Original Article

Cytogenetic and
Genome Research

Elevated sister chromatid exchange frequencies in New Zealand Vietnam War veterans

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Abstract. From July 1965 until November 1971, New Zealand Defence Force Personnel fought in the Vietnam War. During this time more than 76,500,000 litres of phenoxylic herbicides were sprayed over parts of Southern Vietnam and Laos, the most common being known as 'Agent Orange'. The current study aimed to ascertain whether or not New Zealand Vietnam War veterans show evidence of genetic disturbance arising as a consequence of their now confirmed exposure to these defoliants. A sample group of 24 New Zealand Vietnam War veterans and 23 control volunteers were compared using an SCE (sister chromatid exchange) analysis. The results from the SCE study show a highly significant difference ($P < 0.001$) between the mean of the experimental group (11.05) and the mean of a matched control group (8.18). The experimental group also has an exceptionally high proportion of HFCs (cells with high SCE frequencies) above the 95th percentile compared to the controls (11.0 and 0.07%, respectively).

We conclude that the New Zealand Vietnam War veterans studied here were exposed to a clastogenic substance(s) which continues to exert an observable genetic effect today, and suggest that this is attributable to their service in Vietnam.

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During the 1950s South East Asia was in severe political turmoil which resulted in New Zealand becoming embroiled in one of the most bitter wars of the last century outside of the two World Wars: the Vietnam War. New Zealand Defence Force Personnel were based in Vietnam from June 1964 through to November 1971. During the Vietnam War an estimated 76,540,964 litres of phenoxylic herbicides, including Agent Orange, were sprayed over approximately 3.6 million hectares of Vietnamese and Laotian land in order to remove forest cover, destroy crops and clear vegetation from around military installations (Stellman et al., 2003).

There is now firm evidence that New Zealand servicemen were exposed to large quantities of defoliant sprays during the Vietnam War (Chadwick, 2004), a milestone conclusion now accepted by the New Zealand Government. Furthermore, exposure to Agent Orange and other herbicides has been shown to lead to adverse health effects and cause genetic damage in humans (Hardell and Sandstrom, 1979; Eriksson et al., 1981; Schecter et al., 1995; Akhtar et al., 2004; Bukowska, 2004; Palmer, 2005; Duchnowicz et al., 2005).

The rationale for performing the SCE assay in the present study was based on a major report by the IPCS (International Programme on Chemical Safety) which concludes that a significant increase in SCE frequency is a 'sensitive indicator of DNA damage in blood lymphocytes of individuals exposed to genotoxic agents' (Albertini et al., 2000). The above statement endorses numerous studies where this assay has been used successfully as a bioindicator linking chemical exposure with possible genetic damage (Zober et al., 1993; Garaj-Vrhovac and Zeljezic, 2001; Arias, 2003;

