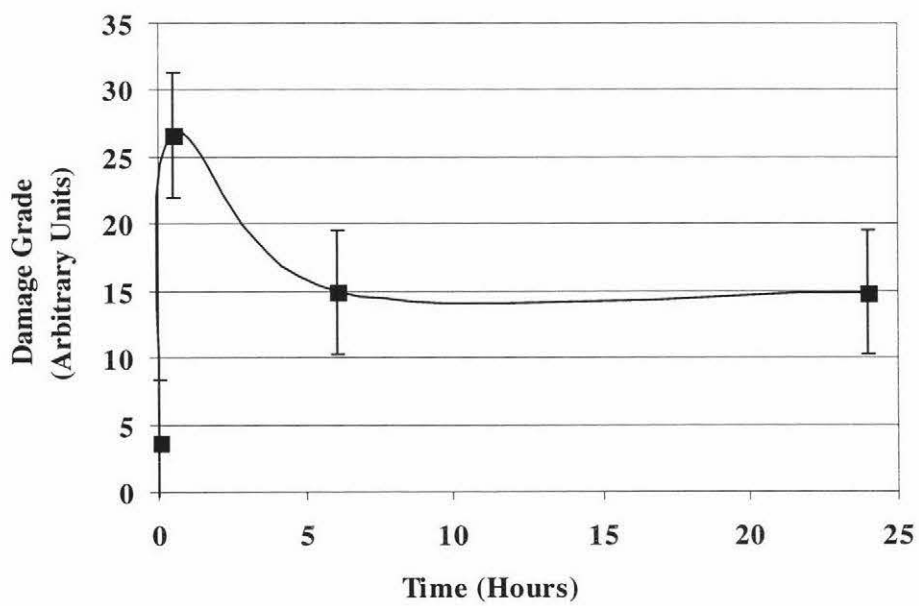

Figure 3.4. Graph of DNA repair measured by the COMET assay over 24 hours. Error bars represent the standard error.

The results for the unexposed sample tested over 24 hours are not shown. However, at no point during the 24-hour period was there a significant deviation from the basal measurement. The results gained from these preliminary experiments indicated that the COMET assay was consistently reproducible and could then be applied to the NTV study.

3.2 DNA DAMAGE IN NUCLEAR TEST VETERANS

The main focus of this thesis was the health of the veterans involved in Operation Grapple. It is the hypothesis of this research that as a result of their participation in the detonation of nuclear weapons they have received significant long-term genetic damage. 6 cytogenetic tests have been employed to test this hypothesis. Only the COMET assay was used in this thesis.

3.2.1 EPIDEMIOLOGICAL ANALYSIS

A preliminary epidemiological study was conducted to determine a possible link between the detonations of nuclear weapons and the health of the veterans who witnessed them, before the cytogenetic study commenced. The data used for this analysis was obtained from the questionnaire data as well as other sources (NZNTVA), the contents of which cannot be republished due to the confidentiality of the individuals involved. Therefore, this information is not available in any of the appendices of this thesis.

3.2.1.1 Mortality Analysis

It is not possible to determine the extent of the radiation doses received by the crewmembers of ships involved in Operation Grapple. We can, however, make assumptions based on the number of detonations an individual was exposed to and its effect on the overall mortality rate of the participants. The analysis of the mortality of the NTVs involved determining whether there is a link between the numbers of weapons each of the 550 veterans of Operation Grapple were exposed to and the rate of mortality in each group. The crew manifests from both the HMNZS Pukaki and the HMNZS Rotoiti were obtained (NZNTVA). These included information regarding the number of detonations witnessed by each individual, as well as whether the individual was now living or deceased. Theoretically, participants who witnessed a large number of detonations could have a higher rate of mortality, possibly due to an

increased exposure to nuclear weapon radiation, compared to an individual who witnessed 1 or 2. It should be noted, however, that factors such as age and other personal data were not factored in to any of the mortality analysis. The analysis presented here is simply an investigation of trends that were observed in data regarding the mortality rate of individuals that were involved in Operation Grapple and may be used to support the finding of the cytogenetic study. If a relationship between the mortality of these individuals and the numbers of detonations witnessed was observed, it may be feasible to conduct a full epidemiological analysis of these data.

Table 3.5 and Figure 3.5 were compiled to determine if the mortality rate of the veterans was related to the total number of detonations each individual witnessed. The mortality data used in this analysis was valid as of June 2002. This analysis is a continuation of the work that was begun by Pearce *et al* (1990).

Table 3.5. The mortality of NTVs compared to the number of detonations witnessed by each individual.

Total No. Of Detonations	Total No. Participants	Total No. Living	Total No. Deceased	Percentage Deceased
1	99	83	16	16.16
2	16	13	3	18.75
3	107	87	20	18.69
4	198	161	37	18.67
5	84	63	21	25.00
6	18	10	8	44.44
7	0	0	0	0.00
8	1	0	1	100.00
9	9	6	3	33.33
Totals	532[4]	423	109	20.49

[4] 18 participants of the Operation Grapple tests are not included in these totals due either to conflicting data or a lack of data regarding these individuals (9), or they served on both the Pukaki and the Rotoiti (9).

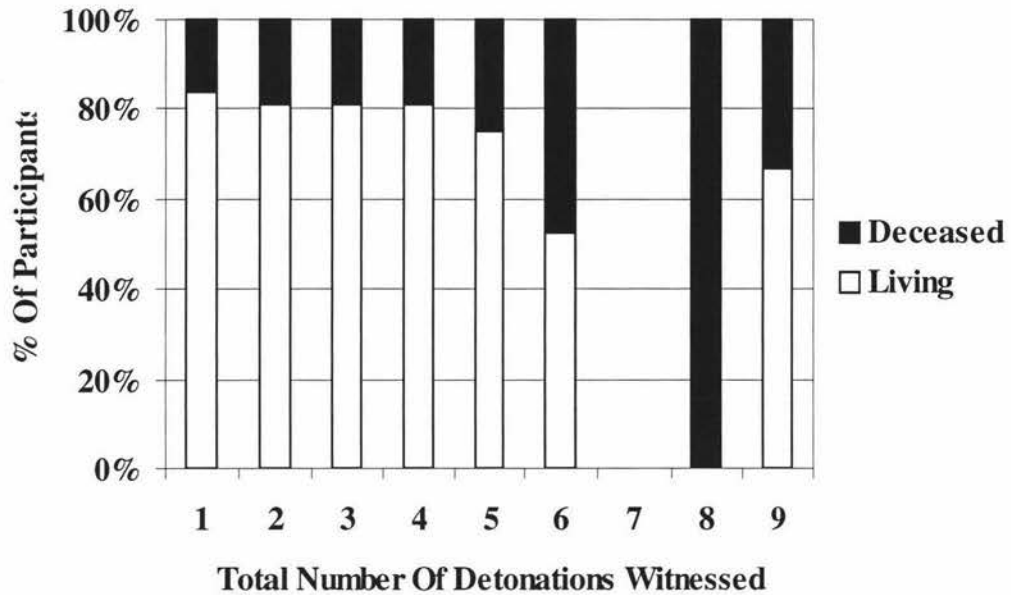

Figure 3.5. Combined mortality rates of crewmen from the HMNZS Pukaki and the HMNZS Rotoiti based on the number of detonations witnessed.

The results shown in Table 3.5 were further subdivided into participants from each of the 2 ships. These data are graphed in Figure 3.6 (Pukaki) and Figure 3.7 (Rotoiti).

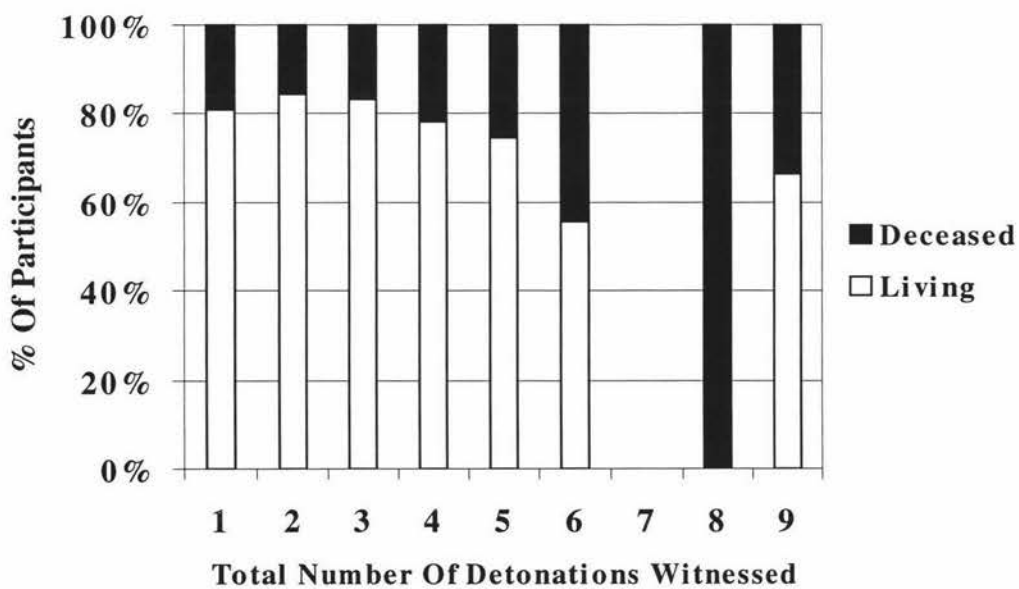

Figure 3.6. Mortality rates of crewmen of the HMNZS Pukaki based on the number of detonations witnessed by each individual.

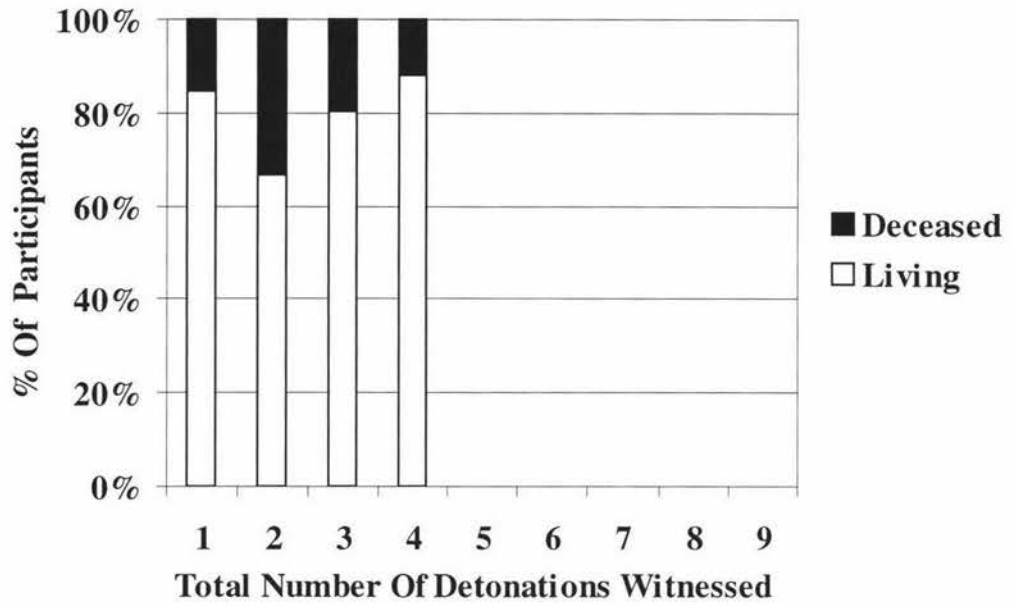

Figure 3.7. Mortality rates of crewmen of the HMNZS Rotoiti based on the number of detonations witnessed by each individual.

An overall chi-squared test of these data was conducted and the difference was found to be not quite significant (P = 0.070). However, a linear-by-linear association chi-squared test (assumes equally ordered data) was very significant (P = 0.013**). A crosstabulation of the observed and expected mortality rates was also clearly significant (P = 0.030*). The overall rate of mortality for these participants appears to increase in response to the number of detonations that were witnessed. If there were no effect as a result of an exposure to the detonation of nuclear weapons one would assume that there would be a uniform death rate regardless of the number of detonations an individual was exposed to.

These data were then pooled in order to show the overall mortality rates of the crewmembers of each ship, regardless of the number of detonations witnessed. If the crew of the Pukaki were present at a higher number of tests than the crew of the Rotoiti, theoretically their mortality rate could be higher due to a potentially higher radiation exposure. This view, however, would be tempered by the yields of the individual bombs. The overall mortality rates for each of these 2 ships are shown in Table 3.6 and graphed in Figure 3.8. A Student's T test of these data showed a statistically significant difference.

Table 3.6. The collective mortality rate data for the crews of each ship that was involved in Operation Grapple.

	Total No. Participants	Total No. Living	Total No. Deceased	Percentage Deceased
Pukaki	308	235	73	23.7
Rotoiti	224	188	36	16.1

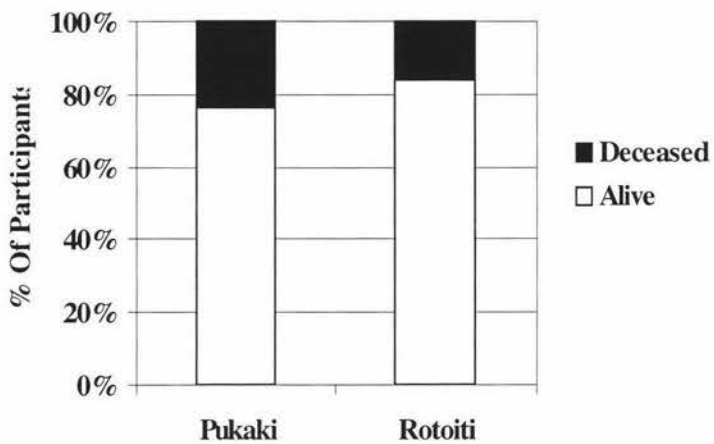

Figure 3.8. The overall mortality rates for the crewmen of the Pukaki and the Rotoiti.

3.2.1.2 Analysis Of Questionnaire data

As part of the selection process for the participants involved in this study, a questionnaire was completed. This questionnaire (Appendix VIII) included relevant questions about the health, lifestyle, and occupation of the participants. While these data were used to select participants, they were also used to determine if the results gained from the participants were in any way affected by factors other than that being investigated. The most important factors that could impact on the results of a cytogenetics study are age, alcohol, cigarettes, and cancer (all cancer sites). The mean data from these 4 factors are shown in Table 3.7. The level of statistical significance is marked with an asterisk (*)[5]. These data shows that these 4 factors are matched (P = 0.050) with the exception of the incidences of cancer.

[5] Levels of significance: * P = <0.050 (significant), ** P = <0.010 (very significant), *** P = <0.001 (extremely significant). This labelling system is used throughout these results hereafter.

Table 3.7. Participant questionnaire data for 4 of the main confounding factors.

	Group[6]	N	Mean	Std. Dev	Std Error	Significance
Age (Years)	C	48	67.17	3.77	0.54	0.285
	E	50	66.41	3.16	0.45	
Alcohol Intake	C	48	3.16	0.991	0.18	0.698
	E	50	2.92	1.064	0.20	
Cigarettes (No. Per day)	C	48	13.80	1.292	0.14	0.900
	E	50	18.40	1.390	0.15	
Incidences Of Cancer (All Sites)	C	48	1	0.14	0.06	0.0005***
	E	50	12	0.43	0.02	

3.2.1.3 Analysis Of Blood Cell Data

A predominant morbidity trait for many of the survivors of the Hiroshima and Nagasaki blasts was the presence of a disease known as leucopoenia, which is characterised by a white blood cell count below 5 x 10^9 cells per L. Leukocytes and neutrophils are the most affected cell types among WBCs. One of the possible means by which damage has occurred in these veterans is the retention of harmful long-lived radionuclides, such as ^{90}Sr, in bones. As part of the sample collection process, cell populations were assayed before each blood sample was received in the laboratory. This information was used to determine the possible incidences of leucopoenia in the participants of this study. The results are shown below in Table 3.8 and Figure 3.9. The diagnosis of leucopoenia is based only on the WBC counts of these individuals. Confirmation of a leucopoenia diagnosis would require a bone marrow analysis and haematological testing.

Table 3.8. Mean WBC counts for control and experimental groups.

Group	No. Of participants	Mean WBC count (x 10^9)	Leukocyte Count (x 10^9)	Neutrophil Count (x 10^9)	No. With WBC counts < 5 x 10^9
C	49[7]	6.90	0.151	4.095	5
E	50	6.97	0.161	3.977	7

[6] C = Control group, E = Experimental (NTV) group.

[7] One outlier was removed from the C group data due to a WBC count that was twice as high as the mean (determined by the Grubbs' test).

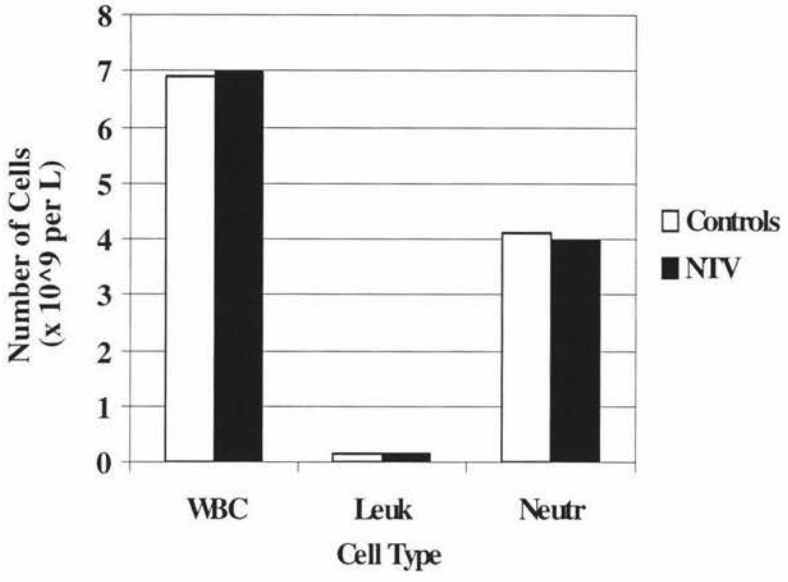

Figure 3.9. A comparison of the 3 main cell types that can be affected by radiation

These data indicate that there are no observable differences in the blood cell populations between the control and experimental groups. A student's T test of the data for the leukocyte, neutrophil, and overall WBC counts showed no statistical differences (P = 0.5919, 0.2764, and 0.8366, respectively).

3.3.2 CYTOGENETIC STUDY

50 Veterans of the nuclear weapons testing during Operation Grapple were studied to determine whether they possessed a higher level of DNA damage in comparison to a control group of 50 ex-navy personnel. Peripheral blood lymphocytes from each participant were analysed using the single-cell electrophoresis assay (COMET assay), which is a sensitive and reliable test for determining genetic damage. Images taken of 100 cells from each participant were analysed using the CASP software and the mean result from 3 measured variables: Tail Length, Tail Moment, Olive Tail Moment, were collected. The identities of the coded samples were determined and the results divided into the NTV group and the control group. The results were averaged and compared using statistical methods.

100 participants were chosen from several volunteers through the use of a questionnaire (Appendix VIII) and a face-to-face interview. Over the course of approximately 1 year, blood samples were taken from these participants from several locations around the North Island of

New Zealand. Fresh, whole blood was collected from these 100 donors by venipuncture in 2 heparinized 10 ml tubes (Vacutainer™). The samples were then placed in a custom-made incubator and stored overnight for transport at 25 °C. These samples were distributed amongst the researchers involved in the current study so that 6 cytogenetic tests could be conducted. Approximately 5 ml from each of these samples was used for the COMET assay. Samples were then prepared as outlined in Section 2.2.2.

Slides were prepared using the pre-treatment method (Section 2.2.3.2) and each sample was subjected to the COMET assay (Section 2.2.4) in order to determine the levels of genetic damage in each of the respective participants. Only 48 control samples were assayed using the COMET assay, as 2 samples were missed due to illness and could not be recollected (NTV# 5 and 27).

For the current study it was decided that a quantitative approach (Section 2.2.5.2) for the analysis of these samples should be taken, rather than the qualitative approach (Section 2.2.5.1) that was used in the preliminary experiments, as it is much more accurate and objective than these scoring "by eye" methods. Either technique, however, would have been valid to determine the extent of DNA damage in a given cell sample (Kobayashi *et al,* 1995). When all of the samples had been subjected to the COMET assay and the results were organized for analysis, the samples were decoded to determine the group to which each sample belonged (experimental group or control group).

3.3.2.1 Genetic Damage In The Control And Experimental Groups

Two comparisons were made using the data obtained from the COMET assay: 1) between the pooled data of the veterans and the controls, and 2) between the 2 ships (Pukaki and Rotoiti). The first comparison was made between the results gained from the COMET assay analysis of the 50 experimental participants and the 48 control participants. This was conducted to determine if those involved in the Operation Grapple tests have sustained any long-term genetic effects as a result of their participation. The mean results for the 50 experimental and 48 control samples are shown in Table 3.9 and graphed in Figure 3.10. The mean COMET assay data from the experimental group was compared to the mean data from the control group using a Student's T test to determine if there was a significant difference between the 2

sets of data. The raw data for individual participants is found in Appendix IX. Due to the fact that this was a blind study that was still ongoing at the time this thesis was presented, the identities of the individual participants are not revealed.

Table 3.9. The mean COMET assay data for 50 experimental and 48 control participants involved in the current study.

