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Nutritional treatment of genome instability: a paradigm shift in disease prevention and in the setting of recommended dietary allowances

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The link between genome instability and adverse health outcomes during the various stages of life, such as infertility, fetal development and cancer, is briefly reviewed against a background of evidence indicating that genome instability, in the absence of overt exposure to genotoxins, is itself a sensitive marker of nutritional deficiency. The latter is illustrated with cross-sectional and dietary intervention data obtained using the micronucleus assay, an efficient biomarker for diagnosing genome instability and nutritional deficiency. The concept of recommended dietary allowances for genome stability and how this could be achieved is discussed together with the emerging field of nutritional genomics for genome stability. The review concludes with a vision for a disease-prevention strategy based on the diagnosis and nutritional treatment of genome instability, i.e. 'Genome Health Clinics'.

DNA damage: Genome stability: Micronutrients: Nutritional genomics: Recommended dietary allowances

Introduction

The central role of the genetic code in determining health outcomes such as developmental defects and degenerative diseases such as cancer is well established. In addition it is evident that DNA metabolism and repair is dependent on a wide variety of dietary factors that act as cofactors or substrates in these fundamental metabolic pathways (Ames, 2001; Fenech & Ferguson, 2001; Ames & Wakimoto, 2002). The DNA inherited from our parents is continuously under threat of major mutations from conception onwards by a variety of mechanisms, which include point mutation, base modification due to reactive molecules such as the hydroxyl radical, chromosome breakage and rearrangement, chromosome loss or gain, gene silencing due to inappropriate methylation of CpG at promoter sequences, activation of parasitic DNA expression due to reduced methylation of CpG as well as accelerated telomere shortening (Fenech & Ferguson, 2001). It is true to say that all of the above mechanisms of genome damage occur spontaneously due to the effects of endogenously generated mutagens or due to deficiency in cofactors required for DNA metabolism and repair. However, it is also true that genetic defects in DNA metabolism and repair, the latter involving more than 100 genes in man (Lindahl & Wood, 1999; Thompson & Schild, 2002), are also a key factor. While much has been learnt of the genes involved in DNA metabolism and repair and their role in a variety of pathologies, such as defects in BRCA1 that cause increased risk for breast cancer (Nathanson *et al*. 2001; Thompson & Schild, 2002), much less is known of the impact of cofactor and/or micronutrient deficiency on DNA repair. Put simply, a deficiency in a micronutrient required as a cofactor or as an integral part of the structure of a DNA repair gene (for example, Zn as a component of the DNA repair glycosylase OGG1 involved in removal of oxidised guanine or Mg as a cofactor for several DNA polymerases) could mimic the effect of a genetic polymorphism that reduces the activity of that enzyme (Ames, 2001, 2003). Therefore nutrition has a critical role in DNA metabolism and repair and this awareness is leading to the development of the new field of nutritional genomics of genome stability. The purpose of the present paper is to:

(a) briefly review the link between genome instability and adverse health outcomes during the various stages of life;

Abbreviations: BFB, breakage–fusion–bridge; BN, binucleated; CBMN, cytokinesis-block micronucleus; MN, micronucleus; MNi, micronuclei; MTHFR, methylene tetrahydrofolate reductase; NPB, nucleoplasmic bridge; RDA, recommended dietary allowances.

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(b) examine the evidence for genome instability as a marker of nutritional deficiency;
(c) explain the application of the micronucleus (MN) assay as an efficient biomarker for diagnosing genome instability and nutritional deficiency;
(d) introduce the concept of recommended dietary allowances (RDA) for genome stability and how this could be achieved;
(e) provide some insight into the importance of the emerging field of nutritional genomics for genome stability;
(f) describe, briefly, the framework for a disease-prevention strategy based on the diagnosis and nutritional treatment of genome instability.

The evidence linking genome damage with adverse health outcomes during the various stages of life

Genome damage impacts on all stages of life. There is good evidence to show that infertile couples exhibit a higher rate of genome damage than fertile couples (Trkova *et al*. 2000) when their chromosomal stability is measured in lymphocytes using the MN assay (Fenech, 2000). The infertility may be due to a reduced production of germ cells because genome damage effectively causes programmed cell death or apoptosis, which is one of the mechanisms by which grossly mutated cells are eliminated (Narula *et al*. 2002; Ng *et al*. 2002; Hsia *et al*. 2003). When the latter mechanism fails, reproductive cells with genomic abnormalities may survive leading to serious developmental defects (Liu *et al*. 2002; Vinson & Hales, 2002). There is ample evidence to show that most genotoxic carcinogens are also teratogens (O'Brien *et al*. 1996; Yu *et al*. 1999). Similarly, micronutrient deficiencies that cause genome damage may themselves cause developmental defects in the fetus or increased risk of cancer in the child. Specific examples include: (a) increased oxidation of sperm DNA in individuals with inadequate vitamin C intake (Fraga *et al*. 1991); (b) neural tube defects in folate-deficient fetuses at deficiency levels that coincide with increased genome damage rate (Fenech, 2001; Green, 2002); (c) the observation that Zn deficiency, which induces oxidative damage to DNA and impairs DNA repair, is itself teratogenic (Dreosti, 2001; Ho & Ames, 2003).

