Micronutrients and oxidative stress in the aetiology of cancer

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Innumerable epidemiological studies have revealed what are, on the whole, rather weak associations between types of food and the incidence of cancer (Table 1). In a comprehensive review of cancer epidemiology, Doll & Peto (1981) estimated the contribution of nutritional factors to cancer incidence at 35%, as significant as the contribution from smoking. However, the nature of the links between diet and this disease remains obscure. Only in a very few cases is it possible to identify the causative agent; for example, aflatoxin (a fungal toxin, known to be mutagenic, that occurs in foods such as nuts and grain stored in tropical conditions) is detected at high levels in populations in Africa at risk of liver cancer, apparently acting synergistically with hepatitis B infection (Wild & Montesano, 1991). On the other hand, a high intake of β-carotene is associated with low incidence of various cancers (Gerster, 1993). In general, though, there is little scientific substance in the provision of information to the public concerning foods that are 'good' or 'bad' in terms of cancer risk.

The role of diet is complex. Food contains carcinogenic chemicals; Ames (1983) lists the natural components of food, mostly plant toxins, that have been shown to be mutagenic (i.e. positive in the Ames bacterial mutagenicity assay) or carcinogenic (i.e. cause cancer in laboratory animals). 'Almost everything natural we eat contains

Table 1. *Dietary factors and cancer*

Dietary factor	Site
Dietary factors associated with increased cancer risk	
Alcohol	Mouth, throat, larynx, oesophagus, liver (risk increases with smoking)
Red meat	Colon
Fat (especially animal fat)	Colon, pancreas, breast, ovary, endometrium, prostate (fish oil may be protective)
Energy intake, obesity	Breast, endometrium, colon, kidney, gallbladder, prostate
Salt	Stomach, oesophagus
Carcinogens (e.g. aflatoxin)	Liver (with hepatitis B)
Dietary factors thought to protect against cancer	
Vegetables, fruits Active components: fibre, starch, antioxidant vitamins (vitamin C, vitamin E, carotenoids), flavonoids?	Colon, stomach, oesophagus, lung, pancreas, breast, ovary, prostate
Se	Stomach, lung (in low Se countries)

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carcinogens, mutagens, teratogens, and clastogens' (Ames *et al.* 1990). We consume an estimated 1·5 g natural plant toxins daily, in addition to the pyrolysis products from cooking, and chemicals produced during smoking or other preservation methods, which are experimental mutagens. However, Ames & Gold (1990) caution against using the data from rodent experiments carried out at near-toxic doses of a chemical to estimate the cancer risk to humans exposed to very low doses, since the cell damage and induced cell proliferation that follow high-dose exposure to many chemicals themselves contribute to the carcinogenic process. It seems unlikely that geographical patterns of cancer incidence can be explained fully by differences in exposure to dietary carcinogens, since the plant toxins are so universally distributed. Rather than seeking the cause of cancer in food, perhaps we should be concentrating on the capacity of components of the diet to protect against carcinogenesis.

Fig. 1 is a highly simplified view of the process of carcinogenesis. Although preservation of the integrity of the genetic information is crucially important, DNA is not a particularly stable molecule (for review, see Lindahl, 1993). Spontaneous changes occur, and the DNA is prone to damage by environmental agents, in particular radiation and chemical mutagens. In addition, DNA damage results from attack by free radicals, or reactive O species, present in the cells as a byproduct of respiration. The ·OH radical is thought to be the most potent, and it is generated from peroxides with the help of transition metals (e.g. Fe^{2+}, Cu^{+}) which undergo redox cycling. The extent of oxidative damage is limited by the presence of free-radical scavengers, including the dietary antioxidants, vitamin C, vitamin E, carotenoids (and perhaps also flavonoids). Most damage is dealt with by cellular repair processes. Free-radical damage comprises strand breaks, which are rapidly rejoined, and oxidized bases, repaired more slowly. If damage remains unrepaired when cells come to replicate the DNA, it may be fixed as a permanent change, or mutation, in the daughter DNA. Mutations occurring (in a single cell) in a specific set of genes coding for proteins involved in regulation of cell growth may result in uncontrolled proliferation, i.e. cancer.

This is not the whole story. Certain chemicals act as tumour promoters; not themselves carcinogens, they promote the process of fixation of mutations, for example by stimulating cellular proliferation. The association of dietary fat with cancer may result in part from the conversion by gut bacteria of bile acids (produced from fats) to tumour-promoting substances.