	Group	N	Mean	Std. Dev	Std Error	Significance
TL	C	48	21.0479	9.38801	1.355	0.045*
	E	50	17.4539	3.03411	1.136	
TM	C	48	4.8308	4.65165	0.671	0.084
	E	50	3.3192	3.84693	0.544	
OTM	C	48	3.7144	2.74808	0.397	0.077
	E	50	2.7930	2.32740	0.329	

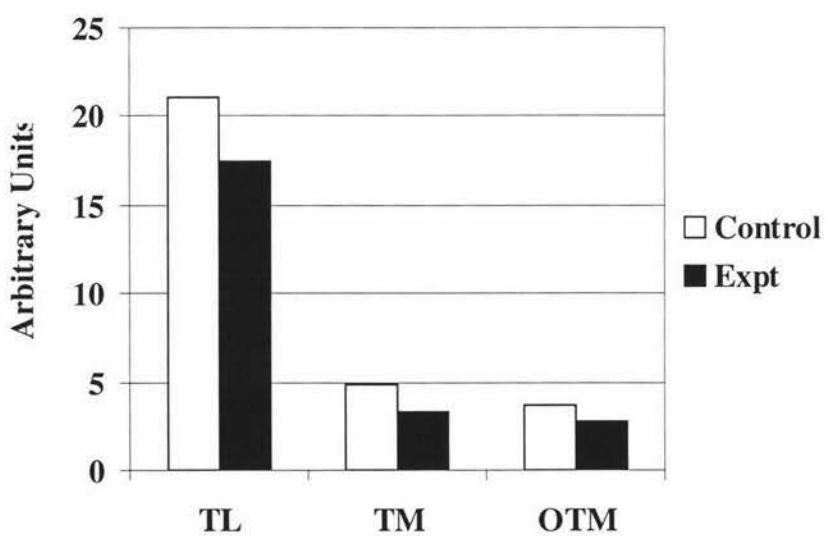

Figure 3.10. Graph showing a comparison of the mean genetic damage in control and experimental groups.

3.3.2.2 Differences In Genetic Damage Between the Two Ships

An analysis of the routes taken by the ships (Write, 2003) showed that after many of the tests, these ships sailed through both the fallout zone and ground zero on several occasions. It is

plausible, although not certain, that one ship, the HMNZS Pukaki, who spent many hours within the fallout zone and ground zero, was exposed to a higher radiation dose than the second ship, the HMNZS Rotoiti. The second comparison of the COMET assay data was made between the participants of the 2 ships. This was conducted to determine any differences between the mean levels of genetic damage found in the participants from each ship. Comparisons were again made using a Student's T test. These results are shown in Table 3.10 and graphed in Figure 3.11.

Table 3.10. Mean COMET assay and questionnaire data of the experimental participants from the Pukaki and the Rotoiti involved in the current study.

Variable	Ship	N[8]	Mean	Std. Dev	Std Error	Significance
TL	P	30	15.8615	6.89886	1.2596	0.194
	R	16	19.5347	9.74729	2.437	
TM	P	30	2.7955	3.30015	0.603	0.314
	R	16	4.2059	4.91293	1.228	
OTM	P	30	2.4384	2.06933	0.378	0.277
	R	16	3.3356	2.84573	0.711	
Age	P	30	66.4656	2.93312	0.536	0.964
	R	16	66.4149	3.89285	0.973	
Alcohol Intake	P	30	1.630	1.066	0.195	0.683
	R	16	1.500	1.033	0.258	
Cigarettes/Day	P	30	19.30	14.61	1.461	0.470
	R	16	16.30	13.10	1.310	
Incidences Of Cancer	P	30	0.200	0.41	0.070	0.333
	R	16	0.310	0.48	0.120	
No. Of Blasts Witnessed	P	30	3.970	1.903	0.347	0.016**
	R	16	2.750	1.342	0.335	

[8] The total number of experimental participants in this study was 50. However, 4 participants served on two different ships during Operation Grapple and were excluded. The inclusion of data from these participants would have introduced complex factors into this analysis.

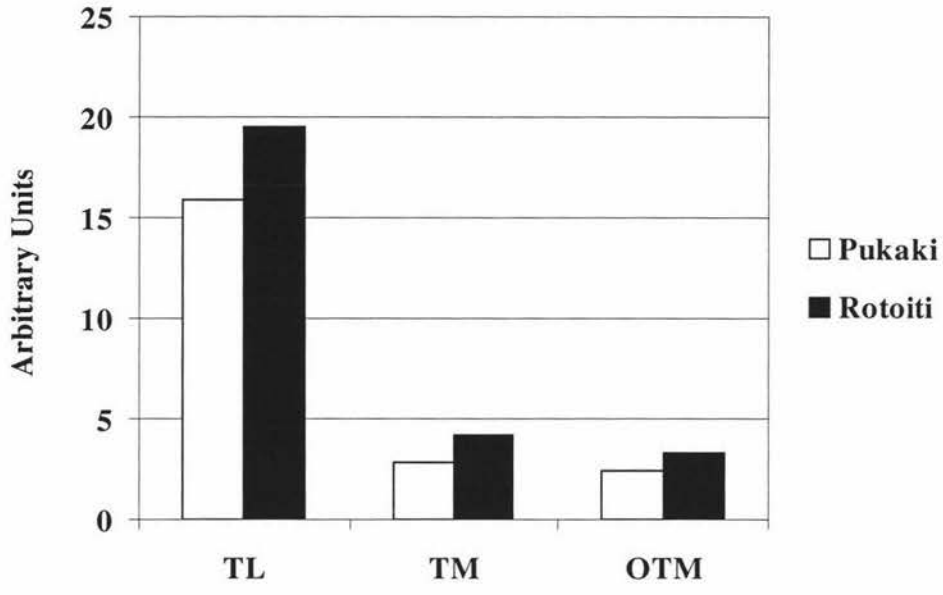

Figure 3.11. Graph showing a comparison of the mean genetic damage in the Pukaki and Rotoiti groups.

3.3 CRYOPRESERVATION PILOT STUDY

The collection of the blood samples used for the current study took over 1 year to complete, and required an individual to travel around the North Island of New Zealand. A pilot study was begun to determine whether human blood samples could be collected from several participants at one time to then be stored cryogenically with the intention that cytogenetic analysis could then be conducted at a later date, without the accumulation of genetic damage as a result of the freezing process. The aim of this study was to freeze and retrieve lymphocytes in a way that would ensure that the levels of genetic damage would not deviate from those of the original sample. The COMET assay was again utilised in this study for the measurement of any such damage.

20 samples, taken from individuals involved in the NTV study, were chosen and stored cryogenically (Section 2.2.7.1). For each of the 20 samples 3 vials were stored, one to be thawed at each of the sampling periods: 2 months (sampling period 1), 4 months (sampling period 2), and 7 months (sampling period 3) after storage. The control sample (sampling period 0) that was used for the comparison was the result gained from the analysis of the fresh sample. At each of the sampling periods samples were retrieved from cold storage (Section 2.2.7.2) and measured for genetic damage using the COMET assay (Section 2.2.4). Analysis

of the samples was conducted using quantitative methods (Section 2.2.5.1). The mean results of the 20 samples for each sampling period, as well as the P values for each after a comparison to the basal levels of DNA damage gained using a Students T test, are shown in Table 3.11. The results are graphed in Figure 3.12. The results for individual samples are found in Appendix X. This table shows the mean accumulated COMET assay data for the 20 samples at each of the 3 sampling periods. These data show that all of the sampling periods yielded results that were statistically different to the original basal results (P = < 0.050).

Table 3.11. Pooled statistical data for Tail Length (TL), Tail Moment (TM), and Olive Tail Moment (OTM) for 20 samples in cryopreservation study.

TL					Sig vs. 0
Sampling Period	**N**	**Mean**	**SD**	**SE**	**(2-tailed)**
0	2031	19.31	23.296	0.517	1.0000
1	1735	39.62	45.254	1.086	0.0000
2	1632	40.75	34.973	34.973	0.0000
3	1590	37.7	38.383	0.962	0.0000

TM					Sig vs. 0
Sampling Period	**N**	**Mean**	**SD**	**SE**	**(2-tailed)**
0	2031	4.403	14.717	0.327	1.000
1	1735	15.31	35.844	0.861	0.000
2	1632	12.78	14.717	23.471	0.000
3	1590	11.24	22.833	0.572	0.000

OTM					Sig vs. 0
Sampling Period	**N**	**Mean**	**SD**	**SE**	**(2-tailed)**
0	2031	3.47	8.377	0.186	1.000
1	1735	9.65	19.314	0.464	0.000
2	1632	8.69	12.549	0.311	0.000
3	1590	6.78	11.129	0.279	0.000

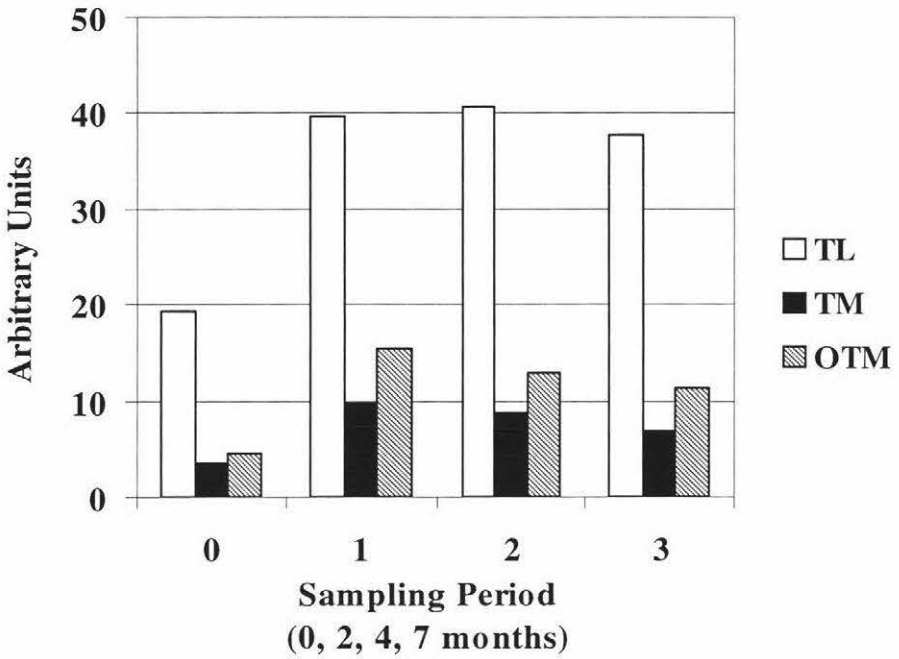

Figure 3.12. Graph showing the mean levels of genetic damage of cryopreserved samples at 3 sampling periods: 2 months, 4 months, and 7 months.

The results observed in Figure 3.12 indicated a slight decrease in the levels of genetic damage at each sampling period (1 - 3) compared to the previous period. The cell viability of the 60 samples was seen to increase over time, indicating a refinement of the technique. These results are shown in Figure 3.13. The data used in this analysis is found in Appendix IV.

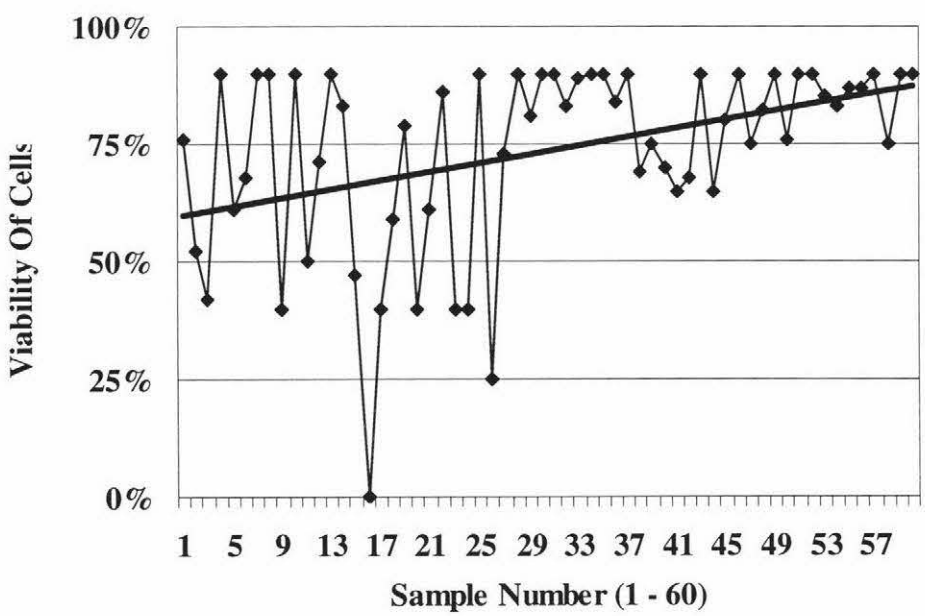

Figure 3.13. Graph showing the changing viabilities of retrieved cryopreserved cells.

No analysis was conducted to compare possible inter-individual changes of genetic damage over time, as this would not give useful information regarding the effectiveness of the cryopreservation protocol. As these samples were obtained from 20 different individuals the levels of genetic damage would naturally be different and so comparisons between individual samples could not be conducted. The only constant for these samples was the cell viability, as this was found to be > 90 % for each participant before the samples were cryopreserved. It should be noted that no cell was described as 100 % viable. This is because cell samples with a viability of above 90 % were labelled > 90 %.

Table 3.12 shows the significance data for each of the 20 individual samples at each of the 3 sampling periods. The data represents the P values of the TL, TM, and OTM for each sampling period when compared to the basal levels of genetic damage of the original sample (Sampling Period 0) through the use of a Student's T test. Statistically insignificant differences (P = > 0.050) are highlighted and represent a sample that achieved a mean level of genetic damage that was statistically similar to the original samples. The mean raw COMET assay data for each of these individual cryopreservation samples is much too extensive to be presented here. These data are found in Appendix X.

Table 3.12. P values of the means for 20 cryopreserved samples. Values indicating no significant difference to the original sample (P = > 0.050) are highlighted.

Sample	2 Months Storage			4 Months Storage			7 Months Storage		
	TL	TM	OTM	TL	TM	OTM	TL	TM	OTM
1	0.000[9]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.001	0.043	0.012	0.000	0.000	0.000
7	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.000	0.342	0.021	0.000	0.009	0.000	0.000	0.000	0.000
9	0.186	0.043	0.226	0.000	0.748	0.078	0.000	0.000	0.000
10	0.000	0.029	0.007	NGS	NGS	NGS	0.248	0.070	0.068
11	0.000	0.006	0.000	0.005	0.037	0.016	0.000	0.018	0.006
12	0.871	0.453	0.691	0.037	0.017	0.003	0.677	0.257	0.180
13	NGS[10]	NGS	NGS	0.000	0.000	0.000	0.000	0.063	0.023
14	NGS	NGS	NGS	0.000	0.000	0.000	0.000	0.011	0.013
15	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.034	0.090
16	0.000	0.001	0.001	0.000	0.009	0.027	0.000	0.007	0.021
17	0.000	0.000	0.000	NGS	NGS	NGS	0.000	0.000	0.000
18	0.311	0.445	0.007	0.000	0.000	0.000	NGS	NGS	NGS
19	0.000	0.000	0.000	0.796	0.758	0.969	0.697	0.470	0.087
20	0.000	0.000	0.000	0.800	0.418	0.358	0.690	0.478	0.240

[9] The P values for this analysis are rounded to 3 decimal places.

[10] NGS - No Gels Survived. On 5 occasions during the cryopreservation study, all of the COMET assay gels for a particular sample were lost during the assay and became unusable. These were not re-assayed.

☢ - CHAPTER 4: DISCUSSION - ☢

Between 1957 and 1958, approximately 550 New Zealand naval personnel were present during the British nuclear weapon tests, known as Operation Grapple, These tests involved participants observing the detonation of 9 nuclear weapons. For many years, several of those who were involved in these tests have claimed that as a result of their participation their quality of life has been affected as a result of possible radiation exposure. Several epidemiological studies have been conducted on this subject and have thus far been unable to support these claims.

For the first time, a major cytogenetic study was conducted that involved the use of 6 specialised cytogenetic tests to analyse blood samples taken from a selected group of these veterans. These were then compared to blood samples that were taken from a selected control group composed of ex-armed forces personnel. The goal of this study was to determine whether there are any significant differences in the levels of genetic damage of these 2 groups. The data presented in this thesis was gained through the use of the single-cell electrophoresis assay, which is more commonly known as the COMET assay.

4.1 PRELIMINARY EXPERIMENTS

4.1.1 MEASUREMENT OF *IN VITRO* OXIDATIVE DAMAGE

Before the COMET assay was used for the current study, it first needed to be trialled to determine its ability to detect genetic damage. These tests were conducted using an *in vitro* exposure of lymphocyte cells to a potent genotoxin, hydrogen peroxide (H_2O_2). Lymphocytes that were exposed to H_2O_2 were found to have statistically significant increases in all of the variables (tail length, tail moment, and Olive tail moment) measured by the COMET assay (Table 3.2 and Table 3.3). The COMET assay was also found to be able to distinguish between differences in the levels of genetic damage caused by small changes in the concentrations of hydrogen peroxide. However, these changes were often not able produce genetic damage that was statistically significant (Section 3.1.1). It should be noted, however, that the COMET assay was able to detect significant damage in samples that were exposed for 20 minutes to a H_2O_2 solution that had been diluted 1,760,000 fold (50 μM) (Table 3.2).

Cells are often at different stages of their life cycle while in the body. If an older cell is exposed to a genotoxic agent, it may be damaged much more than a younger cell. The explanation behind this phenomenon is unknown (Singh *et al*, 1990). Also, when cells are exposed to low doses of a genotoxin, there is a finite chance that a cell will not come in contact with a genotoxic particle (Olive, 1999). These will appear as a cell with relatively intact DNA, surrounded by damaged cells. Cells such as those above can result in a large range of the data, which can affect the significance. This was noted in the current study. Different cells seem to have different tolerances to DNA damage and DNA repair. Singh *et al* (1990) also observed this detail and theorised that these may be due to the presence of several different sub-classes of lymphocytes. Nevertheless, the results from these experiments agree with those found by Visvardis *et al* (1995).

A major problem that arose during these experiments involved the sensitivity of the CASP software. Cells that had received a large amount of genetic damage became difficult for the software to distinguish between those cell's DNA and the background, and as a result they gave a lower than expected measurement. It appears that a cell can be damaged only so much before it is too degraded and spread for the CASP to detect it. When treating with a genotoxic agent, the concentration and exposure time is important. If high levels of genetic damage are expected then the less sensitive neutral COMET assay may be useful (Section 1.2.1.1). In the situation outlined here it appears that a "by eye" scoring system has an advantage. The human eye is able to distinguish the DNA of a severely damaged cell, even if the software cannot. There are COMET assay software packages available that allows the user to manually define the area and length of a cell during analysis. This was not possible during the current study due to limitations in the software utilised. Despite this, the COMET assay was found to be highly reproducible and sensitive for the measurement of genetic damage in lymphocyte cells.

4.1.2 ANALYSIS OF DNA REPAIR

An analysis of the DNA repair kinetics of a donated lymphocyte sample was conducted to establish the ability of the COMET assay to measure a changing level of genetic damage. The analysis of the overall DNA repair kinetics of a cell sample involved an exposure to a genotoxic agent (100 μM H_2O_2), followed by a recovery time in cell culture media at 37 °C, and then sampling at various times over a 24 hours period. The repair of DNA damage and repair was monitored using the COMET assay (Section 3.1.2).

This experiment clearly showed that the levels of DNA damage in these cells, which were initially very high, decreased over a period of at least 6 hours (Figure 3.4). Immediately after a short exposure to H_2O_2, a sharp increase in the levels of genetic damage was observed. This indicated that the DNA had been extensively damage by this powerful oxidant. However, after only 6 hours incubation much of this damaged was quickly repaired. The levels of DNA damage did not change significantly for the remainder of the sampling time. It would be interesting to monitor the changes in the levels of DNA fragmentation within the initial 6-hour period. The results gained in this experiment agree with those obtained in similar studies conducted by Visvardis *et al* (1995) and Singh *et al* (1990). However, in these 2 studies it was observed that the levels of DNA damage returned to a basal level. In the current study it was observed that the levels of DNA damage decreased by almost half during the 6-hour period. It appears that this technique requires refinement for it to be applied to subsequent studies.

From the results of this experiment it appears that the analysis of DNA repair should not proceed beyond 6 hours post-exposure, as it seems to be unnecessary to continue sampling beyond this. As observed in the current study, as well as in the 2 aforementioned studies, the bulk of the DNA repair appears to occur within 6 hours of exposure to a genotoxic agent. This experiment was conducted using manual analysis techniques and was not repeated using the CASP software. This experiment was conducted only as a trial and a refinement of the technique was not sought. The COMET assay appears to be a suitable and valuable technique with which to follow the DNA repair abilities of a cell sample.

4.1.3 CONCLUSIONS

The data that was gathered from these 2 preliminary experiments indicated that the COMET assay could indeed be used for a study such as that involving the NTVs. These experiments indicated that the COMET assay was reproducible and could reliably differentiate different levels of genetic damage. This was shown in the first round of experiments, which involved the exposure of cell samples to different concentrations. The levels of damage measured by the COMET assay increased relative to the concentrations of hydrogen peroxide in both the qualitative and the quantitative experiments. The quantitative experiments were found to give the most reliable and reproducible results.