There is emerging evidence that genome damage or inappropriate gene expression experienced in the fetal stage of development may lead to abnormalities such as neural tube defects or a higher risk of childhood cancers such as leukaemia. The risk for both these conditions is increased when folate is limiting (Thompson *et al*. 2001; Brody *et al*. 2002). The risk of mutations is relatively high during early development and childhood because a much larger proportion of cells are in the DNA synthesis phase, which is more prone to insult by genotoxins or genome damage by micronutrient insufficiency. That an elevated rate of chromosomal damage is a cause of cancer has been proven by ongoing prospective cohort studies in Italy and the Scandinavian countries, which demonstrated a 2- to 3-fold increased risk of cancer in those whose chromosomal damage rate in lymphocytes was shown to be in the highest tertile when measured 10–20 years before cancer incidence was measured (Bonassi *et al*. 2000).

Chromosomal damage is also associated with accelerated ageing and neurodegenerative diseases. Several studies have shown that chromosomal abnormalities, including MN frequency, increase progressively with age in somatic cells (Bonassi *et al*. 2001). Accelerated ageing and cancer-prone syndromes, such as progeria, Bloom's syndrome, Fanconi's anaemia and Werner's syndrome, exhibit increased chromosomal instability and/or accelerated telomere shortening due to defects in a variety of genes essential for DNA repair and telomere maintenance such as ATM, poly(ADP-ribose) polymerase, BRCA1, BRCA2 and DNA helicases (Lansdorp, 2000; Joenje & Patel, 2001, Shen & Loeb, 2001; Thompson & Schild, 2002). Equally interesting is the observation that neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease exhibit much higher rates of MN frequency in peripheral blood lymphocytes (Migliore *et al*. 1999, 2001). In the case of Alzheimer's disease, there is also evidence that the frequency of cells exhibiting trisomy 21 is elevated, which leads to the hypothesis that these individuals may be mosaics for the Down's syndrome phenotype, which is associated with accelerated ageing and increased risk of neurodegenerative disease (Migliore *et al*. 1999).

Increased chromosomal DNA damage may be partly due to inefficient or incorrect DNA repair, which increases the sensitivity of an individual's cells to normal genotoxic stresses. A typical example is cells from individuals with truncation mutations in the BRCA1 and BRCA2 genes, which result in a highly penetrant condition for increased breast cancer risk (Venkitaraman, 2002). These genes are required for the error-free homologous recombinational repair of double-stranded breaks in DNA (Thompson & Schild, 2002). In the absence of normal function of these genes, non-homologous end-joining repair occurs, which leads to exchanges between chromosomes and the formation of abnormal chromosomal structures such as dicentric chromosomes and chromosome fragments (Venkitaraman, 2002). These abnormal chromosomes lead to a chromosomal instability phenotype because of the difficulty in segregating dicentric chromosomes equally between cells leading to the formation of the so-called breakage–fusion–bridge (BFB) cycles, which in turn lead to gene amplification and altered gene dosage (for a detailed account of these mechanisms, see Fenech, 2002). This genome instability phenotype, involving BFB cycles, is typical of most cancer cells (Saunders *et al*. 2000; Gisselson *et al*. 2001). The DNA repair deficiency phenotype is readily diagnosed using a radiation challenge to primary cells and scoring chromosomal damage events using a variety of classical cytogenetic methods that can detect anaphase or nucleoplasmic bridges (NPB) formation (Fenech, 2002). This phenotype of DNA misrepair is associated with elevated risk of a variety of cancers including head and neck cancers, colorectal cancer, breast cancer and myelodysplastic syndrome (Scott *et al*. 1998; Rothfus *et al*. 2000; Baria *et al*. 2002; Kuramoto *et al*. 2002).

Apart from faulty DNA repair, other defects in mechanisms involved in chromosome segregation may lead to abnormal chromosome number or aneuploidy, an event that is increasingly being considered as a potential cancer-initiating event and a definite cause of developmental abnor-

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malities. Important mechanisms include defects in assembly of the spindle, inadequate mitotic cycle checkpoints and abnormal replication of the centrosome, which coordinates the assembly of the spindle (Fenech, 2002). Duesberg and colleagues (Duesberg *et al*. 2000; Li *et al*. 2000) have suggested that induction of aneuploidy (abnormal number of chromosomes) either by a chemical agent or by other means such as a genetic abnormality in the mechanism of chromosome segregation or a defect in microtubule polymerization due to a deficiency in an essential cofactor such as Mg (Hartwig, 2001) may cause altered dosages of oncogenes and tumour suppressor genes. Duesberg's aneuploidy hypothesis of carcinogenesis also predicts that aneuploidy involving chromosomes containing genes that control the mitotic process leads to asymmetric segregation of chromosomes, initiating an autocatalytic karyotypic evolution generating pre-neoplastic and ultimately cancer cells. This hypothesis would explain why cancer cells are often aneuploid; however it would not necessarily explain the high frequency of chromosomal fragment translocations and gene amplification often seen in cancer, which may be better explained by the BFB cycle mechanism of hypermutation (explained later; p. 116). However, a potential unifying mechanism can be discerned from the recent observations that proteins involved in homologous recombination repair of DNA breaks such as XRCC2 and XRCC3 are found to be located at the centriole and that loss of function mutations in these genes leads to abnormal centrosome replication, multipolar mitoses and therefore aneuploidy (Griffin *et al*. 2000; Griffin, 2002). In addition, unequal segregation of dicentric chromosomes caused by asymmetric exchanges following misrepair of double-strand breaks may also lead to aneuploidy. The role of centrosome abnormalities in cancer first proposed by Boveri (1929) is now increasingly being confirmed for a variety of cancers such as prostate cancer in which the level of these abnormalities has been shown to accumulate with progression to a more malignant state (Pihan *et al*. 1998, 2001). Which micronutrients are required for proper centrosome replication and function remains unknown and uninvestigated.