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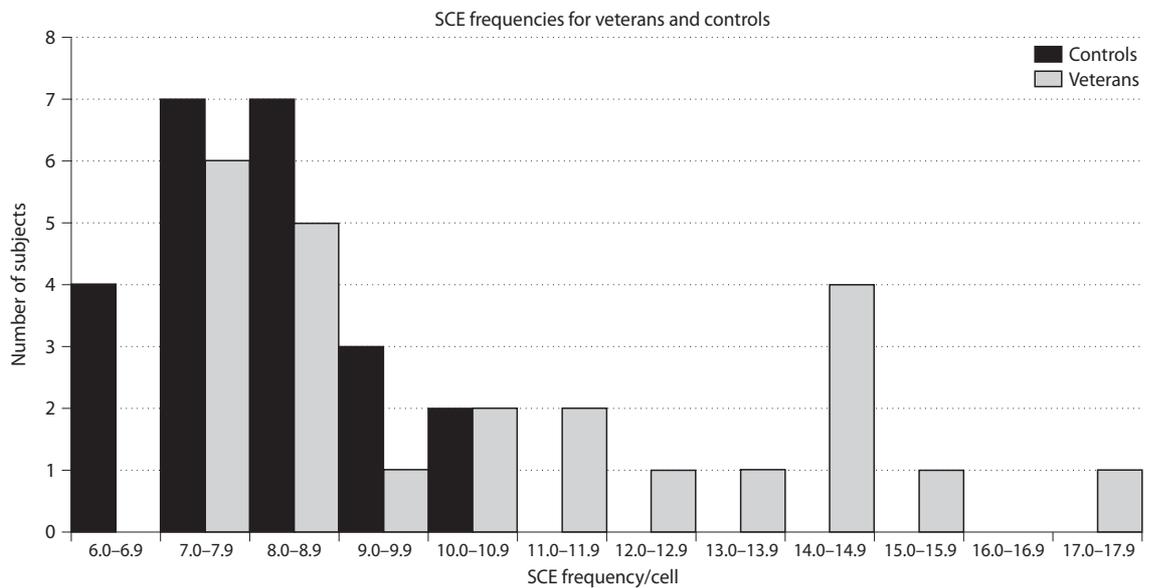e-mail: R.E.Rowland@massey.ac.nz

Fig. 1. Distributions of mean SCE counts/cell for the veterans (grey) and the controls (black). The highest count fell between 10 and 10.9 for the controls, whereas 12 of the 24 veterans had counts between 10 and 17.9.

Iannuzzi et al., 2004; Akin et al., 2005; Bhattacharya et al., 2005). The current study was thus performed in an attempt to determine whether New Zealand Vietnam War veterans have incurred any chromosomal disturbances as a consequence of their service in Vietnam.

Materials and methods

Selection of participants
Prospective participants were sent an information sheet, consent form and personal questionnaire (PQ). The PQ gathered extensive information about participants' past and present occupation, life events, lifestyle and general health. These data were essential for the selection of matched controls and veterans themselves, and to gather as much detail as possible on potential confounding factors. All controls were current or ex-army personnel to eliminate any 'healthy soldier' effect bias, with the important proviso that none served in Vietnam. Of the 50 final participants selected (25 veterans and 25 controls), two failed to return their questionnaire and one returned a questionnaire that was unusable. Thus, the following analyses are based on data from 24 veterans and 23 controls. Once the final participants had been selected, times were arranged for blood collection by an independent person. The mean age for the veterans group was 62.5 years, while the mean age for the controls group was 57 years.

Data collection methods
A 10-ml blood sample was obtained from each participant and coded with a unique numerical code to identify the individual without the researcher knowing the sample's original source (veteran or control). The researcher thus conducted the study blind. Decoding occurred when the final analysis was completed.

Lymphocyte culture and chromosome preparation
A lymphocyte count was taken using a Coulter JT Whole Blood Counter, to ensure that the number of lymphocytes in each culture tube was constant. Blood was added to previously prepared culture tubes to a final concentration of 2.96×10^6 lymphocytes per 6 ml culture tube.

0.01 M BrdU was then added to each culture tube, adjusted to give a final concentration of 20 μM. After approximately 72 h, 100 μl of 0.05% colchicine was added to each tube for 1 h prior to harvest. Chromosome preparation followed established procedures.

Sister chromatid exchange
The fluorescence-plus-Giemsa-staining protocol originally devised by Wolff and Perry (1975) was followed. 50 consecutive second mitotic metaphase cells that showed good chromosome morphology, the complete complement of 46 chromosomes, differentially-stained sister chromatids, no overlapping chromosomes and no indistinguishable sister chromatids, were selected from each participant. C-metaphases were viewed using an Olympus BX51 microscope and images captured using an Optronics MagnaFIRE SS99802 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.

In order to eliminate bias in cell selection, the scanning for metaphase spreads commenced at one end of the slide and progressed sequentially to the other side, with digital images of all identified metaphase spreads taken, until the required number was reached. If a metaphase spread did not meet the specified criteria, no further analysis was performed.