The second experiment in this series involved the exposure of a cell sample to a solution of hydrogen peroxide and then following the repair of the induced damage using the COMET assay. The COMET assay was effective in determining the changing levels of genetic damage in these samples. On the basis of these experiments it was determined that the COMET assay could be effectively applied to the current study involving the Nuclear Test Veterans (NTVs).

4.2 NUCLEAR TEST VETERANS STUDY

A study such as that presented in this thesis is affected by many variables. Variables such as the differences in individual exposure, personal tolerances to genetic damage, and 50 years of personal experiences between the event and the current study are severe problems when attempting to determine whether there is a possible effect. Also, due to the relatively small number of participants taking part in the study only a very large and consistent difference would have appeared as significant. For the current study outlined in this thesis, 2 approaches were taken. The first was an epidemiological analysis of the mortality and morbidity of the individuals involved, and the second was a cytogenetic study comparing a selection of these individuals to a matched control group to determine the presence of any differences in the levels of genetic damage. If such a difference was found, this could indicate that these individuals were indeed affected by their involvement in Operation Grapple.

4.2.1 EPIDEMIOLOGY STUDY

4.2.1.1 Mortality Analysis

An analysis of mortality was conducted due to the unavailability of data obtained from film badges that were used to monitor individual exposures. By using a known gauge for personal exposures (the number of detonations each crewmember witnessed), it is possible to make assumptions regarding the radiation doses that each individual may have received. The results from the mortality study (Section 3.3.1.1) seem to indicate that as a result of witnessing the detonations during Operation Grapple there has been an increase in the mortality rates of these veterans. When the mortality data was organised according to the number of detonations witnessed, there was an increased rate of mortality for those who were present at a higher number of detonations in comparison to those who were present at a lower number.

These data show a possible relationship between the number of detonations witnessed and the mortality rate of the participants of Operation Grapple. Proportionally, those who witnessed a higher number of detonations seem to have a higher rate of mortality (Table 3.5). An overall chi-squared test of the results was not quite significant (P = 0.070). A linear-by-linear association chi-squared test of the mortality rate for the numbers of detonations witnessed, however, was very significant (P = 0.013**). A crosstabulation of the observed and expected counts vs. the number of detonations was also clearly significant (P = 0.030*). The overall rate of mortality for these participants appears to increase in response to the number of detonations that were witnessed. This may indicate that an accumulation of harmful radiation exposure has occurred due to their presence at a higher number of detonations, which has resulted in an increase in the mortality rate of these veterans, possibly due to radiation-induced afflictions. If there were no health effects as a result of witnessing a nuclear detonation, then there should be no differences in the mortality rates of these veterans. Those who witnessed all 9 of the detonations should have the same mortality rate as those who witnessed 1. Moreover, the mortality rates of the crewmen of the Pukaki and the Rotoiti should be similar.

The HMNZS Pukaki was present at all 9 detonations. However, The HMNZS Rotoiti was present at only 4 of the smaller detonations. On average, the Rotoiti was stationed further away from ground zero at the time of detonation, and did not enter the fallout zones for extended periods, in contrast to the Pukaki, which entered the fallout zones and ground zero on several occasions (Write, 2003). Theoretically, there could be differences in the rates of mortality in crewmembers of each ship, as the crew of each ship was exposed to a different number of detonations. When these data were grouped according to the ship that each participant was assigned to and the same statistical method used for the individual data above was applied, only the Pukaki was found to have a significant result (P = 0.030) (Table 3.6 and Figure 3.8), cementing the possibility that an increased exposure to the radiation from these weapons has affected the health of these veterans. It would be difficult to find a possible effect in the veterans from the Rotoiti due to the lower number of detonations.

These data show that the crew of the Pukaki have a higher mortality rate than the crew of the Rotoiti (Table 3.6). The crew of the Pukaki each witnessed an average of 3.970 detonations while the crew of the Rotoiti witnessed an average of 2.750 (Table 3.10). Since the end of Operation Grapple in 1958, 23.77 % of the crew of the Pukaki have died, compared to 16.7 %

of the Rotoiti crew (Table 3.6). The difference between these mortality rates of the 2 ships is extremely significant (P = <0.0001***). 18 participants of the Operation Grapple tests are not included in this analysis due either to a lack of data regarding these individuals, or they were assigned to both ships. These 2 groups of NTVs have been matched, and there were no significant differences with regards to the age, smoking/drinking, and cancer histories observed (P = > 0.050). A study by Pearce et al (1990) of the morbidity of this group of veterans, however, did not find any significant differences from their control group. They did, however, find a possible increase in leukaemia and multiple myelomas, but not mortality.

It should be noted, however, that the work presented here is only a preliminary analysis of these data. Further analysis will be required to validate these results. Moreover, the yields of the nuclear weapons and the distance that the veterans were stationed from the ground zero were not taken into consideration due to the complexities and multiple variables of individual exposures. Personal information such as the age of the participants was not considered, and those who witnessed the most detonations may have been senior officers, and thus older. However, anecdotal evidence (Roy Sefton, Personal Communication) would indicate that those who witnessed the most detonations might have been younger crewmembers.

This work may be a research project in itself and an experienced epidemiologist should carry out such a study. Nevertheless, these results are encouraging with respect to the hypothesis of the current study and should be regarded as possible evidence to support the claims of the New Zealand nuclear test veterans.

4.2.1.2 Questionnaire Data

The questionnaire data was collected for the purpose of the selection of participants for the current study. It also serves as an indication of the possible confounding factors that may affect the results of such a study. The data from these questionnaires were accumulated and the means of 4 major factors were compared between the control group and the experimental group. These factors represent the main confounding factors that can impair a cytogenetic study, and are: the age; the smoking history; the drinking history; and the cancer history of the individual. A comparison of these data was conducted using a Students T test. These data are displayed in Table 3.7.

The most significant difference observed during the analysis of the questionnaire data is with regard to the prevalence of cancer in the participants. 12 participants have at least 1 type of cancer diagnosed during their lifetime, while only 1 of the control participants has been diagnosed with cancer at some point. This was shown to be an extremely significant difference (P = 0.0005***). While this result appears to supports the hypothesis of this study, it is not sufficient evidence. As this is a volunteer study, the incidences of cancer in these veterans may have provided an incentive for the involvement in this study. Therefore, an unrealistic selection of the participants may have resulted. This may be where an epidemiological study has an advantage; such a study can observe the mortality and morbidity statistics of every veteran that was involved in Operation Grapple, whereas research such as that presented in the current study can observe only those who have volunteered. It was not clear if this difference would affect the results gained from a cytogenetic study. The 3 remaining major factors that could impact on the results gained from the COMET assay, such as age, and the smoking/drinking history of the participants, all showed statistically insignificant differences (P = > 0.050), indicating that the 2 groups of participants in this study had been successfully matched.

4.2.1.3 Blood Cell Data

One of the most prevalent disorders seen amongst the survivors of the Hiroshima and Nagasaki nuclear weapon blasts is leucopoenia. Leucopoenia is characterised as a decrease in the numbers of WBCs below 5 x 10^9 cells per litre. This disorder usually arises due to an exposure to several physical and chemical agents; the most common of these is radiation. If the participants of Operation Grapple have indeed ingested long-lived radionuclides as a result of their presence at these tests, then this may have resulted in a lower than normal WBC count when compared to the control group.

Cell population assays for each of the participants were used to determine the presence of any anomalous data in the 3 cell types of interest, possibly due to internal radiation exposure. These data (Table 3.8) show no differences in the cell types of interest. The WBC, neutrophil, and leukocyte counts of the control and experimental groups showed no statistically significant differences (P = 0.8366, 0.2764, and 0.5919, respectively) and the incidences of possible leucopoenia in the controls and the experimental group were only 5 and 7,

respectively. If a significant difference in this analysis had been observed, this may have indicated the presence of radioisotopes present in the skeletons of the NTVs. Nevertheless, the data reported do not rule out the possibility. Dosimetric testing of bone samples would determine the levels of radioactive materials, if any, that are present in these individuals.

One blood sample from the control group was found to have an abnormally high WBC count (Grubbs' outlier test) and resulted in a skewed result. This participant was removed from the analysis of blood cell data for statistical purposes. From the data presented here, it appears that if there are radionuclides present within the skeletons of the participants it has not affected the cell populations. However, this may indicate that 50 years of radioactive decay, coupled with the natural calcium turnover rates in these individuals, has returned the levels of radionuclides to a normal level. The presence of any abnormal levels of radionuclides present in these individuals has not been tested experimentally.

4.2.2 CYTOGENETIC STUDY

The data obtained from the COMET assay have given results that are difficult to explain. Samples obtained from 50 veterans of the nuclear weapon tests during Operation Grapple and 48 matched controls were subjected to the COMET assay to determine the levels of basal genetic damage in the respective individual. When data from the NTV participants were compared to the results gained from the control group, these data showed that there is a statistically significant difference between the 2 groups in only 1 of the 3 variables that were tested. Only the Tail Length was found to be significant ($P = 0.045$). In preliminary experiments, however, this was also found to be the most inconsistent factor and may not be a valid result. All the results gained from the COMET assay show that the experimental group has a mean level of genetic damage that is lower than that seen in the control groups. For the hypothesis of this study to be supported, one would predict that the results would have been reversed. However, these results beg the question, why do these individuals have lower levels of genetic damage than matched controls?

A second comparison was also made using the data from the COMET assay. 2 ships from the New Zealand navy were involved in Operation Grapple, the Pukaki and the Rotoiti. The Pukaki was involved in all 9 of the Operation Grapple detonations, while the Rotoiti was only

involved in 4. The Rotoiti was also stationed further away on average, and did not spend extended periods inside the fallout zone, as opposed to the Pukaki, which was stationed closer to the detonations, and spent extensive periods in the fallout zone and ground zero. The mortality study (Table 3.6) indicated that the crew of the Pukaki had a higher mortality rate than those from the Rotoiti. However, the data from the COMET assay appears to dispute this. Interestingly, the data from the COMET assay again gave results that are difficult to explain. The data obtained from the COMET assay (Table 3.10) indicate that the crew of the Rotoiti have a higher rate of genetic damage than the crew of the Pukaki. One would assume that the data obtained from the COMET assay would support the mortality data. Although the differences are not statistically significant ($P = > 0.050$) it does raise the possibility that other complex factors may be involved.

A possible explanation for these two results is that the most affected individuals of the Operation Grapple tests may have already died as a result of their radiation exposure. This would have removed them from the sampling group so it would appear that the experimental group is actually healthier than the control group. This would explain why the epidemiology and cytogenetic data disagree, as the epidemiological study presented in this thesis took into account all of the veterans, whether they were living or deceased, while the cytogenetic study only took into account those who had volunteered and were obviously living. In such a case, a cytogenetic study may be much too late to detect any possible residual effects from Operation Grapple.

Another possible explanation for these results takes into account the possibility of the presence of radionuclides in the bodies of these individuals. It has been previously shown that high levels of reactive oxygen species (ROSs) are produced in individuals suffering from diabetes mellitus through a mechanism that is unclear (Jakus, 2000). It is thought to be the result of an increase in non-enzymatic and auto-oxidative glycosylation, as well as metabolic stress resulting from changes in energy metabolism. However, this evidence applies only to the insulin-dependent diabetes mellitus (IDDM) form, but not the non-insulin dependent (NIDDM) form.

Anderson *et al* (1998) used the COMET assay to measure the amount of DNA strand breakages that occur in patients diagnosed with IDDM or NIDDM. This study used a control group of 20 participants, with an experimental group of 22 IDDM patients, and 23 NIDDM

patients. It was found that lymphocyte cells taken from those with either form of the disease, when subjected to the COMET test, had a level of DNA strand breakage *lower* than that of the normal control group. These results are contradictory to what would be expected from a study such as this. A possible explanation for this was that, over time, normal lymphocytes might accumulate DNA damage due to endogenous and exogenous factors, such as metabolic by-products or toxins. The lymphocyte may not be stimulated to repair this damage until a certain level has been exceeded, as lymphocytes are replaced after a normal period of 6 months to 2 years. However, lymphocytes in individuals with diabetes mellitus would receive larger than normal levels of oxidative genetic damage for extended periods, and their cells may be stimulated to repair this damage, as well as the normal background damage, that was detected in the control group.

Such an observation could be applied to the current study and may explain the results obtained from the cytogenetic study. If the WBCs from the individuals involved in the Operation Grapple tests were continually bombarded with radiation from radionuclides, such as strontium-90, that may be present within their skeletons, their DNA may be continually repaired, such as in the diabetics studied by Anderson *et al* (1998). As the samples are received in the laboratory approximately 1 day after they were taken from the participants then this may be ample time for any damage that was received within the body to be repaired.

As was seen in the DNA repair study in this thesis, DNA is quickly repaired after 6 hours and may continue to be repaired over the remaining 18 hours to a degree of fragmentation lower than what would normally be expected if the samples were assayed immediately after blood collection (within 3 hours). This would explain why the participants that were thought to have the higher level of genetic damage have in fact a lower level than the control group. However, the presence of a normal WBC count in the experimental group appears to dispute, although not exclude, this possibility. Dosimetric testing of bone and teeth samples could determine the existence of such radionuclides and would confirm the possibility that the veterans have been affected as a result of their participation in Operation Grapple.

On the other hand, the increase may simply be due to several elevated results in the last of the received samples that may have caused a skewed outcome (Appendix IX). As all of these were control subjects the overall mean of the control group may have been erroneously increased. Also, as this is a relatively untested and extremely sensitive assay, the results may

be due to experimental error as there was no standard sample to ensure consistent results over such a long-term study. The outcomes of the remaining 5 cytogenetic tests will determine if this is true and will either support or not support the hypothesis of the current study. These tests are not based on the same DNA fragmentation principles of the COMET assay and involve the analysis of the overall DNA repair mechanism of a cell.

4.2.3 CONCLUSIONS

The mortality rate of the veterans appears to have been affected by the numbers of detonations that they witnessed. Statistical analysis of the data presented in this thesis showed that the mortality rate of the veterans significantly increases as the level of weapon exposures increase. Due to the unavailability of the data from the film badges, which would indicate the extent of the radiation doses that these veterans were exposed to, this was the only avenue available to determine a possible dose-related effect. A similar effect was seen when the data were divided between the two ships involved. The Pukaki had a significant dose-related mortality rate than the second ship, the Rotoiti, which was present at fewer detonations. However, the cytogenetic data obtained from the COMET assay appears to dispute this. The COMET assay showed that the individuals who theoretically received the most radiation, the total nuclear test veterans group and just the crew of the Pukaki, have a lower level of DNA damage than the comparison group. It is therefore difficult to come to a definitive conclusion.

It may be that the most affected of the individuals involved in Operation Grapple have died as a result of their exposure to the detonations. This would have removed them from the sampling group, thus giving the results seen in the current study; an increased overall mortality rate with a decreased level of genetic damage in the surviving participants.

Based on the cytogenetic data obtained from the COMET assay it appears that the NTVs who participated in this study have not been affected by exposure to nuclear weapons during their involvement in Operation Grapple. The data gained from this study indicate that these individuals seem to be healthier than the control group. However, the data gained from the mortality analysis, which would be more accurate, do indicate a possible health effect in the overall Operation Grapple veteran population. Nevertheless, the data outlined in this report is only 1/6 of the total study. A team of researchers conducted 5 other tests during this study and

the results from such tests were yet to be analysed at the time this thesis was presented. Therefore, the conclusions reached in this thesis should not reflect on the NTV study as a whole. Only when the final report is completed can an ultimate conclusion be reached.

4.3 CRYOPRESERVATION

4.3.1 CRYOPRESERVATION PILOT STUDY

The cryopreservation study presented in this thesis was conducted to determine whether blood samples could be collected and stored for an extended period of time, without the accumulation of genetic damage as a result of the cryopreservation procedure. The hypothesis for this study stated that a human lymphocyte sample could be stored cryogenically and then recovered, with a level of genetic damage that was not significantly different to that of the original sample. COMET assay data from individual samples presented in this report indicate that this is possible.

60 samples taken from 20 individuals were stored and recovered at 3 regular intervals (2 months, 4 months, and 7 months after storage) to determine if long-term storage affected the genetic integrity of the cell's DNA. DNA damage was measured using the COMET assay. When the cells were recovered from storage, it was observed that the mean levels of genetic damage at each of these sampling periods was up to twice the mean of the original samples, indicating that the cryogenic procedure had damaged the DNA of these cells. For each of the samples assayed, all 3 of the variables that were measured by the COMET assay (tail length, tail moment, and the Olive tail moment) showed a significant difference to the fresh samples (Table 3.11). This would have affected a cytogenetic study had these samples been used, and may have given a false positive. The significance was found using a Student's T test, and only samples with a P value greater than 0.050 were considered to be acceptable.

Individually, very few samples achieved a mean result that was statistically similar to the original sample (Table 3.12). Indeed, only 3 of the 20 samples yielded statistically insignificant differences to the original means for the TL, TM, and OTM in at least 2 sampling periods, and no sample in this trial yielded these results for all 3 sampling periods. The presence of 3 undamaged samples show that this protocol can be used for the long-term

preservation of human lymphocyte samples, however, more work is required to refine the technique.

Samples taken from the 3 different individuals show a consistent replication in their similarity to their respective original samples. This is encouraging with respect to the hypothesis of the study signifying that there is a possibility that a protocol could be found to maximise the maintenance of genetic viability. Initial attempts to establish cell cultures of a small number of these samples for use in other cytogenetic tests (such as those assays outlined in Appendix V) have proven unsuccessful. Research has been conducted in order to achieve this, as all of the techniques currently used, barring the COMET assay, require cell culture as part of their protocol. Cell cultures have since been established using cryopreserved blood samples for the purpose of cytogenetic testing (Ruth Major, personal communication, 2003).

The possibility of refinement of these cryopreservation techniques has been supported by the trend in the levels of overall genetic damage. Figure 3.12 showed that, over the three sampling periods, the levels of the pooled levels of genetic damage induced by the cryopreservation techniques have decreased. This indicated that as the protocol for both the freezing and thawing of the samples was repeated the technique was being continually improved. This was further supported by an increase over time of the cell viabilities measured after the cells were thawed (Figure 3.13). One should bear in mind that the samples in Figure 3.13 are not arranged in the order that they were thawed, but rather in the order that they were frozen. This indicates that the storage of the samples may be more important than the retrieval of the samples, which presents itself as an overall increase in cellular integrity of the samples. When these data were organised in the order that they were thawed, no such pattern was observed.

4.3.2 CONCLUSIONS

It appears that blood samples can be stored and retrieved with minimal genetic damage, verifying the hypothesis of the cryopreservation pilot study. However, the success achieved in this study seemed to be sporadic. It is unlikely that the cryopreservation of blood cells will be applied to a study in the near future as the analysis of fresh blood is much more reliable. Nevertheless, with further refinement of the protocols used for the storage of blood samples, it may one day be possible to use such techniques routinely in a cytogenetic study. The results gained from this research are promising

☢ - CHAPTER 5: FUTURE WORK - ☢

5.1 THE COMET ASSAY METHODOLOGY

Several improvements to the methodology of the COMET assay were made as a result of this study, and should be taken under the advisement of any researcher attempting to use the assay in a subsequent study.

The most important change is with regards to the alkaline solution. It was discovered during the course of this study that a separate alkaline solution is not required, and can be substituted by simply immersing the slide in the electrophoresis buffer directly after immersion in the lysis solution. This change in the protocol benefits the cells, as they are not exposed to light and temperature during the transfer. Moreover, the stability of the gel is increased, as less movement aids the adherence of the gel to the slide. Implementation of this change increased the number of viable gels significantly in several trials at the conclusion of this study. The duration of the immersion should also be decreased from 40 minutes to 30 minutes.

The purchase of ready-made lysis solution is costly. However, this solution can be made from materials found in most molecular biology laboratories. Researchers using the COMET assay in future studies should consider making the lysis solution themselves to decrease the costs of conducting the COMET assay. The protocol for making the lysis solution is outlined in Section 2.1.5 of this thesis.

As seen in the current study, variations in the replication of the COMET assay can result in measurements that are statistically different from one another. Differences in climate, variations in the concentration of materials, and differences in the timings of the steps of the protocols can affect results gained by the COMET assay in a long-term study due to its ability to detect minute changes in DNA damage. It is often recommended, although not often implemented, to have a standard sample. This is a cell sample from a single donor that is included during every run of the COMET assay. The purpose of this standard is to detect small variations between different applications of the COMET assay, and to act as a form of quality control. The use of such a control is recommended in the IPCS guidelines for the

monitoring of genotoxic effects of carcinogens in humans (Albertini *et al*, 2000). However, this practice does not appear in many studies.

The final revision to the protocol presented in this thesis is with regard to the capturing of images. Problems that were encountered with the analysis software (Section 3.1.1) were resolved by increasing the exposure time of the digital camera. This revision could not be implemented during the current study, as the problem was not found until partway through the experimental work. Thus, any change to the protocol would have invalidated the previous results. However, it should be cautioned that the background of the slide will also be intensified by the change and may negate the difference. If these problems persist it may be appropriate to implement the use of a different DNA stain.