The concept of genome damage as a marker of nutritional deficiency

There is overwhelming evidence that a large number of vitamins and minerals are required as cofactors for enzymes or as part of the structure of proteins (metalloenzymes) involved in DNA synthesis and repair, prevention of oxidative damage to DNA as well as maintenance methylation of DNA. The role of micronutrients in the maintenance of genome stability has recently been extensively reviewed (Fenech & Ferguson, 2001; Ames & Wakimoto, 2002). Examples of micronutrients involved in various genome stability processes are given in Table 1. The main point is that micronutrient deficiency can cause genome damage and as explained later (pp. 111–116) the increments in genome damage caused by micronutrient deficiency are of the same order of magnitude, if not greater, than the genome damage levels caused by exposure to significant doses of environmental genotoxins such as chemical carcinogens, u.v. radiation and ionising radiation. An example from our laboratory is the observation that the chromosomal damage rate caused by reducing folate concentration from 120 to 12 nmol/l is equivalent to that induced by an acute exposure to 0·2 Gy of low linear energy transfer ionising radiation (for example, X-rays), a dose that is approximately ten times greater than the annual allowed safety limit of exposure for the general population (Cruz Suarez *et al*. 2001) (Fig. 1). These results imply that genome damage biomarkers are not only biodosimeters of exposure to man-made or natural genotoxins, but are also biodosimeters of deficiency in micronutrients required: (a) for the prevention of oxidation to DNA (i.e. antioxidants); (b) for the prevention of uracil incorporation into DNA (i.e. folate); (c) for the maintenance methylation of CpG in DNA (methionine, choline, folate, B_{12}); (d) as cofactors or as components of DNA repair enzymes (for example, Zn, Mg); (e) for maintenance of telomere length (for example, niacin, folate). The sensitivity of DNA damage to micronutrient deficiency is underscored by the fact that the eight known human DNA repair glycosylases are dedicated to the removal of the type of DNA base damage that is produced when either antioxidant micronutrients (such as Zn, vitamins C and E) or folate, methionine and vitamin B_{12} are deficient (Table 2).

The micronucleus assay as a biomarker for diagnosing genome damage and nutritional deficiency

Micronuclei (MNi) originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division (Fig. 2) (Fenech *et al*. 1999; Fenech, 2002). The MN index in rodent and/or human cells has become one of the standard cytogenetic tests for genetic toxicology testing of chemicals and radiation *in vivo* or *ex vivo*. The MN assay can be practically measured

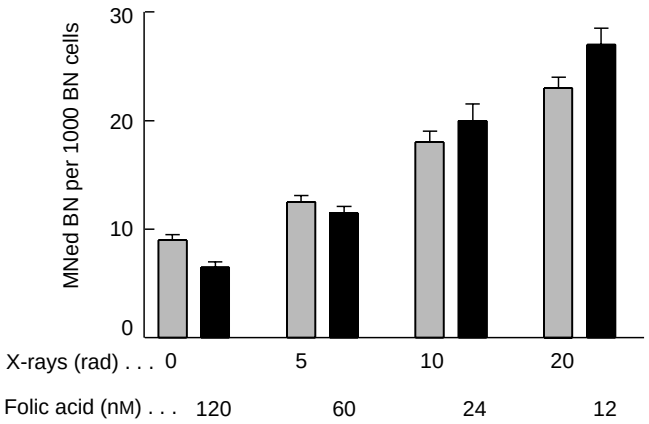

Fig. 1. A comparison of the dose–response effect on micronucleus induction in cytokinesis-blocked cultured lymphocytes caused by acute exposure to X-rays (■ ; *n* 6) up to a maximum dose of 20 rad (0·2Gy), equivalent to 100 times the annual exposure safety limit for the general public (Cruz Suarez *et al*. 2001), and folic acid deficiency within the 'normal' physiological range of 12–120 nM concentration (■; *n* 20). Results are mean values, with standard errors of the mean represented by vertical bars. MNed, micronucleated; BN, binucleated (data from Fenech & Morley, 1986; Crott *et al*. 2001*a*,*b*).