Results and discussion

2,500 metaphase spreads from dividing peripheral blood lymphocytes were located, photographed, and scored. Fifty spreads were analysed for each participant to give a mean SCE count/cell. The number of participants as a function of average SCE counts/cell is shown in Fig. 1. The distributions for veterans and controls reveal that 12 of the 24 veterans produced an equal or higher mean SCE count/cell than did the control participant with the highest count. This resulted in a highly skewed distribution for the veterans.
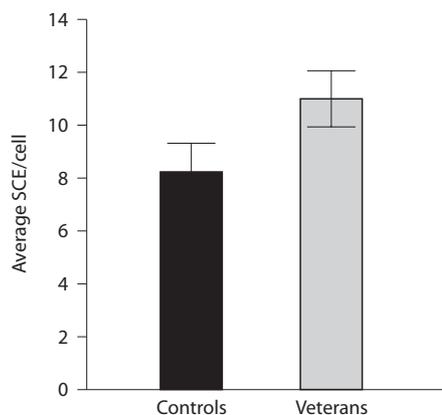
Fig. 2. Graphical representation of the mean differences between the average SCE counts/cell for veterans and controls. Error bars give the 95% confidence interval around the means.
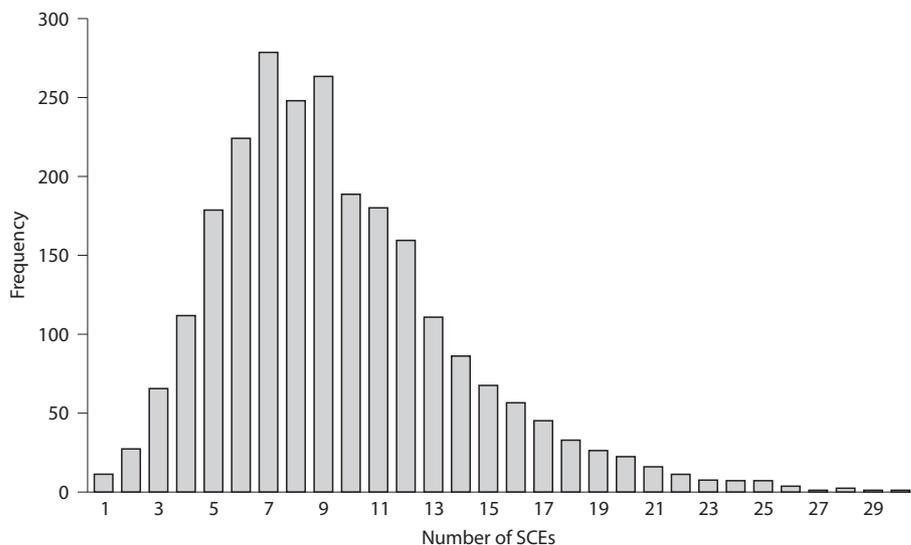

Fig. 3. The distribution shows the frequency of SCE counts for all cells assessed (n = 2,450) across both veterans and controls.

The average differences in SCE counts/cell for veterans and controls is shown graphically in Fig. 2. An independent samples *t*-test (equal variances not assumed) showed that there was a highly statistically significant difference between the mean SCE counts/cell for the veterans (10.99, SD = 3.05) compared with the controls (8.24, SD = 1.10), $t(45)$ = 4.07, $P < 0.001$. This mean difference represents a very large effect size of 1.27 (Cohen, 1988). A previous major report states that the baseline SCE frequency in human peripheral lymphocytes of nonexposed individuals averages 7–10 (Carrano and Natarajan, 1988).

It is possible that the observed SCE difference between veterans and controls was influenced by factors other than presumed exposure to toxic substances in the Vietnam War. Three of the factors most likely to have confounded the outcome of the preceding analysis are participant age, smoking rate, and alcohol consumption (Lazutka et al., 1992; Karaoguz et al., 2005).