5.2 NUCLEAR TEST VETERANS

5.2.1 THE CURRENT STUDY

Despite the fact that this cytogenetic study of the New Zealand Nuclear Test Veterans outlined in this thesis did not find any evidence to support the view that these individuals have been affected at the genetic level as a result of their participation in Operation Grapple, this does not rule out that possibility. Only the COMET assay was used in this thesis. However, 5 tests still remained to be completed in the current study before a final conclusion could be made. It may be that the COMET assay was not suited to such an analysis. The final analysis of the results gained from the entire programme will be presented in a final report and only then should a conclusion be reached regarding the genetic health of these individuals.

5.2.2 DOSIMETRY OF BONES

The main rationale for this study is based on the possibility that the individuals who participated in Operation Grapple may have been exposed to long-lived radionuclides in the fallout from nuclear detonation during their involvement. Bone-seeking isotopes, such as Strontium-90, may have been retained in the skeletons of these individuals. These could have irradiated the surrounding tissues where they were located, causing damage to the genetic material of these cells. Dosimetric testing of bone and teeth samples has successfully detected

radioactive contamination in studies involving individuals in close proximity to sources of radionuclides such as nuclear power plants (Tolstykh *et al,* 2003) and nuclear weapon detonations (Kawamura *et al*, 1987). The possibility of retention of radionuclides could be confirmed or ruled out using these techniques. However, it may be too late for such a study, as the levels of any possible radionuclides present in the bones of these individuals may have been decreased substantially over the past 50 years due to natural calcium turnover rates and radioactive decay. Nevertheless, the levels could still be sufficiently high to make a comparison to a control group.

5.2.3 ANALYSIS OF OFFSPRING

If the claims of the NTVs were found to be valid in the current study, the next step to take would be to study the children of these individuals. The purpose of such a study would be to investigate the possibility that genetic disorders within the veterans of Operation Grapple have been passed on to the offspring. A study of the offspring would be conducted in a similar fashion to the current study. Samples would again be collected from an experimental group (first generation offspring of the participants of Operation Grapple) and a control group (matched offspring from servicemen not involved in Operation Grapple). A battery of cytogenetic tests would again be applied to these samples. However, the COMET assay may not be appropriate for such a study. The COMET assay is usually applied to samples that have been exposed to a genotoxic agent. It is unlikely that radionuclides have been passed from father to offspring. Nevertheless, the COMET assay may be able to detect the presence of a defective DNA repair mechanism in these individuals. In this case, normal genetic damage would accumulate in these individuals, thus giving a higher level of fragmentation than may be seen in a control group.

As outlined in the current study, several epidemiological studies have been conducted regarding the health of the offspring of numerous groups of Nuclear Test Veterans. However, there has been no data reported to support a link between the morbidity and mortality amongst offspring, to their parent's participation in a nuclear weapon testing programs. Therefore, it is unknown if such a study will be possible.

5.2.4 MORTALITY ANALYSIS

An analysis of the mortality rates of these veterans successfully found a possible link between the number of detonations witnessed and the number of veterans who are now deceased. This analysis clearly and significantly showed that the higher the number of detonations an individual witnessed, the higher their mortality rate. The mortality rate seemed to be also affected by the proximity of the individual to ground zero (analysis of the Pukaki vs. Rotoiti mortality data). However, this was only a basic preliminary analysis. The analysis of these data did not take into account the changing distances of the individuals from the blasts, or the differences in the estimated weapon yields. An in-depth statistical analysis of these results is required to verify these observations before the conclusions reached in this research can be accepted. Nevertheless, these results are encouraging with respect to the hypothesis of the current study.

5.3 CRYOPRESERVATION

The second part of this thesis involved an investigation of the cryopreservation of human blood samples, with the intent of recovering them from storage for cytogenetic analysis. The results from this study indicate that while this is possible, further investigation is required. The protocol used in this research must be refined to maximise the genetic integrity of the stored cells. This must be achieved consistently to ensure that the results gained from a cytogenetic study would not be significantly affected by the storage procedure.

Cell culture is a major part of many of the cytogenetic techniques at the disposal of researchers. As reported in this thesis, an attempt was made to culture several samples cryopreserved during this study. However, this attempt proved to be unsuccessful. A researcher is currently seeking to achieve the culture of cryopreserved cells for the purpose of conducting sister-chromatid exchange analysis and the micronucleus assay using these samples.

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APPENDICES

- APPENDIX I -

LETTERS OF APPROVAL FROM THE HUMAN ETHICS COMMITTEE

Manawatu Whanganui Ethics Committee

C/- Palmerston North Hospital
P.O. Box 5203
Palmerston North
Phone/Fax (06) 356 7773
Email: mwethics@xtra.co.nz

20 October 2000

Dr. John Podd
School of Psychology
Massey University
Private Bag 11222
PALMERSTON NORTH

Dear Dr. Podd,

CHROMOSOME STUDIES OF NUCLEAR TEST VETERANS
ETHICS REGISTER: 50/00

Thank you for your response to the points raised by the Committee. The study is approved by the Manawatu-Whanganui Ethics Committee. It I suggested that the Information Sheet would be clearer if the last sentence on the first page was amended to read ... level of chromosomal damage observed **in one group as compared to another".**

Certification

The Committee certifies that it is satisfied this trial is not conducted principally for the benefit of the manufacturer or distributor of the medicine or item in respect of which the trial is carried out. This certification is for the purposes of the Accident Insurance Act 1998, Section 35(5).

Accreditation

The Manawatu-Whanganui Ethics Committee is accredited by the Health Research Council and is constituted and operates in accordance with the National Standards for Ethics Committees July 1996.

Progress Reports

The study is approved until December 2002. It is the Primary Investigator's responsibility to forward a final report at the completion of the study and a preliminary report after the first 12 months. A form to assist with this is available from the administrator.

General

All correspondence, protocol amendments, SAE reports and progress reports should be forwarded to the Manawatu-Whanganui Ethics Committee. Sufficient copies for circulation would be appreciated. Please quote the above ethics committee reference number in all correspondence.

It should be noted that Ethics Committee approval does not imply any resource commitment or administrative facilitation by any healthcare provider within whose facility the research is to be carried out. Where applicable, authority for this must be obtained separately from the appropriate manager within the organization.

Yours sincerely,

Jenny Maher
Chairperson

Massey University Campus Human Ethics Committee: Palmerston North (HEC: PN)
Old Main Building, Turitea Fax: 64 6 350 5622 http://www.massey.ac.nz/~muhec

Professor Sylvia V Rumball, Chair Secretary
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Email: S.V.Rumball@massey.ac.nz Email:

Massey University

Private Bag 11 222,
Palmerston North,
New Zealand
Telephone: 64 6 356 9099

11 February 2003

Dr. Al Rowland
Department of Molecular BioSciences
TURITEA

Dear Dr. Rowland,

Re: HEC: PN Protocol – 02/145
NEW ZEALAND NUCLEAR TEST VETERANS STUDY - EXTENSION TO INCLUDE THE COMET ASSAY AND CRYOPRESERVATION STUDY.

Thank you for your letter dated 30 January 2003 and the amendments requested.

The amendments you have made now meet the requirements of the Massey University Human Ethics Committee and the ethics of your protocol are approved.

Any departure from the approved protocol will require the researcher to return this project to the Massey University Campus Human Ethics Committee: Palmerston North for further consideration and approval.

A reminder to include the following statement on all public documents "This project has been reviewed and approved by the Massey University Human Ethics Committee, PN Protocol No. 02/145. If you have any concerns about the conduct of this research, please contact Professor Sylvia V Rumball, Chair, Massey University Campus Human Ethics Committee: Palmerston North, telephone 06 350 5249, email S.V.Rumball@massey.ac.nz"

Yours sincerely

Sylvia Rumball.

Professor Sylvia V Rumball, Chair
Massey University Campus Human Ethics Committee: Palmerston North

- APPENDIX II -

RANDOM MICROSCOPE COORDINATES

Microscope coordinates were created using the random number generator function of Microsoft Excel™. The areas of the 2 gel areas depicted in Figure 2.1 were used as the parameters for the generator.

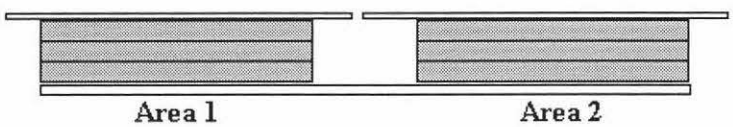


Figure 2.1. Figure showing the 2 areas of the microscope slide in which random coordinates corresponded to. Image not to scale

The Coordinates shown in Table 2.1 were used in all microscope work during this study.

Table 2.1. The set of random X and Y microscope coordinates used in this thesis.

Slide Area 1			Slide Area 2		
Cell #	**X Direction**	**Y Direction**	**Cell #**	**X Direction**	**Y Direction**
01	170	13	**01**	117	13
02	169	10	**02**	117	17
03	171	06	**03**	120	13
04	171	13	**04**	116	10
05	164	10	**05**	106	16
06	162	14	**06**	114	10
07	170	18	**07**	117	14
08	171	10	**08**	113	21
09	158	21	**09**	118	07
10	156	08	**10**	110	19
11	164	07	**11**	109	10
12	154	08	**12**	110	21

13	167	05	**13**	107	10
14	165	07	**14**	119	16
15	156	09	**15**	111	18
16	165	10	**16**	109	18
17	165	21	**17**	119	23
18	167	14	**18**	104	21
19	159	10	**19**	108	21
20	160	13	**20**	112	08
21	158	20	**21**	108	16
22	155	16	**22**	104	09
23	163	17	**23**	118	03
24	156	22	**24**	120	11
25	171	19	**25**	105	21
26	154	17	**26**	113	11
27	153	15	**27**	121	05
28	165	08	**28**	107	09
29	154	08	**29**	114	07
30	169	14	**30**	111	15
31	169	22	**31**	122	03
32	166	11	**32**	116	06
33	158	14	**33**	119	08
34	158	23	**34**	115	12
35	165	09	**35**	115	03
36	171	21	**36**	102	15
37	172	14	**37**	111	07
38	157	03	**38**	118	19
39	161	19	**39**	117	03
40	164	13	**40**	102	08
41	155	07	**41**	105	12
42	167	08	**42**	116	17
43	152	10	**43**	109	18
44	157	03	**44**	110	13

45	154	02	**45**	109	08
46	172	17	**46**	107	10
47	167	21	**47**	112	10
48	167	09	**48**	103	04
49	158	14	**49**	111	12
50	171	22	**50**	103	14

☢ - APPENDIX III - ☢

CASP SOFTWARE OPERATION

3.1 SETTING THRESHOLDS

Before analysing comet images, the sensitivity thresholds were set. Thresholding involves altering the sensitivity of the software, which will influence the measurements. All values outlined in this section are optimal values that were used throughout the study. The manufacturer determined these values. The following parameters can be adjusted:

Head Centre Threshold (HCT): regulates the detection of the head centre. Reducing the threshold will move the head centre towards the tail. Optimal value assessed = 0.8. HCT determines points to detection of the head centre, which is the centre of gravity of found points (pixels). Value 0.8 will mean that all points of intensity greater then 80% of maximal intensity will be used to calculate the head centre.

Tail Threshold (TT): reducing this threshold will reduce the tail size. Optimal value assessed = 0.05. A value of 0 gives the background intensity and a value 1 gives the maximum intensity.

Head Threshold (HT): reducing this threshold will reduce the head size. Optimal values assessed = 0.05. A value of 0 gives the background intensity and 1 gives maximum intensity. This threshold was not used during analysis.

Comet Thresholds (CT): combined head and tail thresholds. This can be activated if both thresholds have the same value. Reducing it will reduce both the head and tail size. Optimal value assessed = 0.05. A value of 0 gives the background intensity and 1 gives maximum intensity. This threshold was not used during analysis

Profiles: This option allows the user to choose between 2 methods of image analysis: profile 1 and profile 2. Differences between these 2 profiles apply only to detection of the tail.

Profile 1 is based on the method of percolation. This means that only those pixels are considered as belonging to the tail, which forms an uninterrupted "carpet" of pixels touching the head region. In other words this is the solid area and there is a connection between any 2 points in the tail. This process eliminates pixel debris that does not belong to the tail. In addition, only pixels right of the head centre are considered. According to the manufacturer, this is the optimal profile setting. This was the choice used during analysis.

In profile 2, all pixels that are outside the head region and are located between the head centre and end of the tail and are enough intensive are identified as belonging to the tail. This was not used during analysis.

The main control panel of the CASP software is shown in Figure 3.1.

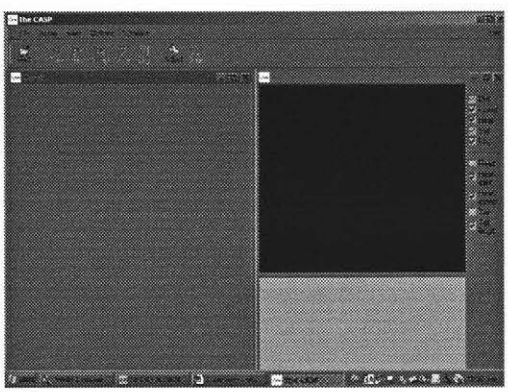


Figure 3.1. The main control panel of the CASP software analysis tool.

The tool for the setting of thresholds is outlined in Figure 3.2.

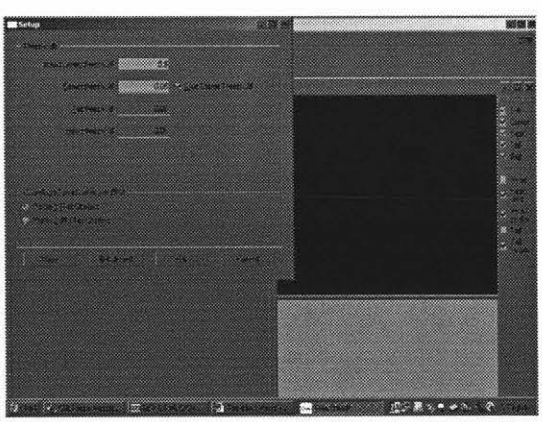


Figure 3.2. Configuring the thresholds of the CASP software.

3.2 SOFTWARE OPERATION

At least 100 cell images for each individual assayed were selected, as outlined in Figure 3.3 and Figure 3.4.

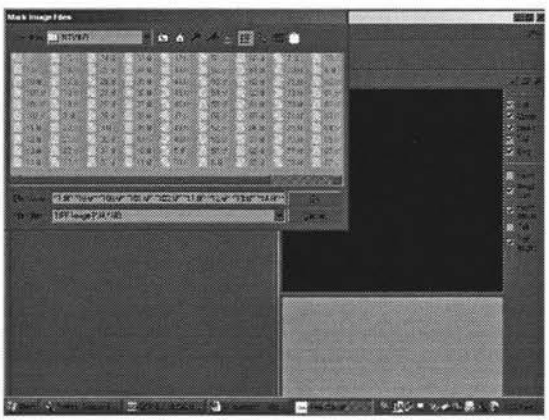


Figure 3.3. The marking of comet cell images for analysis.

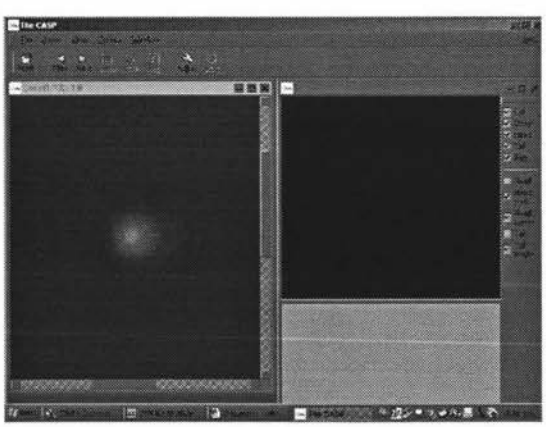


Figure 3.4. The first image selected for analysis.

Using the mouse, a box was drawn over the cell of interest. This box was required to be large enough to encapsulate a cell with a long tail, as this box was used for all of the cells in this particular operation. An example of this action is shown in Figure 3.5. Please note the smaller box above the cell. This was drawn independent of the main box by the program to act as a background sample. Both of the boxes must be as free of debris as possible to achieve an accurate measurement.

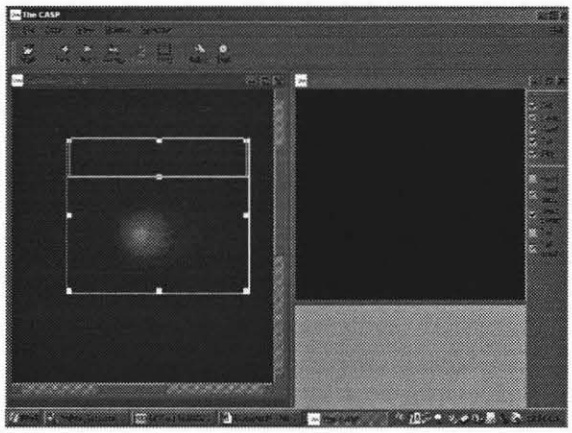


Figure 3.5. Boxes drawn around the cell of interest for analysis.

When the box was of a suitable size the "START" feature was selected, which locked the size of the boxes. This ensured consistency throughout the analysis procedure. The "ASSAY" feature was then selected, which measures the values for each of the 13 variables that the software can quantify. These values were then displayed in the right-hand panel along with a DNA intensity graph of the cell. An example of this is outlined in Figure 3.6. Note that all of the checkboxes on the far right-hand side have been selected to obtain the view seen below.

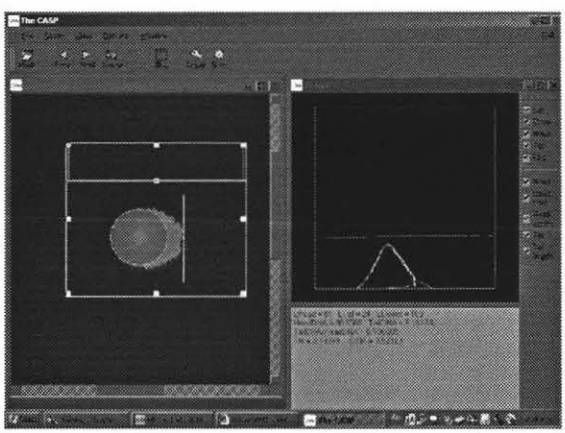


Figure 3.6. A cell that has been measured for genetic damage.

The "STORE" function was then selected, which stores all 13 variables for each cell in an internal spreadsheet. An example of such a spreadsheet is shown in Figure 3.7.

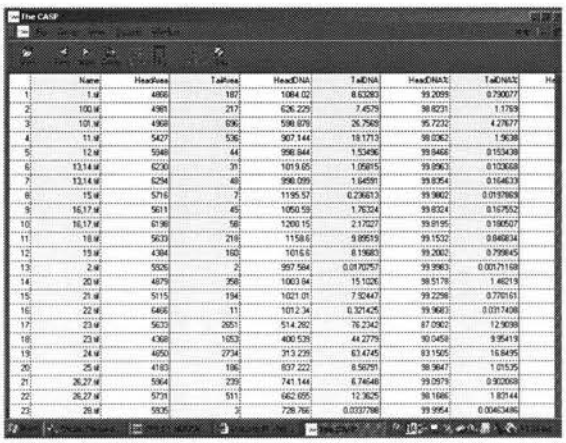


Figure 3.7. Spreadsheet function in which all of the data that has been measured by the software is stored.

These data cannot be directly copied from the CASP's internal spreadsheet. After all cells had been assayed using The CASP software, results were exported to a text document by activating the file menu and selecting "export results". In the window that appeared, the file selected for export to, was "text_results.txt". After saving the file, it was opened, the entire contents copied and then pasted into a new Microsoft Word file. These data were in ASCII format that needed to be converted to Microsoft Excel™ format (Figure 3.9) in order for data analysis to be carried out. A macro was created for this using Microsoft Word™.

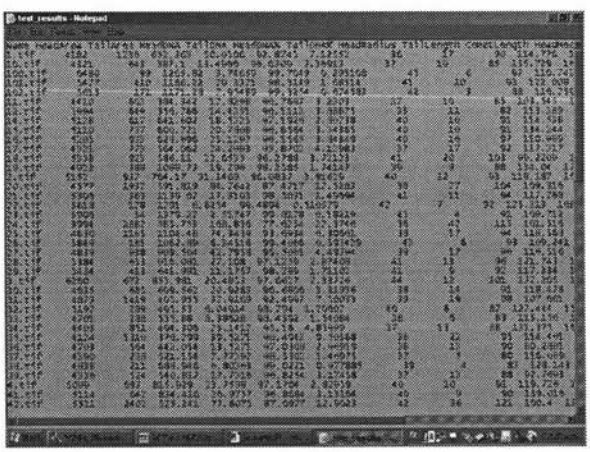


Figure 3.8. Exported CASP data in ASCII format.

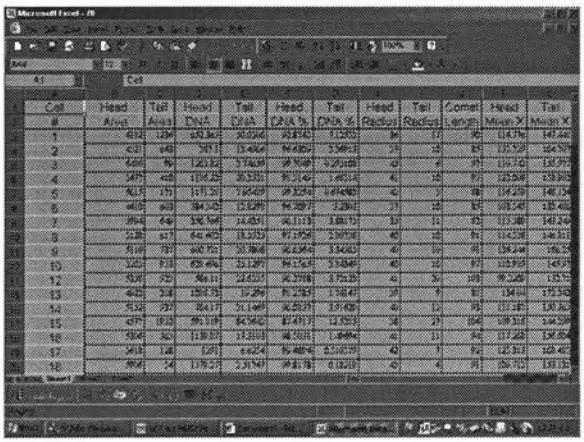


Figure 3.9. Converted CASP data in an Excel™ Spreadsheet.