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Table 1. Examples of the role and the effect of deficiency of specific micronutrients on genomic stability*

Micronutrient/s	Role in genomic stability	Consequence of deficiency
Vitamin C, Vitamin E	Prevention of oxidation to DNA and lipid oxidation†	Increased base-line level of DNA strand breaks, chromosome breaks and oxidative DNA lesions and lipid peroxide adducts on DNA†
Folate and Vitamins B_2, B_6 and B_{12}	Maintenance methylation of DNA; synthesis of dTTP from dUMP and efficient recycling of folate‡	Uracil misincorporation in DNA, increased chromosome breaks and DNA hypomethylation‡
Niacin	Required as substrate for PARP which is involved in cleavage and rejoining of DNA and telomere length maintenance§	Increased level of unrepaired nicks in DNA, increased chromosome breaks and rearrangements, and sensitivity to mutagens§
Zn	Required as a cofactor for Cu/Zn superoxide dismutase, endonuclease IV, function of p53, Fapy glycosylase and in Zn finger proteins such as PARP ||	Increased DNA oxidation, DNA breaks and elevated chromosome damage rate||
Fe	Required as component of ribonucleotide reductase and mitochondrial cytochromes¶	Reduced DNA repair capacity and increased propensity for oxidative damage to mitochondrial DNA¶
Mg	Required as cofactor for a variety of DNA polymerases, in nucleotide excision repair, base excision repair and mismatch repair. Essential for microtubule polymerisation and chromosome segregation**	Reduced fidelity of DNA replication. Reduced DNA repair capacity. Chromosome segregation errors**
Mn	Required as a component of mitochondrial Mn superoxide dismutase††	Increased susceptibility to superoxide damage to mitochondrial DNA and reduced resistance to radiation-induced damage to nuclear DNA††

PARP, poly(ADP-ribose) polymerase; dTTP, deoxythymidine triphosphate; dUMP, deoxyuridine monophosphate.
*For information on other micronutrients (for example, carotenoids, vitamin D, polyphenols, Se and Cu), refer to other papers in Fenech & Ferguson (2001).
†Halliwell (2001); Claycombe & Meydani (2001).
‡Fenech (2001).
§Hageman & Stierum (2001).
||Dreosti (2001); Ho & Ames (2003).
¶Walter *et al*. (2002).
**Hartwig (2001).
†† Keen & Zidenberg-Cherr (1996); Ambrosone *et al*. (1999).

Table 2. DNA glycosylases in human cell nuclei (from Lindahl & Wood, 1999)

Enzyme	Altered base removed
UNG	U and 5-hydroxyuracil
TDG	U or T opposite G, ethenocytosine
hsMUG1	U (preferentially from SS DNA)
MBD4	U or T opposite G at CpG sequences
hOGG1	8-OxoG opposite C
MYH	A opposite 8-oxoG
hNTH1	Thymidine glycol, cytosine glycol, dihydrouracil, formamidopyridine
MPG	2-Me adenine, ethenoadenine, hypoxanthine

in erythrocytes, buccal cells or lymphocytes to obtain a measure of genome damage induced *in vivo*. MNi in erythrocytes originate from genome damage events in their precursors (normoblasts) in the bone marrow (for more detailed explanation, see Fig. 3). MNi in buccal cells originate from genome damage events in the basal layer of the oral mucosa (for more detailed explanation, see Fig. 4). In the case of lymphocytes it is possible to score MNi expressed *in vivo* directly (Fenech *et al*. 1999); however, a more comprehensive approach is to culture the lymphocytes because this allows a measure of genome damage that is accumulated while lymphocytes circulate around the body in the quiescent phase (Fig. 5). Lymphocytes have a half-life of 3–6 months and travel throughout the body, integrating genotoxic events across body tissues. In comparison, buccal cells and erythrocytes turn over every 21 and 120 d, respectively.

The earliest studies on the relationship between MN induction and micronutrient deficiency were performed using the erythrocyte MN assay. In fact MNi were first noted by haematologists and they are still referred to as Howell–Jolly bodies in recognition of the scientists who first described the relationship between megaloblastic anaemia and the prevalence of MNi in erythrocytes or in their immature stage, i.e. reticulocytes (Lessin & Bessis, 1972). This was the first evidence that folate and/or vitamin B_{12} deficiency induces chromosomal instability. It eventually became evident that the sensitivity to dietary deficiency and inter-individual variability of the erythrocyte MN assay could be improved if the subject being investigated happened to be splenectomised because, in man, the spleen removes micronucleated erythrocytes from circulation (Everson *et al*. 1988). Using splenectomised human subjects, it was shown that supplementation with folic acid and the vitamins A, C or E was associated with a lower MN frequency and that drinking more than five cups of coffee or tea per d and consumption of Ca supplements were associ-