A further point at which diet can influence carcinogenesis is in the metabolism of xenobiotics by cytochrome P450 and related enzymes. Many chemicals become carcinogens only after metabolic activation by this system. Classes of cancer-protective compounds found in cabbage and its relatives, and others in the onion family, are thought to operate via an inhibition of this processing of carcinogens. Equally, other chemicals may stimulate the activation.

EVIDENCE FOR ENDOGENOUS FREE-RADICAL ACTIVITY

The kinds of damage induced in DNA by free-radical attack have been characterized by experimental treatment of DNA or cells *in vitro* with ionizing radiation or agents such as H_2O_2. There is a great variety of oxidation products, over twenty derivatives of thymine alone (Téoule & Cadet, 1971). 8-OH-Guanine is a particularly abundant purine product, potentially important because of the alteration in base-pairing properties.

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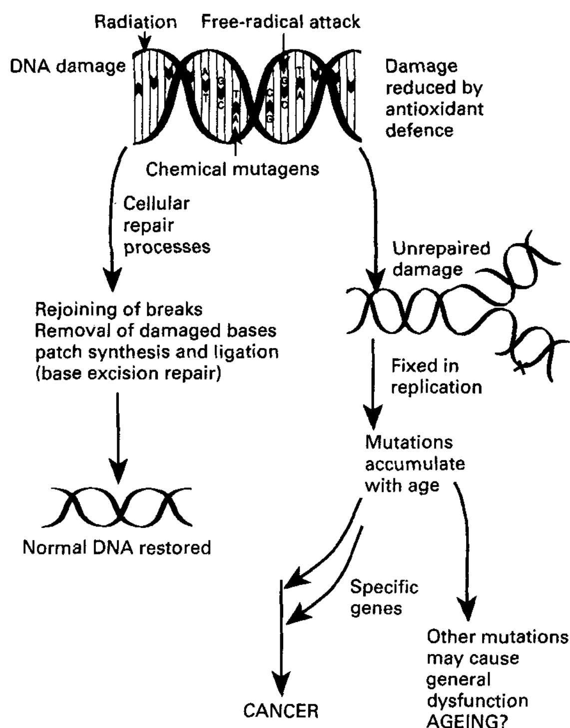

Fig. 1. Biological consequences of DNA damage.

The levels of DNA damage expected in cells within the organism as a result of normal exposure are much less than those in cells treated *in vitro* with DNA-damaging agents. Direct detection of such damage, against the overwhelming background of unaltered bases, is generally very difficult. In the case of oxidative damage, HPLC methods of high sensitivity, using electrochemical detection, have been developed, and various oxidation products have been measured in DNA from human cells. Kasai *et al.* (1991) report the presence of tens of thousands of 8-OH-guanine residues per cell in human lymphocytes; and similar levels of oxidized pyrimidines are claimed by Wagner *et al.* (1992). These findings have recently been questioned by Lindahl (1993). He suggests that since these lesions are potentially mutagenic and can be effectively repaired they would be unlikely to accumulate to such an extent in normal cells. Instead, the observed damage products may result from oxidation during isolation of the DNA with air-saturated buffers and solvents such as phenol–chloroform.

We have recently developed an alternative approach to measure oxidative base damage, using lesion-specific enzymes *in vitro* (Collins *et al.* 1993). The bacterial enzyme endonuclease III (*EC* 3.1.25.1) is specific for oxidized pyrimidines, making a break in the DNA at sites of damage. DNA breaks are readily measured; the method we use, single-cell gel electrophoresis (see legend to Fig. 2), is based on the fact that breaks cause relaxation of the supercoiled loops of DNA in the salt-extracted nucleus. Loops that are relaxed are free to be pulled towards the anode during electrophoresis, and they form the 'tail' of a 'comet'. Normal lymphocytes, not treated with any DNA-damaging agent *in vitro*, nevertheless contain significant numbers of endonuclease III-sensitive sites (Fig. 2)