- APPENDIX IV -

FREEZING SCHEDULE

Table 4.1. The cryopreservation study sample-freezing schedule. N.B. all of the cells used in this study had an initial viability of > 90 % before cryopreservation.

Vial #	NTV# (Sample)	[Cell] Initial (x 10^6)	[Cell] Final (x 10^6)	Date Frozen	Date Thawed	Length Of Time	% Of Viable Thawed Cells
1	86 (1)	0.43	0.50	8/04/2003	8/06/2003	2 Months	76%
2	86 (2)	0.43	0.54	8/04/2003	8/08/2003	4 Months	52%
3	86 (3)	0.43	0.34	8/04/2003	8/11/2003	7 Months	42%
4	87 (1)	0.26	0.26	8/04/2003	8/06/2003	2 Months	> 90%
5	87 (2)	0.26	0.66	8/04/2003	8/08/2003	4 Months	61%
6	87 (3)	0.26	1.22	8/04/2003	8/11/2003	7 Months	68%
7	88 (1)	0.12	0.20	8/04/2003	8/06/2003	2 Months	> 90%
8	88 (2)	0.12	0.20	8/04/2003	8/08/2003	4 Months	> 90%
9	88 (3)	0.12	0.40	8/04/2003	8/11/2003	7 Months	40%
10	89 (1)	0.09	0.20	8/04/2003	8/06/2003	2 Months	> 90%
11	89 (2)	0.09	0.22	8/04/2003	8/08/2003	4 Months	50%
12	89 (3)	0.09	0.34	8/04/2003	8/11/2003	7 Months	71%
13	90 (1)	0.20	0.06	8/04/2003	8/06/2003	2 Months	> 90%
14	90 (2)	0.20	0.24	8/04/2003	8/08/2003	4 Months	83%
15	90 (3)	0.20	0.26	8/04/2003	8/11/2003	7 Months	47%
16	11 (1)	0.27	0.04	29/04/2003	29/06/2003	2 Months	0%
17	11 (2)	0.27	0.16	29/04/2003	29/08/2003	4 Months	40%
18	11 (3)	0.27	0.34	29/04/2003	29/11/2003	7 Months	59%
19	17 (1)	1.00	0.14	29/04/2003	29/06/2003	2 Months	79%
20	17 (2)	1.00	0.40	29/04/2003	29/08/2003	4 Months	40%
21	17 (3)	1.00	0.72	29/04/2003	29/11/2003	7 Months	61%
22	18 (1)	0.68	0.07	29/04/2003	29/06/2003	2 Months	86%

23	18 (2)	0.68	0.10	29/04/2003	29/08/2003	4 Months	40%
24	18 (3)	0.68	0.40	29/04/2003	29/11/2003	7 Months	40%
25	19 (1)	0.64	0.04	29/04/2003	29/06/2003	2 Months	> 90%
26	19 (2)	0.64	0.08	29/04/2003	29/08/2003	4 Months	25%
27	19 (3)	0.64	0.30	29/04/2003	29/11/2003	7 Months	73%
28	3 (1)	0.57	0.60	13/05/2003	13/07/2003	2 Months	> 90%
29	3 (2)	0.57	0.42	13/05/2003	13/09/2003	4 Months	81%
30	3 (3)	0.57	2.10	13/05/2003	13/12/2003	7 Months	> 90%
31	8 (1)	0.28	0.44	13/05/2003	13/07/2003	2 Months	> 90%
32	8 (2)	0.28	0.60	13/05/2003	13/09/2003	4 Months	83%
33	8 (3)	0.28	1.14	13/05/2003	13/12/2003	7 Months	89%
34	9 (1)	0.55	1.16	13/05/2003	13/07/2003	2 Months	> 90%
35	9 (2)	0.55	2.16	13/05/2003	13/09/2003	4 Months	> 90%
36	9 (3)	0.55	2.08	13/05/2003	13/12/2003	7 Months	84%
37	2 (1)	0.68	1.54	20/05/2003	20/07/2003	2 Months	> 90%
38	2 (2)	0.68	0.70	20/05/2003	20/09/2003	4 Months	69%
39	2 (3)	0.68	3.12	20/05/2003	20/12/2003	7 Months	75%
40	6 (1)	0.71	1.14	20/05/2003	20/07/2003	2 Months	70%
41	6 (2)	0.71	0.86	20/05/2003	20/09/2003	4 Months	65%
42	6 (3)	0.71	2.42	20/05/2003	20/12/2003	7 Months	68%
43	7 (1)	0.55	0.12	20/05/2003	20/07/2003	2 Months	> 90%
44	7 (2)	0.55	0.92	20/05/2003	20/09/2003	4 Months	65%
45	7 (3)	0.55	0.70	20/05/2003	20/12/2003	7 Months	80%
46	10 (1)	1.51	3.22	20/05/2003	20/07/2003	2 Months	> 90%
47	10 (2)	1.51	2.14	20/05/2003	20/09/2003	4 Months	75%
48	10 (3)	1.51	1.14	20/05/2003	20/12/2003	7 Months	82%
49	91 (1)	0.65	0.11	20/05/2003	20/07/2003	2 Months	> 90%
50	91 (2)	0.65	0.50	20/05/2003	20/09/2003	4 Months	76%
51	91 (3)	0.65	1.86	20/05/2003	20/12/2003	7 Months	> 90%
52	92 (1)	0.36	0.12	20/05/2003	20/07/2003	2 Months	> 90%
53	92 (2)	0.36	0.54	20/05/2003	20/09/2003	4 Months	85%
54	92 (3)	0.36	1.42	20/05/2003	20/12/2003	7 Months	83%

55	93 (1)	0.85	1.58	27/05/2003	27/07/2003	2 Months	87%
56	93 (2)	0.85	1.08	27/05/2003	27/09/2003	4 Months	87%
57	93 (3)	0.85	2.78	27/05/2003	27/12/2003	7 Months	> 90%
58	94 (1)	0.44	0.64	27/05/2003	27/07/2003	2 Months	75%
59	94 (2)	0.44	0.88	27/05/2003	27/09/2003	4 Months	> 90%
60	94 (3)	0.44	1.76	27/05/2003	27/12/2003	7 Months	> 90%

- APPENDIX V -

CYTOGENETIC TESTS

6 cytogenetic tests were used by researchers who were involved in the current study, for the purpose of determining if the levels of genetic damage in nuclear test veterans were different to that of the selected control group. Only the COMET assay was used in this thesis. However, the principles behind the remaining 5 tests are outlined below.

5.1 THE MICRONUCLEUS (MN) ASSAY

The micronucleus assay is an *in vivo* mammalian method, which has been widely used for screening the genotoxic potency of chemical or physical agents. This assay measures the efficiency of a persons DNA repair system following the *in vitro* irradiation of cultured lymphocytes. A micronucleus (MN) is formed during the metaphase/anaphase transition of mitosis (cell division). It may arise from a whole lagging chromosome or from an acentric chromosome fragment from a chromosome after a breakage that are not integrated in to the daughter nuclei. By adding a cytokinesis-blocking agent, all of the nuclei are retained within the same cell membrane. Figure 5.1 shows typical views of micronucleated cells.

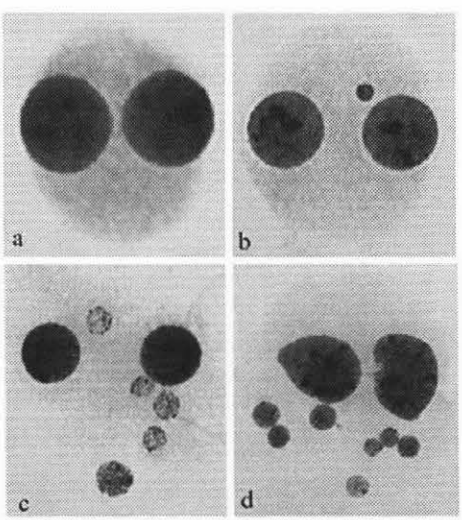


Figure 5.1. The micronucleus assay. Panels show cells with a) no micronuclei, b) 1 micronucleus, c) 5 micronuclei, and d) 7 micronuclei.
Image provided by Mohammed Abdul Wahab.

5.2 THE G2 ASSAY

The G2 Assay is similar to the Micronucleus Assay in that it is interpreted as measuring the efficiency of a person's DNA repair system, but at a different time in a dividing cell's cycle. Whereas one person may be sensitive to the MN technique, they may not be with the G2 Assay and vice versa. Thus it is advisable to apply both techniques in a study of genetic damage.

The G2 Assay procedure also involves breaking the DNA of lymphocytes in a blood sample with a powerful dose of radiation, but at the G2 stage of the cell cycle. Subjects with an inefficient DNA repair system exhibit a higher number of both chromosome and chromatid breaks. The G2 assay is scored based on the number of breakages observed.

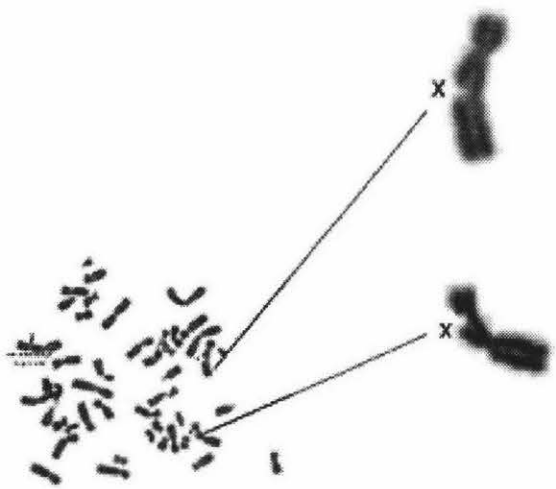


Figure 5.2. The G2 assay. The enlarged views show chromosomes with breakages. Image provided by Liz Nickless

5.3 THE SISTER-CHROMATID EXCHANGE

Sister chromatid exchanges (SCEs) involve breakage of both DNA strands, followed by an exchange of whole DNA duplexes. This occurs during S phase and is efficiently induced by mutagens that form DNA adducts or that interfere with DNA replication. The formation of SCEs has been correlated with recombinational repair and the induction of point mutations, gene amplification and cytotoxicity. This technique is widely used as an indicator of clastogenicity, with a concomitant increase in ill health.

The SCE test is usually performed on human peripheral blood lymphocytes. As peripheral lymphocytes are in the resting G0 stage of the cell cycle, they have to be stimulated to divide by an aspecific antigen, like phytohaemagglutinin. To collect a sufficient number of mitotic cells, a spindle inhibitor like colcemid may be added shortly before fixation (at 72 hours) in order to block cells in (pro) metaphase of the second mitosis.

BrdU (5-bromo-2-deoxy-uridine) is added to the culture medium for the duration of 2 complete cell cycles to allow for a differential staining that enables the researcher to distinguish both chromatids. Chromatids in which only 1 strand of DNA incorporated BrdU show a normal dark Giemsa staining, whereas those with 2 substituted strands stain less darkly. If an exchange occurred, this can be seen as the dark part changes to the other arm, known as "harlequin chromosomes". Figure 5.3 shows a chromosome spread that has undergone 19 sister-chromatid exchanges.

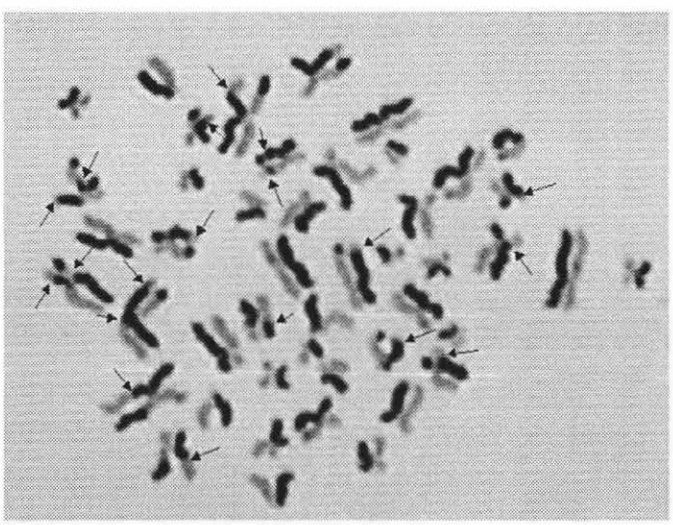


Figure 5.3. The Sister-chromatid Exchange showing 19 exchanges.
Image provided by Mohammed-Abdul Wahab

5.4 CHROMOSOME ABERRATION TEST

The chromosome aberration test is a classical genetic test that has been used for many to detect large chromosome aberrations such as translocations, deletions, or extra chromosomes. Structural chromosome aberrations can result from direct DNA breakages, replication using a damaged DNA template, inhibition of DNA synthesis, and other mechanisms. Such DNA breaks may rejoin such that the chromosome is restored to its original state, rejoin incorrectly,

or not rejoin at all. The latter 2 cases may be observable on microscopic preparations of metaphase cells.

Many of these gross changes probably will not allow cell survival after division, however they serve as indicators for the induction of smaller, not readily observable changes, which do allow cell survival but may have deleterious consequences for the organism. Structural aberrations that can be detected using the chromosome aberration test are gaps, breaks, dicentric chromosomes, and ring chromosomes. Numerical aberrations that can be detected are polyploidy, hypodiploidy (< 46 chromosomes), hyperdiploidy (> 46 chromosomes). A human karyotype that is used for the detection of chromosome aberrations is shown in Figure 5.4.

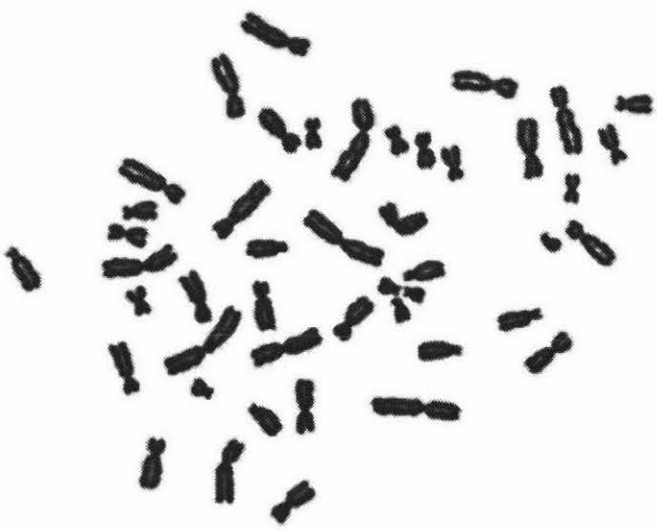


Figure 5.4. A human karyotype showing 46 chromosomes.
Image provided by Liz Nickless.

5.5 FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

Fluorescence *in situ* hybridisation (FISH) is a sensitive and useful addition to cytogenetic testing for the detection of abnormalities of chromosomal structure or numbers such as deletions, translocations, duplications, and aneuploidy and is often the method of choice for detection of microdeletions. FISH involves the use of fluorescently labelled DNA probes that are specific for a DNA sequence. These probes can be used to label a specific gene, chromosome region, or entire chromosome and can be used to show translocation events between labelled and unlabelled chromosomes. Figure 5.5 shows an example of FISH.

Probes for chromosomes 2, 3, and 5, as well as a probe specific for centromeric DNA have been used to label the DNA. The remaining chromosomes have been stained blue using DAPI.

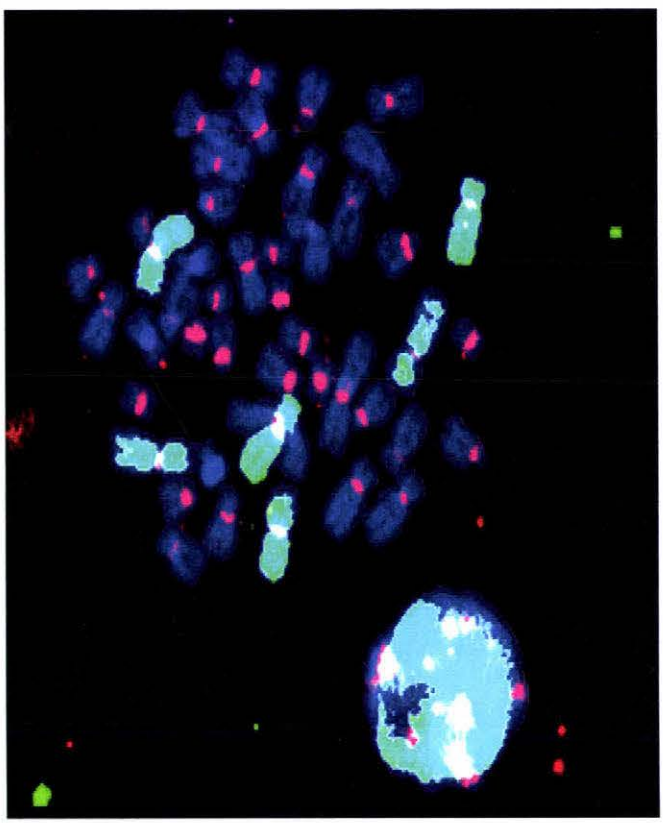


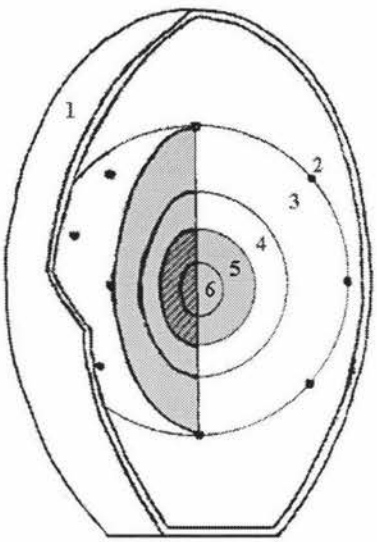
Figure 5.5. Fluorescence *In Situ* Hybridisation (FISH).
Image provided by Liz Nickless.

☢ - APPENDIX VI - ☢

NUCLEAR WEAPON PRINCIPLES

It is prudent to give some background information of the mechanisms and effects of a nuclear weapon. This section gives relevant data on the types of weapons that were detonated during the Operation Grapple tests. The weapons detonated during the tests at Malden Island and Christmas Island were 7 fission bombs, also known as atomic bombs, and 2 small fusion bombs, also known as Hydrogen bombs or thermonuclear bombs. These weapons release immense amounts of energy in several different forms, all of which are potentially biological hazards.

Fission bombs derive their energy from the splitting of heavier elements, such as uranium, into smaller nuclides. Multiple detonators simultaneously initiate detonation of high explosives. The explosive shock front compresses and transits the pusher, which facilitates transition of the shock wave from low-density high explosive to high-density core material. The shock front in turn compresses the reflector, tamper, and fissile core inward, and a neutron burst initiates a fission chain reaction in the fissile core. A neutron splits a plutonium/uranium-235 atom, releasing perhaps 2 or 3 neutrons to do the same to other atoms, and so on and so forth. The energy released from this reaction increases geometrically. The basic structure of a fission bomb is shown below in Figure 6.1.



Numbered Parts

1. Bomb casing
2. Detonators
3. Conventional high explosive
4. Pusher (aluminium, others) and reflector (beryllium, tungsten)
5. Tamper (uranium-238)
6. Fissile core (plutonium or uranium-235)

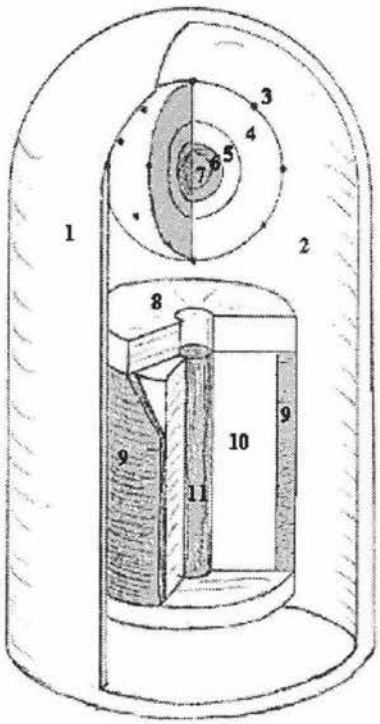
Adapted from http://www.johnstonsarchive.net

Figure 7.1. A simplified schematic diagram of a nuclear fission implosion weapon. Key indicates the identity of the numbered component.

Fusion bombs, on the other hand, are more complicated and derive a large portion of their energy from the nuclear fusion of hydrogen isotopes into helium nuclei. A conventional fission reaction, such as that described above, is required to initiate the reaction. Gamma radiation from the fission explosion superheats the filler material, turning it into plasma. Compression reaches the fusion fuel, which has been partially protected from the gamma radiation by a radiation shield and the fissile sparkplug is compressed to a super-critical mass. Neutrons from the fission reaction reach the fissile sparkplug through the channel in the radiation shield, initiating a fission chain reaction causing it to explode outward.

The fusion fuel is super-compressed between the fusion pusher/tamper from without and the spark plug from within, turning it into superheated plasma. Lithium and deuterium nuclei collide in the fusion fuel to produce tritium (^{3}H), and tritium and deuterium (^{2}H) nuclei engage in fusion reactions: nuclei fuse by pairs into helium nuclei, producing a large energy release of gamma rays, neutrons, and heat. The large release of neutrons from fusion in the fusion fuel causes transmutation of uranium-235 atoms in the fusion pusher/tamper, releasing additional energy.