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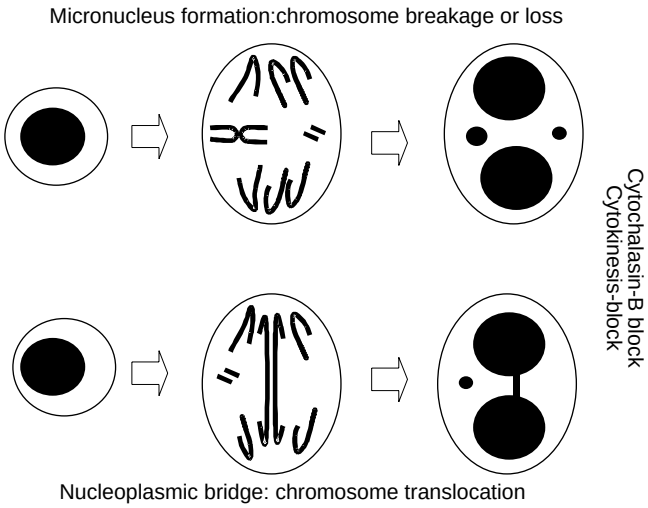

Fig. 2. Expression of micronuclei (MNi) and nucleoplasmic bridge (NPB) during nuclear division. MNi originate from either (a) lagging whole chromosomes (top panel) that are unable to engage with the mitotic spindle due to a defect in the spindle, or a defect in the centromere–kinetochore complex required to engage with the spindle or (b) an acentric chromosome fragment originating from a chromosome break (top and bottom panels) which lags behind at anaphase because it lacks a centromere–kinetochore complex. Misrepair of two chromosome breaks may lead to an asymmetrical chromosome rearrangement producing a dicentric (i.e. two centromeres) chromosome and an acentric fragment (bottom panel); frequently the centromeres of the dicentric chromosome are pulled to opposite poles of the cell at anaphase resulting in the formation of an NPB between the daughter nuclei. NPB are frequently accompanied by a micronucleus originating from the associated acentric chromosome fragment. Because MNi and NPB are only expressed in cells that have completed nuclear division it is necessary to score these genome instability biomarkers specifically in once-divided cells. This is readily accomplished by blocking cytokinesis using cytochalasin-B (for a more detailed explanation, see Fenech, 2002; Thomas *et al*. 2003).

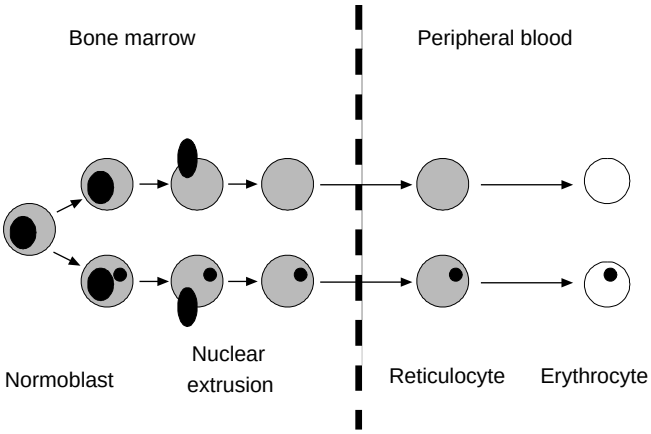

Fig. 3. Micronucleus formation in erythrocytes. Genome instability events in the normoblasts in the bone marrow lead to chromosome breakage or loss and micronucleus formation in the precursor cells of erythrocytes, the reticulocytes. During the maturation process to the reticulocyte stage, nuclei are excluded but micronuclei remain in the reticulocyte. The reticulocyte (with or without a micronucleus) eventually enters the peripheral blood and becomes a fully mature erythrocyte (with or without a micronucleus). Erythrocytes containing a micronucleus are eventually eliminated by the spleen; therefore the assay is best performed in individuals that have been splenectomized or by using a method that allows isolation of nascent reticulocytes identified by the presence of the transferrin receptor. The reticulocyte is distinguishable from the erythrocyte by its larger size and much higher RNA content. Micronucleus frequencies in reticulocytes and erythrocytes are usually recorded separately (for a more detailed explanation, see MacGregor *et al*. 1990; Abramsson-Zetterberg *et al*. 2000).

ated with an increase in MN frequency (Everson *et al*.1988; Smith *et al*. 1990). Using the same model in mice MacGregor and colleagues showed that MN induction by folate deficiency was aggravated by increased caffeine intake and that Mg deficiency doubled the MN frequency in fetal and maternal blood (MacGregor, 1990; MacGregor *et al*. 1990). Recently, a flow cytometric method for scoring MNi in nascent (immature) erythrocytes has been described, making this method practical for dietary studies in which small alterations in genome damage rate may be expected (Abramsson-Zetterberg *et al*. 2000). In the latter study it was observed that MN frequency in erythrocytes was significantly and negatively correlated with vitamin B_{12} status.