Data for age, the number of cigarettes/cigars smoked per day, and the number of standard alcoholic drinks imbibed each week were controlled for using Analysis of Covariance (ANCOVA) (Tabachnik and Fidell, 2001). This analysis allowed for the mean SCE counts/cell to be adjusted for any effects of these three covariates.

Correlations among the three covariates were low, the highest being between rate of smoking and alcohol consumption, $r = 0.39$. Therefore, all three covariates were entered into the ANCOVA. The analysis revealed no statistically significant effect of any of the three covariates (all *F*s <1). Table 1 shows the mean SCE counts/cell for both veterans and controls adjusted for the aggregate effects of the three covariates.

Table 1. Adjusted mean SCE counts/cell following ANCOVA with the aggregate effects of age, smoking rate, and alcohol consumption controlled for in the analysis. Standard error 95% confidence intervals are nonoverlapping between veterans and controls.

	Mean	Standard error	95% Confidence interval	
			lower bound	upper bound
Veterans	11.05	0.53	9.99	12.12
Controls	8.18	0.54	7.08	9.27

These means, 11.05 for the veterans and 8.18 for the controls, barely differ from the unadjusted means of 10.99 and 8.24, respectively. It can be safely concluded that participant age, smoking rate, and level of alcohol consumption had no effect on the mean SCE counts/cell, either individually or in the aggregate.

Figure 3 shows the distribution of SCE counts across all cells assessed for both veterans and controls. Of particular interest is the ratio of veteran to control cells for the High Frequency SCE count Cells (HFCs). All cells with an SCE count >17 fall at or beyond the 95th percentile for the distribution shown in Fig. 3. A total of 141 HFCs fell into this part of the distribution. Interestingly, 132 of these (93.6%) were cells drawn from the veteran sample. Clearly, the veterans have fewer than expected low frequency SCE count cells (89.0%) and more than expected HFCs (11.0%), compared to 99.3 and 0.07%, respectively, for the controls $(\chi^2(1) = 110.65, P < 0.001)$.

A key question that warrants addressing is whether the observed difference can be attributed to an event which oc-

curred such a long time ago. It is an accepted paradigm that SCEs arise during the period of lymphocyte culture, which would suggest that the veterans have a persistent clastogen still in their bloodstream, or that they possess a common instability. Dioxin, a contaminant of Agent Orange, is known to be an inducer of SCEs (Zober et al., 1993). It is also very persistent and has an average half-life in humans of 7.78 years (Geyer et al., 2002), which suggests that New Zealand Vietnam veterans who were exposed to defoliants more than 30 years ago are still very likely to have elevated dioxin levels when compared to non-veterans. They have not been tested. As recently as 1995, dioxin blood levels were found to be between 25 and 170 times higher in people living in sprayed areas of Vietnam, compared to people living in unsprayed villages in Northern Vietnam (Palmer, 2005). Furthermore, a recently released report (Fowles et al., 2005) on current dioxin levels in residents who lived in the suburb of Paritutu in New Plymouth, New Zealand, during the 1960s, 70s and 80s, near an agricultural plant that manufactured ingredients identical to Agent Orange, records elevated dioxin levels in the residents with an approximate mean 7-fold higher than the expected norm. All told, a long-lived chemical such as dioxin may be a candidate for explaining the elevated SCE frequencies observed today in our Vietnam War veterans' sample.

The discovery of a highly significant difference in SCE frequency between the Vietnam War veterans and matched controls, together with an exceptionally high HFC count in the veterans, leads us to conclude that the sample of New Zealand Vietnam War veterans in the present study were exposed to a harmful clastogenic agent(s) as a result of service in Vietnam, which continues to exert an observable genetic effect today. Within the strictures of interpreting the biological significance of this particular assay (Albertini et al., 2000), there is evidence that these men may have incurred persistent genetic damage. The result strongly indicates that further research on possible adverse genetic consequences in Vietnam War veterans is required.

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