All reactions end as the superheated remnants expand under the energy release; the entire weapon is vaporized. The time required for this reaction to occur is approximately 0.00002 seconds. The basic structure of a hydrogen bomb is shown below in Figure 6.2.



Numbered Parts

1. Bomb casing
2. Interior filling (plastic material)
3. Detonators
4. Conventional high explosive
5. Pusher (aluminium, others) and reflector (beryllium, tungsten)
6. Tamper (uranium-238)
7. Fissile core (plutonium or uranium-235)
8. Radiation shield (tungsten, others)
9. Fusion pusher/tamper (uranium-235 sleeve)
10. Fusion fuel (solid lithium-deuteride)
11. Sparkplug (uranium-235 or plutonium)

Adapted from http://www.johnstonsarchive.net

Figure 6.2. A simplified schematic diagram of a multistage thermonuclear weapon. Key indicates the identity of the numbered component.

An immense amount of energy is released from a nuclear detonation. The total (100 %) of the energy released from a nuclear weapon comes in 4 primary categories: Blast, thermal radiation, ionizing radiation, and residual radiation (nuclear fallout). Table 6.1 (Gladstone *et al*, 1977) shows the approximate quantity of each type of energy that is released during an atomic detonation.

Table 6.1. Sources of energy released during an atomic detonation.

Source	Percent Of Total Energy Released
Blast	40-60 %
Thermal Radiation	30-50 %
Ionizing Radiation	5 %
Residual Radiation (Nuclear Fallout)	5-10 %

Of course the energy released in each of the above will depend on design of the weapon, and the environment in which it is detonated. Any one of these energy types can cause damage to an organism

☢ - APPENDIX VII - ☢

MECHANISMS OF RADIOACIVE DECAY

7.1 ALPHA (α) DECAY

Alpha (α) decay involves the release of a helium molecule (2 protons and 2 neutrons) such as those involved in a fusion reaction. The relatively large size of these molecules affects their mobility, thus they will only travel short distances at approximately 5 % of the speed of light. Unless ingested, alpha particles cannot penetrate the epithelial layer of human skin and so are not detrimental to human health. However, if material that decays through the release of an alpha particle is ingested, it can cause tremendous damage. Due to an α-particles large size, its action is analogous to that of a cannonball, compared to other smaller particles, and can result in serious genetic damage wherever it resides (Varney, 2000). These particles are not important with respect to the current study.

7.2 BETA (β) DECAY

Beta (β) decay involves the release of a high-speed electron or positron. Due to their small size these particles can travel approximately 1.2 metres through air or, depending on the particle's maximum energy, can penetrate several mm through solid objects, including human tissue, at velocities close to the speed of light. Low energy beta particles cannot easily penetrate the skin, and so do present an internal hazard. High-energy beta particles can penetrate living tissue and will also produce harmful x-rays when absorbed by a target. This type of decay is important with regard to its effect on human health, and the current study.

7.3 GAMMA (γ) DECAY

Gamma (γ) decay is the most important type of radioactive decay and is a concern when considering human health, with the exception of neutron decay, which will not be considered. γ-decay involves the release of photons. These particles can travel hundreds of metres through air at the speed of light, and can easily penetrate several centimetres into lead and tens of

centimetres into concrete, but most importantly they can pass straight through an organism and can cause tremendous genetic damage if a particle comes in contact with DNA

A summary of these decay products and their penetrating power is illustrated in Figure 7.1 and Figure 7.2, respectively.

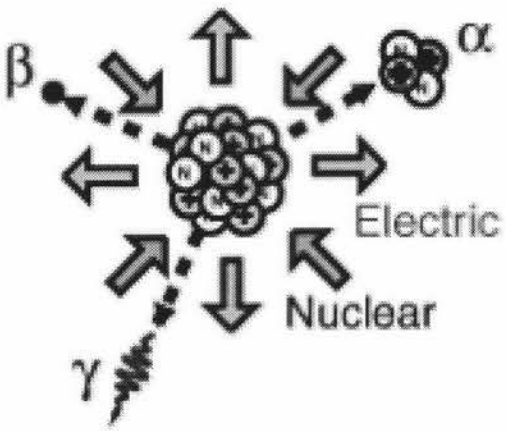


Figure 7.1. A summary of the 3 main mechanisms of radioactive decay.

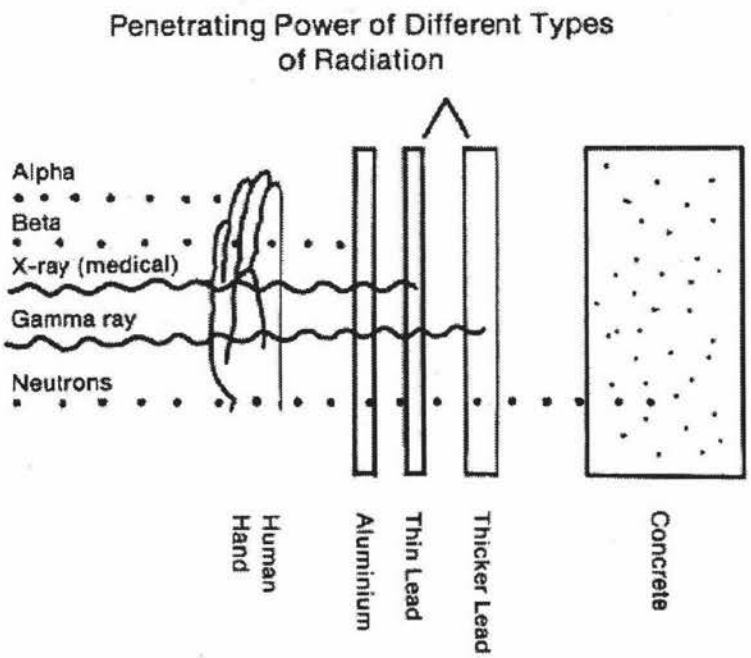


Figure 7.2. A simplified diagram of the penetrating power of X-rays, neutrons, and α, β, and γ particles (Bertell, 1986).

☢ - APPENDIX VIII - ☢

QUESTIONNAIRE SENT TO ALL PARTICIPANTS DURING THE SELECTION OF THE CONTROL AND EXPERIMENTAL GROUPS

New Zealand Nuclear Test Veterans Postal Survey: A Pilot Study

A research project conducted on behalf of the New Zealand Nuclear Test Veterans Association by independent researchers from Massey University

Please read the following instructions carefully:

- **All the information you give us is in confidence and will be used only for the purposes of this study.**
- **Please attempt every question and be careful not to skip any pages.**
- **There are no right or wrong answers; we want the response which is best for you.**
- **It is important that you give your own answers to the questions. Please do not discuss your answers with others.**
- **Do not linger too long over each question; usually your first response is best.**
- **The survey is comprehensive and appears long; however, we have used a large print size to make the text easier to read.**
- **We suggest that you plan to answer the questions over a few sittings. You will find a bookmark inside the front cover, to help you mark your place, as you progress through the survey. Each of the six parts of the survey is also printed in a different colour, to help you monitor your progress.**

						Mail out date: (from Massey)							
(Shaded area for Office Use Only)						Date received: (at Massey)							

After you have returned this survey, you will be telephoned by a member of the research team to arrange the time and venue for a face-to-face interview.

Please enter your telephone number below:

	STD CODE	NUMBER
Telephone number:		

Interviews will take place within a month of receiving this completed postal survey. If you know that you will be away at any time during this period, or have regular commitments on specific days, please specify <u>the dates you will be away/otherwise occupied</u> below, in order to assist us setting up the interviewing schedule:

_____

_____

_____

_____

_____

Thank you. Please complete the consent form on the next page, which will be removed, together with this page, when your completed postal survey is returned to Massey.

There are SIX PARTS to this survey. PART 1 begins on the page following the consent form. ☞

Massey University
COLLEGE OF HUMANITIES & SOCIAL SCIENCES

School of Psychology
Private Bag 11 222,
Palmerston North,
New Zealand
Telephone: 64 6 356 9099
Facsimile: 64 6 350 5673

NEW ZEALAND NUCLEAR TEST VETERANS: A PILOT STUDY

CONSENT FORM

I have read the Information Sheet and have had the details of the study explained to me in written form. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I understand that I have the right to withdraw from the study at any time, and to decline to answer any particular questions.

I agree to provide a sample of blood for chromosomal analysis on the understanding that I have access to my results, and that I be advised if any abnormalities are found. I understand that my blood sample will not be used for any other research, and will be disposed of sensitively.

I agree to provide information to the researchers on the understanding that my name will not be used without my permission.

(*The information will be used for this research and publications arising from this research project.*)

I agree to participate in this study under the conditions set out in the Information Sheet.

Signed .....

Name: ***(please print)*** .....

Date: .....

If you would like to receive feedback from this project in the form of a brief written report please tick the appropriate box below:

YES ☐ **NO** ☐

☐☐☐☐☐

New Zealand Nuclear Test Veterans Postal Survey

PART 1

Firstly, we would like some general background information about you. Please tick the circle ✔ next to the answer which you believe gives an accurate indication of your CURRENT situation, or write details in the spaces provided.

Office Use Only

1 **What is your date of birth?** *(Please state* **day / month / year**)

				1	9		

day month year

2 **What is your gender?**

○ Male ○ Female

3 **Which ethnic group/s do you belong to?**
(You may tick more than one circle.)

○ 1 New Zealander of European descent
○ 2 New Zealander of Maori descent
○ 3 Pacific Islander
○ 4 Asian
○ 5 Other (Please specify)

_____

4 **Which of the following best describes the area where you live?**
(Please tick one circle.)

○ 1 Main Urban Area:	A city with population of 30,000 or more e.g. Palmerston North
○ 2 Secondary Urban Area:	A town / city with a population of between 10,000 & 29,999
○ 3 Minor Urban Area:	A town with a population of between 1,000 & 10,000
○ 4 Rural Centre:	A town with a population of between 300 & 1,000
○ 5 Rural Area:	Outside a town / city boundaries

Please tick the circle ✔ which you believe gives an accurate indication of your CURRENT situation, or write details in the spaces provided.

5 **Do you live . . . .** *(You may tick more than one circle.)*

- ◯ 1 with your spouse / partner and no one else?
- ◯ 2 with your spouse / partner and family?
- ◯ 3 with relatives?
- ◯ 4 alone?
- ◯ 5 with other adults?
- ◯ 6 in a rest home / nursing home / veterans' home?
- ◯ 7 Other *(Specify in the space provided below)*

_____

6 **Are you retired?**

◯ Yes *(please continue)* ◯ No *(please go to Q8 below)*

7 **IF** you **ARE RETIRED** what **was** your main occupation?

_____

8 **IF** you **ARE NOT RETIRED** what **is** your main occupation?

_____

9 **What is your highest educational qualification?**
(Please tick one circle.)

- ◯ 1 **Less than 3 years** at secondary school
- ◯ 2 **From 3 to 5 years** at secondary school
- ◯ 3 School qualifications, University Entrance and above
- ◯ 4 Trade certificate or Professional certificate or diploma
- ◯ 5 University degree, diploma, or certificate
- ◯ 6 Other (Specify in the space provided below)

_____

Office Use Only

32 38 39 40 41 42

Please tick the circle ✓ which you believe gives an accurate indication of your CURRENT situation, or write details in the spaces provided.

10 **In which branch of the service were you employed?**
(You may tick more than one circle.)

Office Use Only

1 ◯ NZArmy — 43

2 ◯ RNZNavy — 44

3 ◯ RNZAirForce — 45

4 ◯ Other *(Please specify, for example, Royal Navy)*

_____

_____

_____ — 46

11 **What is/are your service number/s?**
(Please enter details in the boxes provided, below.
*Start entering data from the **LEFT hand side** of the boxes.*
When you have entered your number leave any spare boxes blank.
***If you have MORE THAN ONE service number**, please ensure you specify **WHICH BRANCH** each number is associated with, in the space provided **BENEATH** the boxes.)*

↓ *Start here*

Service Number: | | | | | | | | | — 49–55
associated with ...

(Specify branch) _____

↓ *Start here*

Service Number: | | | | | | | | | — 58–64
associated with ...

(Specify branch) _____

↓ *Start here*

Service Number: | | | | | | | | | — 67–73
associated with ...

(Specify branch) _____

Please tick the circle ✓ which you believe gives an accurate indication of your CURRENT situation, or write details in the spaces provided.

Office Use Only

12 **Have you ever been in a situation where you have been exposed to a nuclear blast?**

○ **Yes** *(please continue)*

○ **No** *(please go to PART 2 on page 9)*

1

13 **Did you serve in OPERATION GRAPPLE?**

○ **Yes** *(please continue)*

○ **No** *(please go to Q17 below)*

2

14 **When did you serve in OPERATION GRAPPLE?**

From: ☐☐☐☐ (month / year) **To:** ☐☐☐☐ (month / year)

8

14

15 **What ship(s) did you serve on, in OPERATION GRAPPLE?**

_____

Ship(s)

15

In what branch did you serve during OPERATION GRAPPLE?

16 _____

Branch

16

17 **How many blasts were you exposed to?**

_____

number of blasts

18

If you are NOT an Operation Grapple veteran, but have been exposed to a nuclear blast, please turn to page 8 to record your responses to the questions.

The instructions that follow apply ONLY to OPERATION GRAPPLE VETERANS:

Pages 5, 6 & 7 list NINE Operation Grapple blasts in order (by operation name, place & date). Please record your responses to the questions (such as the type of protective clothing worn; where you were at the time of the blast; how long you remained in the exclusion zone etc.) for EACH OPERATION GRAPPLE blast that you were exposed to.

Once you have completed listing details related to ALL the blasts that you were exposed to, please go to PART 2 on page 9.

Record if you were present (or not) at <u>EACH</u> blast listed below.

Office Use Only

1 **Did you serve on GRAPPLE 1?** ***(Malden Island on 15 May 1957)***

◯ **Yes** *(continue)* ◯ **No** ***(go to Q2 below)***

[1a] **WHERE were you at the time of the blast?** *(Specify below)*

_____

[1b] **What protective clothing did you wear at that time?** *(Specify below)*

_____

[1c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No**

[1d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days**

[1e] **What were you doing during this time?** *(Specify below)*

_____

2 **Did you serve on GRAPPLE 2?** ***(Malden Island on 31 May 1957)***

◯ **Yes** *(continue)* ◯ **No** ***(go to Q3 below)***

[2a] **WHERE were you at the time of the blast?** *(Specify below)*

_____

[2b] **What protective clothing did you wear at that time?** *(Specify below)*

_____

[2c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No**

[2d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days**

[2e] **What were you doing during this time?** *(Specify below)*

_____

3 **Did you serve on GRAPPLE 3?** ***(Malden Island on 19 June 1957)***

◯ **Yes** *(continue)* ◯ **No** ***(go to Q4 on page 6)***

[3a] **WHERE were you at the time of the blast?** *(Specify below)*

_____

[3b] **What protective clothing did you wear at that time?** *(Specify below)*

_____

[3c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No**

[3d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days**

[3e] **What were you doing during this time?** *(Specify below)*

_____

Record if you were present (or not) at <u>EACH</u> blast listed below.

Office Use Only

4 **Did you serve on GRAPPLE X?** ***(Christmas Is. on 8 Nov. 1957)***

◯ **Yes** *(continue)* ◯ **No** ***(go to Q5 below)***

[4a] **WHERE were you at the time of the blast?** *(Specify below)*

[4b] **What protective clothing did you wear at that time?** *(Specify below)*

[4c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No**

[4d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days**

[4e] **What were you doing during this time?** *(Specify below)*

5 **Did you serve on GRAPPLE Y?** ***(Christmas Is. on 28 April 1958)***

◯ **Yes** *(continue)* ◯ **No** ***(go to Q6 below)***

[5a] **WHERE were you at the time of the blast?** *(Specify below)*

[5b] **What protective clothing did you wear at that time?** *(Specify below)*

[5c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No**

[5d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days**

[5e] **What were you doing during this time?** *(Specify below)*

6 **Did you serve on GRAPPLE Z1?** ***(Christmas Is. on 22 August 1958)***

◯ **Yes** *(continue)* ◯ **No** ***(go to Q7 on page 7)***

[6a] **WHERE were you at the time of the blast?** *(Specify below)*

[6b] **What protective clothing did you wear at that time?** *(Specify below)*

[6c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No**

[6d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days**

[6e] **What were you doing during this time?** *(Specify below)*

You should record if you were present (or not) at <u>EACH</u> blast.

Office Use Only

→ **7** **Did you serve on GRAPPLE Z2?** ***(Christmas Is. on 2 Sept. 1958)*** 0 7 57

◯ **Yes** *(continue)* ◯ **No** ***(go to Q8 below)***

[7a] **WHERE were you at the time of the blast?** *(Specify below)* 60

[7b] **What protective clothing did you wear at that time?** *(Specify below)* 63

[7c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No** 64

[7d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days** 69

[7e] **What were you doing during this time?** *(Specify below)* 72

→ **8** **Did you serve on GRAPPLE Z3?** ***(Christmas Is. on 11 Sept. 1958)*** 0 8 3

◯ **Yes** *(continue)* ◯ **No** ***(go to Q9 below)***

[8a] **WHERE were you at the time of the blast?** *(Specify below)* 6

[8b] **What protective clothing did you wear at that time?** *(Specify below)* 9

[8c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No** 10

[8d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days** 15

[8e] **What were you doing during this time?** *(Specify below)* 18

→ **9** **Did you serve on GRAPPLE Z4?** ***(Christmas Is. on 23 Sept. 1958)*** 0 9 21

◯ **Yes** *(continue)* ◯ **No** ***(go to Q10 on page 8)***

[9a] **WHERE were you at the time of the blast?** *(Specify below)* 24

[9b] **What protective clothing did you wear at that time?** *(Specify below)* 27

[9c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No** 28

[9d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days** 33

[9e] **What were you doing during this time?** *(Specify below)* 36

The entries below provide for responses from any participants who have been exposed to nuclear blasts OTHER THAN THOSE ASSOCIATED WITH OPERATION GRAPPLE. If this does not apply to you please go to PART 2 on page 9.

For <u>EACH BLAST</u> please record below:

Office Use Only

10 **Where the blast occurred?** _____ | 1 | 0 | | | 3

When? _____ (day & month) | 1 | 9 | | | (year) | | | 9

[10a] **WHERE were you at the time of the blast?** *(Specify below)* 12

_____

[10b] **What protective clothing did you wear at that time?** *(Specify below)* 15

_____

[10c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No** 16

[10d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days** 21

[10e] **What were you doing during this time?** *(Specify below)* 24

_____

11 **Where the blast occurred?** _____ | 1 | 1 | | | 27

When? _____ (day & month) | 1 | 9 | | | (year) | | | 33

[11a] **WHERE were you at the time of the blast?** *(Specify below)* 36

_____

[11b] **What protective clothing did you wear at that time?** *(Specify below)* 39

_____

[11c] **Did you leave the exclusion zone <u>immediately</u> after your exposure to *<u>this</u>* blast?** ◯ **Yes** ◯ **No** 40

[11d] **If you did NOT leave the zone immediately afterwards, how long did you remain in the area (in days)?** _____ **days** 45

[11e] **What were you doing during this time?** *(Specify below)* 48

_____

Do you need any extra formatted sheets to complete the list of nuclear blasts to which you have been exposed?

◯ **Yes** ◯ **No** 49

(Extra sheets will be brought for you to complete during your face-to-face interview.)

PART 2 questions, related to your occupational history, begin on the next page. ☞

New Zealand Nuclear Test Veterans Postal Survey

PART 2

The first section focuses on your past occupational history. Please write details in the spaces provided.

A **Please list below ALL the occupations that you have had <u>from 1950 until the present</u>. For each entry record the start and end dates (month & year), and a <u>brief</u> description of the type of work. If you need to record <u>more than 24</u> entries you can request that extra formatted pages be provided at your face-to-face interview.**

When you have completed recording your list of occupations <u>turn to page 12</u>.

	<u>Occupation & type of work</u>:	<u>From date</u>: month year	<u>To date</u>: month year	Office Use Only
[1]				3 – 9 – 11
[2]				14 – 20 – 22
[3]				25 – 31 – 33
[4]				36 – 42 – 44
[5]				47 – 53 – 55
[6]				58 – 64 – 66
[7]				69 – 75 – 77

A [contd.] **Please continue to list below ALL the occupations that you have had from 1950 until the present. For each entry record the start and end dates (month & year), and a brief description of the type of work.**

Please turn to page 12 when you have completed recording your list of occupations.

	Occupation & type of work:	From date: month year	To date: month year	Office Use Only	
[8]					3
					9
					11
[9]					14
					20
					22
[10]					25
					31
					33
[11]					36
					42
					44
[12]					47
					53
					55
[13]					58
					64
					66
[14]					69
					75
					77
[15]					3
					9
					11
[16]					14
					20
					22

A [contd.] **Please continue to list below ALL the occupations that you have had from 1950 until the present. For each entry record the start and end dates (month & year), and a brief description of the type of work.**

Please turn to page 12 when you have completed recording your list of occupations.