Although the buccal cell MN assay has been successfully applied to demonstrate elevated spontaneous genome damage rate in individuals with inherited genome instability syndromes such as Bloom's syndrome (Rosin & German, 1985; Honma *et al*. 2002) and in those exposed to chemical genotoxins (Kassie *et al*. 2001) or ionising radiation (Moore *et al*. 1996), much less is known about the impact of dietary deficiency on this index with only three studies reporting on the effect of diet. The first report of a dietary intervention using buccal mucosal cells was that of Stich *et al*. (1984) in which a β-carotene and retinol supplement given to betel-nut chewers was shown to decrease MN frequency by 66 %, while the unsupplemented group showed no change. The second by Piyathilake *et al*. (1995), a cross-sectional study on smokers and non-smokers, showed a three-fold increment in MN frequency in smokers who also had lower buccal mucosal folate and B_{12} when compared with non-smokers. The third by Titenko-Holland *et al*. (1998), a depletion–repletion study of nine post-menopausal women, showed a reduction in MN frequency in the buccal exfoliated cells after dietary supplementation with 516 μg folate/d. None of these studies made allowance for the possible effects of supplements on cell division kinetics, which may influence MN expression. For an extensive review of the application of the MN assay in buccal cells as well as other exfoliated cells (for example, cervical epithelium), refer to Majer *et al*. (2001).

The cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring MNi in cultured human lymphocytes because scoring is specifically restricted to once-divided cells. These cells are recognised by their binucleated (BN) appearance after inhibition of cytokinesis by cytochalasin-B (Fenech & Morley, 1986; Fenech, 2002). Restricting scoring of MNi in BN cells prevents confounding effects caused by altered cell division kinetics, which is a major variable in this *ex vivo* assay. Over the past 17

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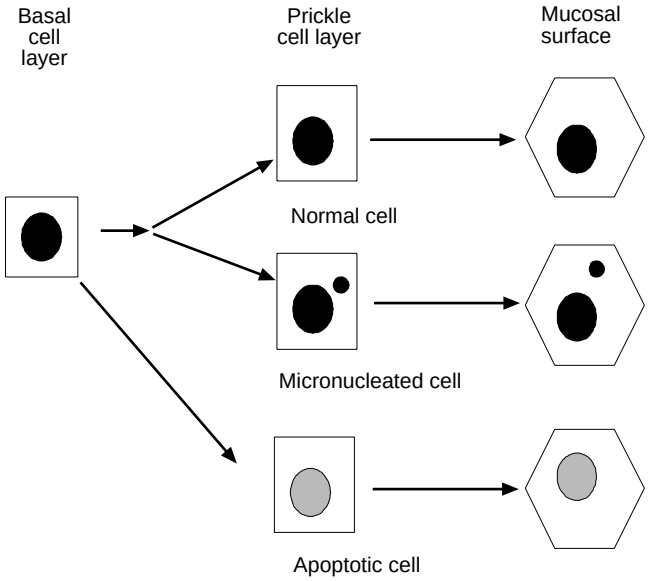

Fig. 4. Micronucleus (MN) formation in buccal cell mucosa. Genome instability in the basal cells leads to chromosome breakage or loss and MN formation. Some cells with genome damage may be eliminated via the apoptotic process. The daughter cells from the basal layer differentiate into 'prickle cells' which are eventually differentiated into the flattened and keratinised surface mucosal cells. Each of these cell types may contain an MN. The frequency of micronuclei observed may depend on the proportion of the various cell types scored (for a more detailed account of mucosal cell biology and micronucleus expression, see Rosin & German, 1985; Wertz & Squier, 1991; Titenko-Holland *et al*. 1998).

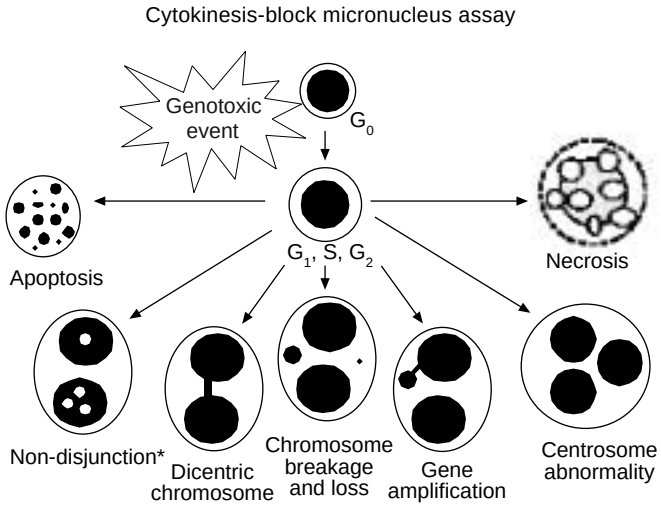

Fig. 5. The various possible fates of cultured cytokinesis-blocked lymphocytes following exposure to cytotoxic and genotoxic agents or micronutrient deficiency. Using these biomarkers within the cytokinesis-blocked micronucleus assay it is possible to measure the frequency of chromosome breakage (micronucleus), chromosome loss (micronucleus), chromosome rearrangement, for example, dicentric chromosomes (nucleoplasmic bridge), gene amplification (nuclear buds), necrosis and apoptosis. In addition, cytostatic effects are readily estimated from the ratio of mono-, bi- and multinucleated cells. Chromosome loss can be distinguished from chromosome breakage using pancentromeric probes or anti-kinetochore antibodies. In addition, non-disjunction (malsegregation of chromosomes) can also be measured in binuclear cells using chromosome-specific centromeric probes. In the example shown, non-disjunction results in one nucleus containing only one chromosome (i.e. one less than normal diploid state; monosomy) and the other nucleus containing three homologous chromosomes (i.e. one more than the normal diploid state; trisomy). Both monosomy and trisomy of specific chromosomes are associated with developmental defects and cancer risk (Fenech, 2002).

years the CBMN assay has evolved into a comprehensive method for measuring chromosome breakage, chromosome loss, non-disjunction, gene amplification, necrosis, apoptosis and cytostasis (Fig. 5).