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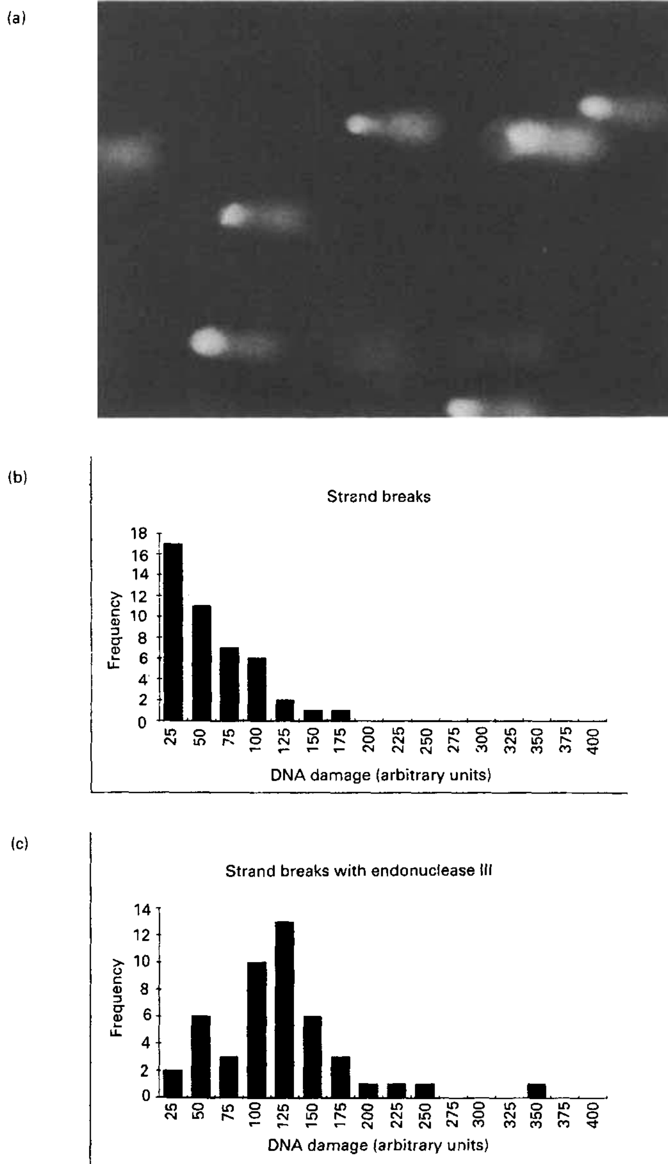

Fig. 2. DNA damage revealed by single-cell gel electrophoresis. (a) Typical 'comets' from HeLa human epithelial cells containing DNA breaks. Cells embedded in agarose are lysed in Triton X-100 and 2·5 M-NaCl to remove membranes, cytosol and almost all nuclear proteins, leaving the DNA as a compact, supercoiled mass. Breaks relax the supercoiling in DNA loops which are then free to extend towards the anode during alkaline electrophoresis, forming distinctive comet 'tails' seen here after staining with the fluorescent dye, DAPI. The percentage of DNA released into the tail reflects the extent of DNA damage. For more details, see Collins *et al.* (1993). (b, c) Combined results of single-cell gel electrophoresis analysis on lymphocytes of forty-five human volunteers (male non-smokers). (b) Indicates that most subjects have low levels of DNA strand breakage (assessed by estimating tail DNA content of 100 comets from each subject). (c) Illustrates the effect of digesting DNA, in-gel, with endonuclease III (*EC* 3.1.25.1) to reveal oxidized pyrimidines as breaks. The extra breaks result in more pronounced comet tails and the distribution of damage levels shifts to the right.

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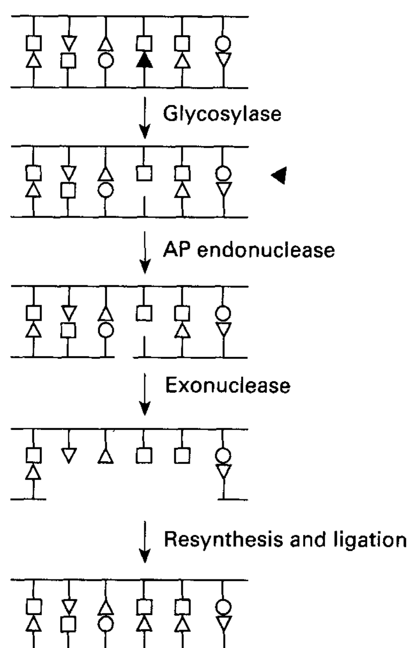

Fig. 3. Base excision repair. ▲, A damaged base (for example, 8-OH-guanine) in a DNA molecule.

estimated at several hundred per cell (Collins *et al.* 1993). In contrast, cultured human epithelial cells of the HeLa cell line reveal no enzyme-sensitive sites, perhaps reflecting the more efficient repair process in these cells. Lymphocytes contain very little by way of DNA precursors, since they are not engaged in proliferation, and the transient DNA breaks present during cellular base excision repair (Fig. 3) apparently remain open for a long time because of the slowness of the repair synthesis step.