	Occupation & type of work:	From date: month year	To date: month year	Office Use Only
[17]				25 – 31 – 33
[18]				36 – 42 – 44
[19]				47 – 53 – 55
[20]				58 – 64 – 66
[21]				69 – 75 – 77
[22]				3 – 9 – 11
[23]				14 – 20 – 22
[24]				25 – 31 – 33

B **Do you need any extra formatted sheets to complete your list of occupations since 1950 to be provided during your face-to-face interview?**

◯ **Yes** ◯ **No**

36

Now we would like some specific information about any substances that you have been exposed to since 1950, in your *WORK, HOME OR ANY OTHER ENVIRONMENT*. Please tick the circle ✔ next to the answer which you believe gives an accurate indication of your situation, and where appropriate, write further details in the spaces provided.

Since 1950 have you EVER been exposed, either by breathing or direct skin contact, to any of the substances listed below? Please answer 'Yes' or 'No' to each substance that is listed. If you answer 'Yes' to a substance, try and remember when you were first and last exposed to that particular substance, and the total length of time that you were exposed to it (which should be recorded in months wherever possible, but otherwise specified – e.g. 10 days).

Example: *If you were exposed to Asbestos you would tick 'Yes'; then*

- *record the date you were first exposed – May 1976;*
- *record the date you were last exposed – Oct 1987;*
- *record the total length of time exposed during that entire period – 3 & a half months (which could represent more than one occasion when you were exposed to the substance during the stated period).*

Office Use Only

1 Asbestos?

○ Yes ○ No

First exposed:		Last exposed:		Length of time exposed:
month	year	month	year	in months, if possible

2 Radiation (*EXCLUDING OPERATION GRAPPLE*)?

○ Yes ○ No

First exposed:		Last exposed:		Length of time exposed:
month	year	month	year	in months, if possible

3 Coal products?

○ Yes ○ No

First exposed:		Last exposed:		Length of time exposed:
month	year	month	year	in months, if possible

A			3
			9
			15
R			18
			24
			30
C			33
			39
			45

Since 1950 have you EVER been exposed, either by breathing or direct skin contact, to any of the substances listed below? Please answer 'Yes' or 'No' to each substance that is listed. If you answer 'Yes' to a substance, try and remember when you were first and last exposed to that particular substance, and the total length of time that you were exposed to it (which should be recorded in months wherever possible, but otherwise specified – e.g. 10 days).

Office Use Only

D			48
			54
			60

4 Dust (such as wood or leather)?

○ Yes ○ No

First exposed: month / year **Last exposed:** month / year **Length of time exposed:** in months, if possible

H			3
			9
			15

5 Pesticides or herbicides?

○ Yes ○ No

First exposed: month / year **Last exposed:** month / year **Length of time exposed:** in months, if possible

P			18
			24
			30

6 Petroleum products?

○ Yes ○ No

First exposed: month / year **Last exposed:** month / year **Length of time exposed:** in months, if possible

D			33
			39
			45

7 Dyes?

○ Yes ○ No

First exposed: month / year **Last exposed:** month / year **Length of time exposed:** in months, if possible

S			48
			54
			60

8 Solvents?

○ Yes ○ No

First exposed: month / year **Last exposed:** month / year **Length of time exposed:** in months, if possible

9 **Since 1950 have you EVER been exposed, either by breathing or direct skin contact, to <u>ANY OTHER</u> chemicals/substances?**

Office Use Only

63

○ Yes *(please continue)*

○ No *(please go to PART 3 on page 16)*

If you answered 'Yes' to Q9 above can you think of the names of <u>ANY OTHER</u> specific chemicals/substances (other than the eight already identified on pages 12 & 13) which you know, or suspect that you were exposed to, by breathing or direct skin contact, in your *<u>WORK, HOME OR ANY OTHER ENVIRONMENT,</u>* since 1950? Please first write down the substance, and then the dates of your exposure to <u>that</u> substance, including the total length of time of your exposure, in the spaces provided below.

When you have completed recording your list of substances <u>please go to PART 3 on page 16</u>.

Office Use Only

10a Substance: _____

<u>First exposed:</u>		<u>Last exposed:</u>		<u>Length of time exposed:</u>
month	year	month	year	in months, if possible

3 9 15

10b Substance: _____

<u>First exposed:</u>		<u>Last exposed:</u>		<u>Length of time exposed:</u>
month	year	month	year	in months, if possible

18 24 30

10c Substance: _____

<u>First exposed:</u>		<u>Last exposed:</u>		<u>Length of time exposed:</u>
month	year	month	year	in months, if possible

33 39 45

10d Substance: _____

<u>First exposed:</u>		<u>Last exposed:</u>		<u>Length of time exposed:</u>
month	year	month	year	in months, if possible

48 54 60

If you need to continue recording more names of <u>OTHER</u> specific chemicals/substances (other than the eight already identified on pages 12 & 13) which you know, or suspect that you were exposed to, by breathing or direct skin contact, in your *<u>WORK, HOME OR ANY OTHER ENVIRONMENT,</u>* since 1950, space is provided below. Please first write down the substance, and then the dates of your exposure to <u>that</u> substance, including the total length of time of your exposure.

When you have completed recording your list of substances <u>please go to PART 3 on page 16</u>.

Office Use Only

10e **Substance:** _____

<u>First exposed</u>: month _____ year

<u>Last exposed</u>: month _____ year

<u>Length of time exposed</u>: _____ in months, if possible

3 9 15

10f **Substance:** _____

<u>First exposed</u>: month _____ year

<u>Last exposed</u>: month _____ year

<u>Length of time exposed</u>: _____ in months, if possible

18 24 30

10g **Substance:** _____

<u>First exposed</u>: month _____ year

<u>Last exposed</u>: month _____ year

<u>Length of time exposed</u>: _____ in months, if possible

33 39 45

10h **Substance:** _____

<u>First exposed</u>: month _____ year

<u>Last exposed</u>: month _____ year

<u>Length of time exposed</u>: _____ in months, if possible

48 54 60

Thank you for completing PART 2 of the survey.

PART 3 questions, which are related to your health, begin on the next page.

New Zealand Nuclear Test Veterans Postal Survey

PART 3

These questions focus on your health during the PAST 12 MONTHS. For each question, please tick the circle ✓ for the answer that best applies to you.

When you respond 'Yes' to a question you will be asked to list further details. Each question provides for a specific number of listed responses. If there is insufficient space to accommodate your entire list of responses, you will be able to indicate this at the end of PART 3. This will ensure that extra response sheets for those specific questions will be given to you for completion during your face-to-face interview.

Please do not skip any questions, and do take your time, as this will ensure that your responses are as complete as possible. If you wish to attach any extra notes of your own, you are welcome to do so.

Please note that "the PAST 12 MONTHS" is the period:

From: _____ ☐☐ **To:** _____ ☐☐

OR (recorded in month and year format)

From: ☐☐ ☐☐ **To:** ☐☐ ☐☐

month **year** **month** **year**

Office Use Only ☐☐ 24

You should ONLY refer to this time period whilst completing questions 1 to 6. To assist you remember these dates, they will be repeated at the top of each page.

1 Have you had any surgery during the past 12 months?

Office Use Only ☐☐☐ 7

◯ **Yes** *(please complete the list below, then go to Q2 on page 18)*

◯ **No** *(please go to Q2 on page 18)*

If you have had any surgery during the past 12 months, please list below, for EACH time that you had an operation, the reason for the surgery, and the date when you had it (recorded in month and year format).

	Surgery:	**Date:** (month / year)	
1a	**Reason:** _____	☐☐ ☐☐	☐☐☐ ☐☐☐ 13

Please note that "the PAST 12 MONTHS" is the period:

From: | | | | | **To:** | | | | |
month year month year

1 contd. ***If you need to list any further operations you had during the past 12 months, please continue to record the reason for each operation, and the date when you had it (specify the month & year). When you have completed entering your responses please go to Q2 on page 18.***

Surgery: **Date:** **Office Use Only**

1b Reason: _____ month year 19

_____

_____

1c Reason: _____ month year 25

_____

_____

1d Reason: _____ month year 31

_____

_____

1e Reason: _____ month year 37

_____

_____

1f Reason: _____ month year 43

_____

_____

1g Reason: _____ month year 49

_____

_____

1h Reason: _____ month year 55

_____

_____

1i Reason: _____ month year 61

_____

_____

Please note that "the PAST 12 MONTHS" is the period:

From: | | | | | **To:** | | | | |
month year month year

2 Have you had any fevers during the past 12 months?

◯ **Yes** *(please complete the list below, then go to Q3 on page 20)*

◯ **No** *(please go to Q3 on page 20)*

If you have had any fevers during the past 12 months, please list below, for EACH time that you had a fever:

- **the illness that was associated with that fever**
- **the date when you had the fever (recording the month & year each time)**
- **the medication you took for the fever**

2a Illness: _____

_____

Date: | | | | | (month year)

Medication: _____

_____

2b Illness: _____

_____

Date: | | | | | (month year)

Medication: _____

_____

2c Illness: _____

_____

Date: | | | | | (month year)

Medication: _____

_____

Office Use Only

3
6
10
13
16
20
23
26
30
33

Please note that "the PAST 12 MONTHS" is the period:

From: [ ][ ][ ][ ] (month year) **To:** [ ][ ][ ][ ] (month year)

2 ***Please continue to list any fevers you have had during the past 12 months.***
contd. ***When you have completed entering your responses please go to Q3 on page 20.***

Office Use Only

2d Illness: _____ 36

_____

Date: [ ][ ][ ][ ] (month year)

Medication: _____ 40 43

_____

2e Illness: _____ 46

_____

Date: [ ][ ][ ][ ] (month year)

Medication: _____ 50 53

_____

2f Illness: _____ 56

_____

Date: [ ][ ][ ][ ] (month year)

Medication: _____ 60 63

_____

2g Illness: _____ 3

_____

Date: [ ][ ][ ][ ] (month year)

Medication: _____ 7 10

_____

2h Illness: _____ 13

_____

Date: [ ][ ][ ][ ] (month year)

Medication: _____ 17 20

_____

These next questions are about MEDICATION you have taken over the PAST 12 MONTHS.

Please note that "the PAST 12 MONTHS" is the period:

From: | | | | | (month / year) **To:** | | | | | (month / year)

3 **Have you taken any medication prescribed by a doctor in the past 12 months (for example: blood pressure pills, antibiotics, insulin, tranquillisers, muscle relaxants, etc.)?**

Office Use Only

○ **Yes** *(please complete the list below, then go to Q4 on page 23)*

○ **No** *(please go to Q4 on page 23)* — 3

Please record below ANY PRESCRIBED MEDICATION taken during the PAST 12 MONTHS, & the REASON for taking it.

Type of prescription medication taken: **From date:** (month / year) **To date:** (month / year)

3a _____ | | | | | | | | — 9

_____

Reason: _____ — 15

3b _____ | | | | | | | | — 21

_____

Reason: _____ — 27

3c _____ | | | | | | | | — 33

_____

Reason: _____ — 39

3d _____ | | | | | | | | — 45

_____

Reason: _____ — 51

3e _____ | | | | | | | | — 57

_____

Reason: _____ — 63

Please note that "the PAST 12 MONTHS" is the period:

From: month year To: month year

3 contd. *Please continue to record below ANY PRESCRIBED MEDICATION that you have taken during the PAST 12 MONTHS, & the REASON for taking it. When you have completed entering your responses please go to Q4 on page 23.*

Office Use Only

3f Type of prescription medication taken: _____ From date: month year To date: month year — 69

Reason: _____ 75

3g Type of prescription medication taken: _____ From date: month year To date: month year — 3 6

Reason: _____ 12

3h Type of prescription medication taken: _____ From date: month year To date: month year — 18

Reason: _____ 24

3i Type of prescription medication taken: _____ From date: month year To date: month year — 30

Reason: _____ 36

3j Type of prescription medication taken: _____ From date: month year To date: month year — 42

Reason: _____ 48

3k Type of prescription medication taken: _____ From date: month year To date: month year — 54

Reason: _____ 60

3l Type of prescription medication taken: _____ From date: month year To date: month year — 66

Reason: _____ 72

Please note that "the PAST 12 MONTHS" is the period:

From: [ ][ ] [ ][ ] (month, year) **To:** [ ][ ] [ ][ ] (month, year)

3 *Please continue to record below ANY PRESCRIBED MEDICATION that you have taken during the PAST 12 MONTHS, & the REASON for taking it. When you have completed entering your responses please go to Q4 on page 23.*
contd.

Office Use Only

3m **Type of prescription medication taken:** ______ **From date:** month year **To date:** month year 3 6

Reason: ______ 12

3n **Type of prescription medication taken:** ______ **From date:** month year **To date:** month year 18

Reason: ______ 24

3o **Type of prescription medication taken:** ______ **From date:** month year **To date:** month year 30

Reason: ______ 36

3p **Type of prescription medication taken:** ______ **From date:** month year **To date:** month year 42

Reason: ______ 48

3q **Type of prescription medication taken:** ______ **From date:** month year **To date:** month year 54

Reason: ______ 60

3r **Type of prescription medication taken:** ______ **From date:** month year **To date:** month year 66

Reason: ______ 72

3s **Type of prescription medication taken:** ______ **From date:** month year **To date:** month year 3 6

Reason: ______ 12

Please note that "the PAST 12 MONTHS" is the period:

From: month year **To:** month year

4 **Have you taken <u>any non-prescription</u> medication in the past 12 months (for example: aspirin, antacid, anti-histamine, sedatives, or other drugs)?**

◯ **Yes** *(please complete the list below, <u>then</u> go to Q5 on page 25)*

◯ **No** *(please go to Q5 on page 25)*

Please record below <u>ANY NON-PRESCRIPTION MEDICATION</u> that you have taken during the <u>PAST 12 MONTHS</u> & the <u>REASON</u> for taking it.

Office Use Only 3

4a **<u>Type of non-prescription medication taken</u>:** _____ **<u>From date</u>:** month year **<u>To date</u>:** month year 6 9

Reason: _____ 15

4b **<u>Type of non-prescription medication taken</u>:** _____ **<u>From date</u>:** month year **<u>To date</u>:** month year 18 21

Reason: _____ 27

4c **<u>Type of non-prescription medication taken</u>:** _____ **<u>From date</u>:** month year **<u>To date</u>:** month year 30 33

Reason: _____ 39

4d **<u>Type of non-prescription medication taken</u>:** _____ **<u>From date</u>:** month year **<u>To date</u>:** month year 42 45

Reason: _____ 51

4e **<u>Type of non-prescription medication taken</u>:** _____ **<u>From date</u>:** month year **<u>To date</u>:** month year 54 57

Reason: _____ 63

4f **<u>Type of non-prescription medication taken</u>:** _____ **<u>From date</u>:** month year **<u>To date</u>:** month year 66 69

Reason: _____ 75

Please note that "the PAST 12 MONTHS" is the period:

From: | | | | | month | year | To: | | | | | month | year |

4 contd. ***Please continue to record below ANY NON-PRESCRIPTION MEDICATION that you have taken during the PAST 12 MONTHS & the REASON for taking it that you have not already listed on the previous page. When you have completed entering your responses please go to Q5 on page 25.***

Office Use Only

4g Type of non-prescription medication taken: _____ From date: month year To date: month year

_____ 6

_____ 12

Reason: _____

4h Type of non-prescription medication taken: _____ From date: month year To date: month year

_____ 18

_____ 24

Reason: _____

4i Type of non-prescription medication taken: _____ From date: month year To date: month year

_____ 30

_____ 36

Reason: _____

4j Type of non-prescription medication taken: _____ From date: month year To date: month year

_____ 42

_____ 48

Reason: _____

4k Type of non-prescription medication taken: _____ From date: month year To date: month year

_____ 54

_____ 60

Reason: _____

4l Type of non-prescription medication taken: _____ From date: month year To date: month year

_____ 66

_____ 72

Reason: _____

Please note that "the PAST 12 MONTHS" is the period:

From: month year **To:** month year

5 Do you currently take any vitamins or herbal remedies, or have you done so in the past 12 months?

Office Use Only 23

◯ **Yes** *(please complete the list below then go to Q6 on page 27)*

◯ **No** *(please go to Q6 on page 27)*

Please record below ANY VITAMINS / HERBAL REMEDIES that you have taken during the PAST 12 MONTHS & the REASON for taking it.

5a Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year 9

Reason: _____ 15

5b Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year 21

Reason: _____ 27

5c Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year 33

Reason: _____ 39

5d Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year 45

Reason: _____ 51

5e Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year 57

Reason: _____ 63

5f Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year 69

Reason: _____ 75

Please note that "the PAST 12 MONTHS" is the period:

From: [ ][ ][ ][ ] (month year) To: [ ][ ][ ][ ] (month year)

5 contd. *Please continue to record below <u>ANY VITAMINS / HERBAL REMEDIES</u> that you have taken during the <u>PAST 12 MONTHS</u> (including the <u>REASON</u> for taking them) that you have not already listed on the previous page. When you have completed entering your response please go to Q6 on page 27.*

Office Use Only

5g Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year [ ][ ][ ][ ] [ ][ ][ ][ ] — 6

Reason: _____ — 12

5h Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year [ ][ ][ ][ ] [ ][ ][ ][ ] — 18

Reason: _____ — 24

5i Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year [ ][ ][ ][ ] [ ][ ][ ][ ] — 30

Reason: _____ — 36

5j Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year [ ][ ][ ][ ] [ ][ ][ ][ ] — 42

Reason: _____ — 48

5k Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year [ ][ ][ ][ ] [ ][ ][ ][ ] — 54

Reason: _____ — 60

5l Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year [ ][ ][ ][ ] [ ][ ][ ][ ] — 6

Reason: _____ — 12

5m Type of vitamin/herbal remedy taken: _____ From date: month year To date: month year [ ][ ][ ][ ] [ ][ ][ ][ ] — 18

Reason: _____ — 24

Please note that "the PAST 12 MONTHS" is the period:

From: [ ][ ] [ ][ ] (month / year) **To:** [ ][ ] [ ][ ] (month / year)

6 Have you had a vaccination in the past 12 months?

○ **Yes** *(please complete the list below <u>then</u> go to Q7 on page 28)*

○ **No** *(please go to Q7 on page 28)*

Office Use Only 2

Please record below <u>ANY VACCINATION</u> that you have received in the <u>PAST 12 MONTHS</u>, and the <u>DATE</u> you had it.

<u>Type of vaccination</u>: **<u>Date</u>:**

6a ____________________ [ ][ ] [ ][ ] [ ][ ] (day / month / year) 5 11

<u>Type of vaccination</u>: **<u>Date</u>:**

6b ____________________ [ ][ ] [ ][ ] [ ][ ] (day / month / year) 14 20

<u>Type of vaccination</u>: **<u>Date</u>:**

6c ____________________ [ ][ ] [ ][ ] [ ][ ] (day / month / year) 23 29

<u>Type of vaccination</u>: **<u>Date</u>:**

6d ____________________ [ ][ ] [ ][ ] [ ][ ] (day / month / year) 32 38

<u>Type of vaccination</u>: **<u>Date</u>:**

6e ____________________ [ ][ ] [ ][ ] [ ][ ] (day / month / year) 41 47

7 **Have you ever had any DENTAL X-RAYS?**

◯ **Yes** *(please continue)*

◯ **No** *(please go to Q8 below)*

Did you have a dental X-ray ... ***(please tick ONE only)***

◯ **.. within the last month?**

◯ **.. within the last 6 months?**

◯ **.. within the last 6-12 months?**

◯ **.. over one year ago?**

8 ***(Please note the change in the time period you are asked to refer to in this question: Questions 1-6 all focused on "the past 12 months", but this question covers the period "since 1950".)***

Have you had any diagnostic or therapeutic X-rays (OTHER THAN DENTAL X-RAYS) since 1950?

◯ **Yes** *(please complete the list below then go to Q9 on page 31)*

◯ **No** *(please go to Q9 on page 31)*

Please record below the REASON FOR EACH X-RAY that you have had since 1950, the X-RAY SITE (e.g. chest), and the YEAR (e.g. 1972) that you had that X-Ray.

(Try to remember events in your life that required you to have an X-ray, and then link each event to a date.)

The reason for X-ray: **Year:**

8a **Reason:** _____ □□□□ year

X-Ray Site: _____

8b **Reason:** _____ □□□□ year

X-Ray Site: _____

8c **Reason:** _____ □□□□ year

X-Ray Site: _____

Office Use Only

48 □

49 □

52 □□□

55 □□□

59 □□

62 □□□

66 □□

69 □□□

73 □□

8 contd. *If you need to continue listing any <u>diagnostic or therapeutic X-rays</u> (OTHER THAN DENTAL X-RAYS) that you have had since 1950, please continue as before.*

(Try to remember events in your life that required you to have an X-ray, and then link each event to a date. For each X-ray please record the <u>REASON</u> for having the X-ray, the <u>X-RAY SITE</u>, and the <u>YEAR</u> when you had it. When you have completed your list please go to Q9 on page 31.)

The reason for X-ray: **Year:** **Office Use Only**

8d **Reason:** _____ year (3–7)

X-Ray Site: _____

8e **Reason:** _____ year (10–14)

X-Ray Site: _____

8f **Reason:** _____ year (17–21)

X-Ray Site: _____

8g **Reason:** _____ year (24–28)

X-Ray Site: _____

8h **Reason:** _____ year (31–35)

X-Ray Site: _____

8i **Reason:** _____ year (38–42)

X-Ray Site: _____

8j **Reason:** _____ year (45–49)

X-Ray Site: _____

8 contd. ***If you need to continue listing any diagnostic or therapeutic X-rays (OTHER THAN DENTAL X-RAYS) that you have had since 1950, please continue as before.***

(Try to remember events in your life that required you to have an X-ray, and then link each event to a date. For each X-ray please record the REASON for having the X-ray, the X-RAY SITE, and the YEAR when you had it. When you have completed your list please go to Q9 on page 31.)