The results of cross-sectional studies of vegetarians and non-vegetarians (Fenech & Rinaldi, 1995), older men (Fenech *et al*. 1997*b*) and young adults (Fenech *et al*. 1998; Odagiri & Uchida, 1998) indicated that MN frequency in lymphocytes was negatively correlated with plasma folate and vitamin B_{12}, positively correlated with homocysteine and vitamin C and unrelated to vitamin E status. Placebo-controlled dietary interventions have shown that supplementation with 700 μg folic acid and 2·5 μg vitamin B_{12} (Fenech *et al*. 1998) reduced MN frequency in lymphocytes by 25 %, which is in accordance with the predictions from the cross-sectional data (Fenech & Rinaldi, 1995). Supplementation with vitamin E (Fenech *et al*. 1997*a*) or vitamin C (Crott & Fenech, 1999) did not decrease MN frequency in lymphocytes. A depletion–repletion study in nine post-menopausal women showed a decrease in lymphocyte MN frequency following repletion with 516 μg folic acid/d (Titenko-Holland *et al*. 1998). Daily intake over a 4-month period of a multivitamin, containing 15 mg β-carotene, 75 mg rutin, 3 mg retinyl acetate, 30 mg α-tocopherol, 150 mg ascorbic acid and 0·2 mg folic acid, resulted in a significant reduction of baseline MN frequency in lymphocytes from older subjects (56–80 years) and increased the resistance of lymphocytes to radiation-induced MNi in both younger and older adults (Gaziev *et al*. 1996).

More recently it has been proposed that NPB between nuclei in BN cells should also be scored in the CBMN assay because they provide a measure of chromosome rearrangement, which is otherwise not measured in this assay if only MNi are scored (Thomas *et al*. 2003). The NPB are assumed to occur when the centromeres of dicentric (abnormal, rearranged) chromosomes are pulled to opposite poles of the cell at anaphase. It is rarely possible to observe dicentric anaphase bridges before the nuclear membrane is formed, because cells proceed through anaphase and telophase rapidly, completing cytokinesis and ultimately breakage of the NPB when the daughter cells separate. However in the CBMN assay, BN cells with NPB are allowed to accumulate because cytokinesis is inhibited and the nuclear membrane is eventually formed around the chromosomes allowing an NPB to be observed.

Over the past decade another unique mechanism of MN formation, known as nuclear budding, has emerged. This process has been observed in cultures grown under strong selective conditions (Toledo *et al*. 1992; Ma *et al*. 1993; Shimura *et al*. 1999) that induce gene amplification. Shimizu *et al*. (1998, 2000) showed that amplified DNA is selectively localised to specific sites at the periphery of the

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nucleus and eliminated via nuclear budding to form MNi during the S phase of mitosis. Furthermore, DNA synthesis inhibitors such as hydroxyurea were shown to increase the rate of elimination of amplified DNA via this process. Amplified DNA may be eliminated through recombination between homologous regions within amplified sequences forming minicircles of acentric and atelomeric DNA (double minutes), which localise to distinct regions within the nucleus or through the excision of amplified sequences after segregation to distinct regions of the nucleus. This suggests that the nucleus may have a capacity to sense excess DNA that does not fit well within the nuclear matrix indicating a higher-order DNA repair or nuclear housekeeping process. Shimizu *et al*. (1998, 2000) have suggested that the nucleus eliminates excess amplified DNA by an active process that concentrates the amplified DNA to a peripheral point in the nucleus, following which this surplus DNA is budded out to form a MN and eventually excluded from the cell altogether by extrusion of the MN from the cytoplasm leading to the formation of a 'minicell'.

In a recent study on folic acid deficiency in long-term primary human lymphocyte cultures the inter-relationship between MNi, NPB and nuclear buds was carefully quantified in an attempt to validate the use of these biomarkers and to determine more comprehensively the impact of folic acid deficiency on various aspects of genomic stability (Crott *et al*. 2001*a*,*b*). Briefly, lymphocytes from twenty (eight male, twelve female) asymptomatic volunteers (34–65 years) were cultured in duplicate in RPMI–1640 medium containing 5 % dialysed fetal calf serum, interleukin-2 (10 U/ml) and either 12, 24, 60 or 120 nM-folic acid for 9 d. The medium was refreshed at days 3 and 6, cytokinesis was inhibited on day 8 and cells were transferred to microscope slides 24 h later. The pooled data from this study verified that folic acid concentration correlated significantly ($P < 0{\cdot}0001$) and negatively (r –0·63 to –0·74) with uracil in DNA and micronucleated cells, which were minimised at 60–120 nM-folic acid, the latter being greater than the concentration of folate normally observed in plasma (10–30 nM) (Fig. 6). However, even more interestingly, it was observed that the frequency of NPB and nuclear buds correlated significantly and negatively with folic acid dose, suggesting that chromosome rearrangement and gene amplification are induced by folic acid deficiency. The strong cross-correlation between MN, NPB and nuclear bud frequency (r 0·75 to 0·77, $P < 0{\cdot}001$) suggests a common mechanism initiated by folic acid deficiency-induced DNA breaks, although coincidence of effects with other DNA damage events (for example, folic acid deficiency-induced CpG hypomethylation) cannot be excluded.