Another kind of evidence for endogenous free-radical damage is the presence of products of DNA repair in urine. Ames and colleagues (Park *et al.* 1992) have detected 8-OH-guanine and the corresponding nucleosides, 8-OH-guanosine and 8-OH-deoxyguanosine in human and rat urine, using a monoclonal antibody affinity chromatography step to carry out a preliminary purification of 8-OH compounds, and then analysing them by HPLC. 8-OH-Guanine is the expected product of DNA repair (see Fig. 3); however, it could also come from the breakdown of RNA. 8-OH-Guanosine is unlikely to be released from DNA. 8-OH-Deoxyguanosine, on the other hand, should be specific to DNA, but there is no known DNA repair pathway that releases the oxidized nucleosides. It could originate as 8-OH-dGTP in the DNA precursor pool; a bacterial enzyme is known that breaks down the triphosphate (Maki & Sekiguchi, 1992). However, Lindahl (1993) suggests that 8-OH-deoxyguanosine in the urine merely represents degradation of DNA from dead cells by non-specific nucleases and phosphatases, with oxidation occurring during excretion in the kidney. If this is the case, the urinary meaurements may not be a reliable biomarker for oxidative stress.

A further complication was identified by Park *et al.* (1992). Feeding rats on nucleic acid-free chow greatly decreased the levels of 8-OH-guanine and 8-OH-guanosine in

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the urine (compared with normal chow). Presumably the same would hold for man; in other words, excreted 8-OH-guanine and 8-OH-guanosine largely reflect the presence of nucleic acids in food. 8-OH-deoxyguanosine excretion, however, is not altered by diet.

Assuming, for the moment, that the urinary measurements are reliable, there are about 10^4 oxidative hits/cell per d (Ames & Gold, 1990). Thus, our value of several hundred oxidized pyrimidines present at any time reflects a steady-state between input of damage and its repair, with a turnover for the average lesion of a few hours. The absence of detectable oxidized bases in HeLa cells indicates a lower rate of oxidation, or a very high turnover rate. It is the steady-state level of damage, rather than the rate at which damage occurs, that is significant as an indicator of the potential for mutagenesis.

THE SIGNIFICANCE OF DIETARY ANTIOXIDANTS; A MOLECULAR APPROACH TO EPIDEMIOLOGY

Once the involvement of free radicals in the aetiology of disease is established, it is, in principle, possible to reduce the incidence of the disease by decreasing exposure to oxidative damage. The so-called antioxidant hypothesis states:

(1) endogenous free radicals cause DNA damage which can lead to cancer (as well as lipid damage resulting in heart disease);

(2) antioxidants, such as vitamin C, vitamin E, carotenoids and flavonoids, scavenge free radicals before they have a chance to do damage;

(3) therefore, antioxidants in the diet should decrease the risk of cancer (and also of heart disease).

We have set out to test this hypothesis, in a human population, with an approach described as molecular epidemiology. Exposure to antioxidants is precisely controlled and monitored, and particular biomarkers that indicate the levels of oxidative damage are assayed at the molecular level. Dependence on biomarkers, rather than waiting for disease to develop, means that we can carry out our study using a small population group over a short period (Fig. 4). We have recruited 100 volunteers through the Health Centre in a town near Aberdeen; fifty male smokers and fifty male non-smokers aged 50–59 years. Baseline antioxidant levels were established by measuring vitamin C, uric acid, tocopherols, and various carotenoids in plasma; the volunteers then began a course of daily supplementation with vitamin C (100 mg), vitamin E (400 mg) and β-carotene (25 mg), or a placebo (half of each group). At 5-week intervals, estimation of antioxidant levels is repeated. At the same time, we are measuring glutathione peroxidase (*EC* 1.11.1.9) in plasma and erythrocytes), erythrocyte glutathione, conjugated dienes and lipid hydroperoxides (all relating to antioxidant status); oxidized low-density lipoprotein, triacylglycerol and cholesterol levels in lipoprotein fractions as biomarkers for heart disease risk; and, as indicators of DNA damage, the following:

(1) DNA breaks in lymphocytes, measured by single-cell gel electrophoresis;

(2) oxidized pyrimidines in lymphocytes, converted to breaks with endonuclease III and measured by single-cell gel electrophoresis;

(3) 8-OH-guanine, 8-OH-guanosine and 8-OH-deoxyguanosine in urine, as a measure (subject to the reservations expressed previously) of the repair of oxidative base damage;

(4) the frequency of occurrence of lymphocytes bearing a mutant form of the *hprt* gene.