The reason for X-ray: **Year:** **Office Use Only**

8k **Reason:** _____ year ____ 52

_____ ____ 56

X-Ray Site: _____

8l **Reason:** _____ year ____ 59

_____ ____ 63

X-Ray Site: _____

8m **Reason:** _____ year ____ 66

_____ ____ 70

X-Ray Site: _____

8n **Reason:** _____ year ____ 73

_____ ____ 77

X-Ray Site: _____

8o **Reason:** _____ year ____ 3

_____ ____ 7

X-Ray Site: _____

8p **Reason:** _____ year ____ 10

_____ ____ 14

X-Ray Site: _____

8q **Reason:** _____ year ____ 17

_____ ____ 21

X-Ray Site: _____

8 contd. ***If you need to continue listing any <u>diagnostic or therapeutic X-rays</u> (OTHER THAN DENTAL X-RAYS) that you have had since 1950, please continue as before.***

(Try to remember events in your life that required you to have an X-ray, and then link each event to a date. For each X-ray please record the <u>REASON</u> for having the X-ray, the <u>X-RAY SITE</u>, and the <u>YEAR</u> when you had it. When you have completed your list please go to Q9 on page 31.)

<u>The reason for X-ray</u>: **<u>Year</u>:**

8r **Reason:** _____ year

X-Ray Site: _____

8s **Reason:** _____ year

X-Ray Site: _____

8t **Reason:** _____ year

X-Ray Site: _____

9 Are you aware of any birth defects, or other genetic disorders, or inherited diseases that do / did affect ...

.. your parents? ◯ Yes ◯ No

.. your brothers &/or sisters? ◯ Yes ◯ No

.. children of your brothers &/or sisters? ◯ Yes ◯ No

If you responded 'No' to <u>ALL</u> of the above please go to Q10 on page 32. If you responded 'Yes' to <u>ANY</u> of the above please record brief details below.

_____

_____

_____

_____

_____

_____

Office Use Only

24 28 31 35 38 42 43 44 45 48 54

10 **Are you aware of any birth defects, or other genetic disorders, or inherited diseases that do / did affect ...**

Office Use Only

a) .. your <u>own</u> children? ◯ Yes ◯ No 55 56

b) .. your <u>own</u> grandchildren? ◯ Yes ◯ No

If you responded 'Yes' to (a) or (b) above, please record brief details below about the genetic health problems affecting <u>each</u> off-spring, including: whether that off-spring is your child/grandchild; their date of birth; their gender and the nature of their genetic disorder.

58 62 66 70 74 76

If you were unable to complete any of the PART 3 question response lists due to lack of space, and <u>you require extra formatted sheets</u> to complete during your face-to-face interview, please tick the SQUARE next to the relevant question/s below.

Q 1 – ANY SURGERY in the past 12 months ☐

Q 2 – ANY FEVERS in the past 12 months ☐

Q 3 - PRESCRIPTION MEDICATIONS in the past 12 months ☐

Q 4 - NON-PRESCRIPTION MEDICATIONS in the past 12 months ☐

Q 5 - VITAMINS/HERBAL REMEDIES in the past 12 months ☐

Q 6 - VACCINATIONS in the past 12 months ☐

Q 8 X-RAYS since 1950 ☐

Thank you for completing PART 3. Please ensure you have completed the response lists for <u>every</u> question, before continuing. If you have requested extra formatted sheets (as indicated above) these will be given to you at your face-to-face interview.

PART 4 starts on the next page. ☞

New Zealand Nuclear Test Veterans Postal Survey

PART 4

PART 4 focuses on your diet, starting off with questions about your current eating and drinking habits. For each question please tick the circle ✓ for the answer that best applies to you, or write the details in the spaces provided. If you are unsure about how to answer any question, please give the best answer you can.

Please avoid ticking more than one circle per question, unless asked to do so.

1 Do you eat vegetables?

○ Yes ○ No

2 Do you eat meat?

○ Yes ○ No

3 Do you use diet sweetners?

○ Yes ○ No *(please go to Q4 below)*

How many diet sweetners do you use per day OR per week?

(Please enter the number of diet sweetners in the space provided below, and ALSO indicate whether this is per day OR per week by circling the appropriate description.)

_____ diet sweetners **per day OR per week**

number ***(Circle one of these terms)***

4 Do you drink diet drinks?

○ Yes ○ No *(please go to Q5 on page 34)*

How many units (measured as 250ml, which is equivalent to an average size glass) per day OR per week?

(Please enter the number of 250 ml unit diet drinks in the space provided below, and ALSO indicate whether this is per day OR per week by circling the appropriate description.)

_____ 250ml diet drinks **per day OR per week**

number ***(Circle one of these terms)***

Office Use Only

1 2 3 6 7 10

5 **Are there any other comments that you would like to make regarding your diet that may not have been covered already? (e.g. do you follow a special diet such as low fat, high protein, low carbohydrate, etc.)**

Office Use Only

_____

_____

_____

_____

_____

_____ 13

_____

_____ 19

6 **Do you drink coffee?**

◯ Yes ◯ No *(please go to Q8 below)* 20

How many cups do you drink <u>per day</u> OR <u>per week</u>?

(Please enter the number of 250 ml cups of CAFFEINATED COFFEE in the space provided below, and ALSO indicate whether this is <u>per day</u> OR <u>per week</u> by circling the appropriate description.)

_____ 250ml caffeinated coffee **<u>per day</u> OR <u>per week</u>** 23

number ***(Circle one of these terms)***

7 **How often do you drink decaffeinated coffee?**

◯ 1	◯ 2	◯ 3	◯ 4	◯ 5
all of the time	most of the time	some of the time	a little of the time	none of the time

24

8 **Do you drink tea?**

◯ Yes ◯ No *(please go to page 35)* 25

How many cups do you drink <u>per day</u> OR <u>per week</u>?

(Please enter the number of 250 ml cups of TEA in the space provided below, and ALSO indicate whether this is <u>per day</u> OR <u>per week</u> by circling the appropriate description.)

_____ 250ml tea **<u>per day</u> OR <u>per week</u>** 28

number *(Circle one of these terms)*

The next group of questions are about alcohol consumption.

As a guide a drink is:

- **a can or small bottle of beer (a third of a pub jug)**
- **a small glass of wine**
- **a nip of spirits (a 'single' in a pub)**

For each question, please tick the circle ✓ for the answer that best applies to you. Please do not skip any questions.

Please tick ONLY ONE circle in response to each question.

Office Use Only

1 **Has a relative, or friend, or a doctor, or other health worker been concerned about your drinking, or suggested that you cut down?** 29

○ **No** ○ **Yes - but not in the last year** ○ **Yes - during the last year**

2 **Do you currently avoid drinking ALL alcohol because you have had difficulties in the past limiting the amount of alcohol that you drank?** 30

○ **No** *(please continue)* ○ **Yes** *(please go to Q10 on page 37)*

3 **Have you had a drink containing alcohol in the last year?** 31

○ **Yes** *(please continue)* ○ **No** *(please go to Q10 on page 37)* ○ **Don't know** *(please continue)*

4 **How often do you have a drink containing alcohol?** 32

○ **monthly or less** ○ **2 - 4 times a month** ○ **2 - 3 times a week** ○ **4 or more times a week**

5 **How many drinks containing alcohol do you have on a typical day, when drinking?** ***(Please tick ONLY ONE circle.)*** 33

○ **1 or 2 drinks** ○ **3 or 4 drinks** ○ **5 or 6 drinks** ○ **7 to 9 drinks** ○ **10 or more** ○ **don't know**

As a guide a drink is:

- **a can or small bottle of beer (a third of a pub jug)**
- **a small glass of wine**
- **a nip of spirits (a 'single' in a pub)**

For each question, please tick the circle ✓ for the answer that best applies to you. Please do not skip any questions.

Please tick ONLY ONE circle in response to each question.

6 **How often do you have six or more drinks on one occasion?**
(Please tick ONLY ONE circle.)

never	less than monthly	monthly	weekly	daily or almost daily
○	○	○	○	○

7 **How often during the last year have you found that you were not able to stop drinking once you had started?**

never	less than monthly	monthly	weekly	daily or almost daily
○	○	○	○	○

8 **How often during the last year have you failed to do what was normally expected from you because of drinking?**

never	less than monthly	monthly	weekly	daily or almost daily
○	○	○	○	○

9 **How often during the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session?**

never	less than monthly	monthly	weekly	daily or almost daily
○	○	○	○	○

Office Use Only

34

35

36

37

The following questions are about your smoking history. For each question, please tick the circle ✔ for the answer that best applies to you, or enter details in the space provided. Please do not skip any questions.

10a Do you currently smoke any substance other than tobacco?

○ Yes ○ No

10b Have you EVER smoked any substance other than tobacco?

○ Yes ○ No

11 Does anyone (including yourself) currently smoke tobacco products inside your home every day, or most days?

○ Yes ○ No

12 Do you currently smoke ANY tobacco products?

○ Yes *(please go to Q16 on page 38)*

○ No *(please continue)*

13 Have you ever been a smoker of ANY tobacco products in the past?

○ Yes *(please continue)*

○ No ***(please go to PART 5 on page 40)***

14 Were you ever a regular, daily smoker of tobacco products, before you stopped smoking?

○ Yes ○ No

15a What date did you last smoke? *(please specify month and year)*

Date: ☐☐ month ☐☐☐☐ year **OR** ○ Don't know

15b About how many years did you smoke tobacco products before you stopped? ***(Please specify below)***

_____ *years*

(If you currently DO NOT SMOKE ANY TOBACCO PRODUCTS please go to PART 5 on page 40)

Office Use Only

38 ☐

39 ☐

40 ☐

41 ☐

42 ☐

43 ☐

45 ☐☐

49 ☐☐

51 ☐☐

53

For each question, please tick the circle ✓ for the answer that best applies to you, or enter details in the space provided. Please do not skip any questions.

16 Do you currently smoke one or more tobacco cigarettes a day?

○ Yes *(please continue)* ○ No *(please go to Q19 below)*

17a *Please specify below the MONTH & YEAR you FIRST started smoking one or more cigarettes a day.*

month year

17b About how many years have you been smoking one or more cigarettes per day? *(please specify)* _____ *years*

17c Have you ALWAYS smoked one or more cigarettes per day from the date you specified above, right up until today's date?

○ Yes ○ No

18 About how many cigarettes do you smoke in an average day?

○ 1 to 10 a day? ○ 11 to 20 a day? ○ 21 to 30 a day? ○ 31 or more a day?

19 Do you currently smoke cigars?

○ Yes *(please continue)* ○ No *(please go to Q22 on page 39)*

20a *Please specify below the MONTH & YEAR you FIRST started smoking cigars.*

month year

20b About how many years have you been smoking cigars?

(please specify) _____ *years*

20c Have you ALWAYS smoked cigars from the date you specified above, right up until today's date?

○ Yes ○ No

Office Use Only

54 56 62 63 64 65 67 73 74

For each question, please tick the circle ✓ for the answer that best applies to you, or write details in the space provided. Please do not skip any questions.

21 **How often do you smoke cigars?**

○ only occasionally	○ 1 cigar in an average day?	○ 2 to 3 cigars in an average day?	○ 4 or more cigars in an average day?

22 **Do you currently smoke a pipe?**

○ **Yes** *(please continue)*

○ **No** ***(please go to the final instructions below)***

23a ***Please specify below the MONTH & YEAR you FIRST started smoking a pipe.***

month year

23b **About how many years have you been smoking a pipe?**

(please specify) _____ ***years***

23c **Have you ALWAYS smoked a pipe from the date you specified above, right up until today's date?**

○ **Yes** ○ **No**

24 **How often do you smoke a pipe?**

○ only occasionally	○ 1 pipe full in an average day?	○ 2 to 3 pipes full in an average day?	○ 4 or more pipes full in an average day?

Office Use Only

1 2 4 10 11 12

Please check that you have answered every question that applies to you in PART 4, before turning to PART 5.

New Zealand Nuclear Test Veterans Postal Survey

PART 5

The following questions focus on any long-term health problems that you may CURENTLY have.

Long-term health problems are more severe health problems that you have had for six months or more, or something that is likely to last for at least six months. Please tick the circle ✓ corresponding to the word 'Yes' OR 'No' to indicate if a doctor, nurse, or other health care worker has told you that you have any of the following long-term health problems. Please do not skip any questions.

	(Please tick ONE CIRCLE on each line.)	Yes	No	Office Use Only
1a	Cancer?	○	○	1
1b	If you **DO** suffer from cancer, what type/s of cancer? *(specify below)* _____			5
1c	Have you ever received radiation therapy **OR** chemotherapy to treat your cancer?	○	○	6
2	Diabetes?	○	○	
3	Epilepsy?	○	○	
4	High blood pressure or hypertension?	○	○	
5	Heart trouble e.g. angina or myocardial infarction?	○	○	10
7	Stroke?	○	○	
7	Asthma?	○	○	
8	Other respiratory conditions e.g. bronchitis?	○	○	13
9	Stomach ulcer or duodenal ulcer?	○	○	14
10	Chronic liver trouble e.g. cirrhosis?	○	○	
11	Bowel disorders e.g. colitis or polyps?	○	○	
12	Hernia or rupture?	○	○	
13	Chronic kidney or urinary tract conditions?	○	○	18

Long-term health problems are more severe health problems that you have had for six months or more, or something that is likely to last for at least six months. Please tick the circle ✓ corresponding to the word 'Yes' OR 'No' to indicate if a doctor, nurse or other health care worker has told you that you have any of the following long-term health problems. ***Please do not skip any questions.***

	(Please tick ONE CIRCLE on each line.)	Yes	No	Office Use Only
14	Chronic skin conditions e.g. dermatitis or psoriasis?	○	○	19
15	Arthritis or rheumatism?	○	○	20
16	Hepatitis?	○	○	
17	Sight impairment or loss?	○	○	
18	Hearing impairment or loss?	○	○	
19	Glandular fever (Infectious mononucleosis)?	○	○	24
20	Herpes?	○	○	
21	AIDS?	○	○	
22	Meningitis?	○	○	
23	Bacterial or viral infection?	○	○	28

24 **Do you currently have, OR have you ever had any <u>OTHER</u> MAJOR illness?** 30

○ **Yes** *(please continue)* ○ **No** *(please go to Q25 on page 43)* 31

Please record (in the space provided below) any <u>OTHER MAJOR ILLNESS</u>, stating <u>WHEN</u> you were ill (month & year to month & year), and the <u>TREATMENT</u> for that illness. Provision is made for you to make 7 entries in this booklet. ***If this is insufficient space, extra formatted sheets will be brought to your face-to-face interview. When you have finished recording your entries please go to Q25 on page 43.***

[24a] **Illness:** _____ **From:** ☐☐☐☐ 34

_____ **month** **year**

_____ **To:** ☐☐☐☐ 40

Treatment: _____ 46

_____

[24b] **Illness:** _____ **From:** ☐☐☐☐ 49

_____ **month** **year**

_____ **To:** ☐☐☐☐ 55

Treatment: _____

_____ 61

24 contd. *Please continue recording (in the space provided below) any <u>OTHER MAJOR ILLNESS</u> (not already listed in PART 5 Q1 to Q23), stating <u>WHEN</u> you were ill (month & year to month & year), and the <u>TREATMENT</u> for that illness.*

If there is insufficient space, extra formatted sheets will be brought to your face-to-face interview. When you have finished recording your entries please go to Q25 on page 43.

Office Use Only

[24c] **Illness:** _____ **From:** | | | | | (month / year)

_____ **To:** | | | | |

_____

Treatment: _____

_____ 64 70 76

[24d] **Illness:** _____ **From:** | | | | | (month / year)

_____ **To:** | | | | |

_____

Treatment: _____

_____ 3 9 15

[24e] **Illness:** _____ **From:** | | | | | (month / year)

_____ **To:** | | | | |

_____

Treatment: _____

_____ 18 24 30

[24f] **Illness:** _____ **From:** | | | | | (month / year)

_____ **To:** | | | | |

_____

Treatment: _____

_____ 33 39 45

[24g] **Illness:** _____ **From:** | | | | | (month / year)

_____ **To:** | | | | |

_____

Treatment: _____

_____ 48 54 60

24 contd. **Do you need any extra sheets at your face-to-face interview to record <u>more</u> entries for Q24?**

◯ **Yes** ◯ **No** 61

25 Please list <u>any other illness</u> (including cold and 'flu) that you have experienced in the <u>PAST 12 MONTHS</u>. Record the <u>ILLNESS</u>, <u>WHEN YOU WERE ILL</u> (month & year to month & year), and the <u>TREATMENT</u> for that illness. Provision is made for you to record 9 entries in this booklet.

If there is insufficient space, extra formatted sheets will be brought to your face-to-face interview. When you have completed recording your entries please go to PART 5 on page 45.

Please note that "the PAST 12 MONTHS" is the period

From: | | | | | **To:** | | | | |
month year month year

Office Use Only

[25a] **Illness:** _____ **From:** | | | | | 3
month year
_____ **To:** | | | | | 9
Treatment:
_____ 15

[25b] **Illness:** _____ **From:** | | | | | 18
month year
_____ **To:** | | | | | 24
Treatment:
_____ 30

[25c] **Illness:** _____ **From:** | | | | | 33
month year
_____ **To:** | | | | | 39
Treatment:
_____ 45

[25d] **Illness:** _____ **From:** | | | | | 48
month year
_____ **To:** | | | | | 54
Treatment:
_____ 60

[25e] **Illness:** _____ **From:** | | | | | 63
month year
_____ **To:** | | | | | 69
Treatment:
_____ 75

25 contd. *Please continue to record any other illness (including cold and 'flu) that you have experienced in the PAST 12 MONTHS. Record the ILLNESS, WHEN YOU WERE ILL (month & year to month & year), and the TREATMENT for that illness. Provision is made for you to make 9 entries in this booklet. If there is insufficient space, extra formatted sheets will be brought to your face-to-face interview.*

Please note that "the PAST 12 MONTHS" is the period

From: | | | | | (month / year) **To:** | | | | | (month / year)

Office Use Only

[25f] Illness: _____ **From:** | | | | | (month / year)

_____ **To:** | | | | |

_____

Treatment:

_____ 3 9 15

[25g] Illness: _____ **From:** | | | | | (month / year)

_____ **To:** | | | | |

_____

Treatment:

_____ 18 24 30

[25h] Illness: _____ **From:** | | | | | (month / year)

_____ **To:** | | | | |

_____

Treatment:

_____ 33 39 45

[25i] Illness: _____ **From:** | | | | | (month / year)

_____ **To:** | | | | |

_____

Treatment:

_____ 48 54 60

25 contd. **Do you need any extra sheets at your face-to-face interview to record more entries for Q25?**

◯ **Yes** ◯ **No** 63

Thank you for answering PART 5. Please ensure that you have answered every question, before turning to PART 6. ☞

PART 6

The following two sections have statements that relate to your memory in everyday life. In Section I the statements focus on your ability to remember specific types of information (for example, the name of the person just introduced to you).

I **The statements below give examples of using your memory in everyday situations. Please indicate your ability to remember the specific type of information by writing the appropriate number in the box alongside the statement, using the key provided below.**

Example: ***If you consider that your ability to remember your friends' telephone numbers is good, you should enter 4 (refer to the key below) in the box beside that statement; alternatively, if you think your memory to do this is poor, you should enter 2 (see the key below). Please do not leave out any statements.***

Use the following key for your responses:				
1 very poor	2 poor	3 fair	4 good	5 very good

My ability to remember...

		Office Use Only
1	.. the gifts I have received on special occasions (for instance Christmas or my birthdays) during the past several years is ...	1
2	.. the name of a person just introduced to me is ...	2
3	.. to turn out lights, turn off appliances, and lock doors when leaving home is ...	3
4	.. specific facts from a newspaper or magazine article I have just finished reading is ...	4
5	.. verbal directions to a place given minutes earlier is ...	5
6	.. the details of holidays or special occasions of my childhood is ...	6
7	.. telephone numbers or address codes that I use on a daily or weekly basis is ...	7

Section II statements focus on <u>how often</u> you have trouble remembering specific things (for example, forgetting what you intended to buy at a grocery store or a pharmacy).

II

The statements below list examples of difficulties using your memory in everyday situations. Please indicate HOW OFTEN you have trouble with your memory by writing the appropriate number in the box alongside each statement, using the key provided below.

Complete each statement below by selecting your response from the key provided. Simply write the number of that response in the box beside the statement. Please do <u>not</u> leave out any statements.

Use the following key for your responses:				
1 very often	2 often	3 some-times	4 rarely	5 very rarely

Complete these statements:

		Office Use Only
1	I have difficulty recalling a word I wish to use ...	19
2	I miss the point that someone else is making during a conversation ...	20
3	I go into a room to get something, and forget what I was after ...	
4	I have to stop and think when distinguishing right from left ...	22
5	I forget which waiter took my order in a restaurant ...	
6	I feel that a word or name I want to remember is 'on the tip of my tongue' but cannot recall it ...	24
7	I have difficulty following a conversation when there are distractions in the environment, such as noise from a TV or a radio ...	
8	I forget to bring up an important point in a conversation that I intended to mention ...	26
9	I dial a number and forget whom I was calling before the phone is answered ...	
10	I fail to recognise people who recognise me ...	28