Mutation is a stage further along the route from DNA damage to cancer. The *hprt*

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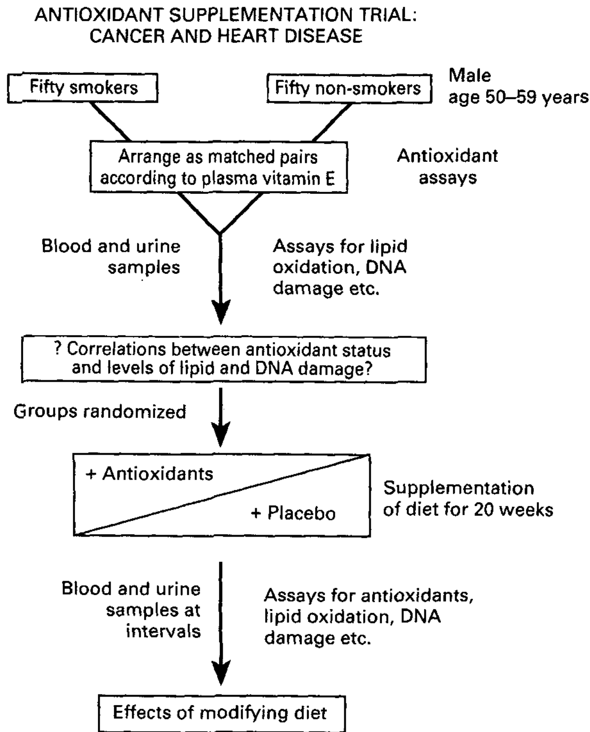

Fig. 4. Antioxidant supplementation trial in progress during 1993. The aim is to examine whether biomarkers of cancer risk (and also of heart disease risk) can be modulated by dietary administration of antioxidants. For details, see p. 72.

marker gene has no direct relevance to carcinogenesis but reflects the overall level of mutations including mutations in the cancer-related genes. Hypoxanthine phosphoribosyltransferase (*EC* 2.4.2.8; HPRT) is a salvage enzyme which recycles purines into the cell's biosynthetic pathway for nucleic acid precursors. It is not essential, so cells acquiring a mutant, inactive *hprt* gene survive; in populations of long-lived stem cells, such mutations accumulate. As the *hprt* gene is X-linked, a single inactivation is sufficient for expression of the mutant phenotype. The frequency of $hprt^-$ mutants is ascertained by plating out lymphocytes (in the presence of mitogen) with 6-thioguanine, a toxic purine analogue which is taken up by $hprt^+$ cells and kills them. The surviving $hprt^-$ cells develop into clones which are counted. The mutant frequency increases markedly with age, and is higher in smokers than non-smokers (Cole *et al.* 1991). If antioxidants have a protective role against DNA damage, we expect to find an elevated mutant frequency in individuals with low antioxidant levels.

Our antioxidant supplementation trial will be completed in late 1993.

CONCLUSIONS

The molecular epidemiological approach can be extended and applied to other areas of nutrition by identifying other biomarkers for disease risk and developing appropriate

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molecular assays. Free-radical damage is implicated in a variety of diseases, either as cause or effect.

In the field of cancer, we intend to refine the analysis of antioxidant effects by supplementing with individual antioxidants over a range of doses; we will also investigate other putative antioxidants, such as flavonoids, although there is a need for research at the cellular level to establish the mode of action of typical flavonoids before they can be employed in human trials.

As well as antioxidant protection, cellular repair of DNA damage is crucially important in controlling carcinogenesis. Whether the capacity for repair of oxidative damage varies between different tissues, or between individuals, and whether it is modulated (induced or suppressed) by dietary factors, are intriguing questions still to be addressed.

An exciting application of the comet assay is in '*ex vivo*' experiments. Lymphocytes, freshly isolated from the blood of subjects on different dietary regimens, can be tested for their response to a damaging agent (such as H_2O_2) administered *in vitro*, to gain information about the levels of protection and repair in relation to factors in the diet.

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