Because folic acid deficiency is known to cause gene amplification and chromosome damage such as double-stranded breaks (Blount & Ames, 1995; Blount *et al*. 1997; Melnyk *et al*. 1999; Fenech, 2001), it is probable that these

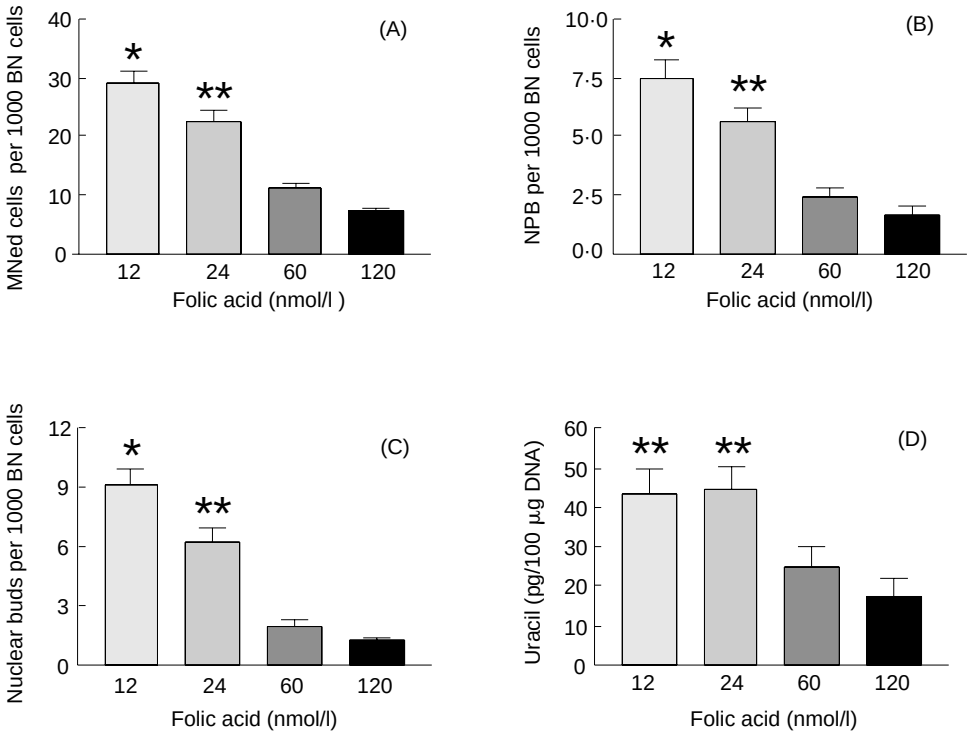

Fig. 6. The effect of medium folic acid concentration on the induction of (A), micronucleated binucleated (MNed BN) cells; (B), nucleoplasmic bridges (NPB); (C), nuclear buds; (D), uracil in primary human lymphocytes *in vitro*. Values are means, with standard errors of the mean represented by vertical bars for twenty human subjects. Results from ANOVA were $P < 0{\cdot}0001$ for (A)–(C) and $P < 0{\cdot}001$ for (D). * Mean value was significantly different from those for 24, 60 and 120 nM ($P < 0{\cdot}01$); ** mean value was significantly different from those for 60 and 120 nM ($P < 0{\cdot}01$) (Tukey's *post-hoc* test). Data from Crott *et al*. (2001*a*,*b*).

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events contribute to the formation of MNi, NPB and nuclear buds in this system. Gene amplification is thought to be a key event in cellular resistance to drugs like methotrexate (Biedler & Spengler, 1976) and in tumour progression (Brison, 1993). Several plausible models of gene amplification have been proposed (Windle & Wahl, 1992; Stark, 1993); however, the presence of NPB in the cytokinesis-blocked cells provides support for the BFB cycle model described in the seminal work of McClintock (1942) in maize. According to this theory, sister chromatids, which have both undergone double-stranded breakage, fuse at a distal position (possibly telomeric) forming a dicentric chromosome that has two copies of homologous genes positioned between the two centromeres. During anaphase these dicentric chromosomes are drawn towards both poles and form (nucleoplasmic) bridges. During cytokinesis these dicentric chromosomes, which span both daughter nuclei, are thought to break unevenly and may form a chromosome with two copies of one or more genes and a chromosome (fragment) with no copies of these genes. The chromatids with multiple copy number of these genes may fuse again during interphase forming a dicentric chromosome (doubling again the gene copy number within the chromosome), which is then replicated during the next nuclear division leading to the next BFB cycle and further gene amplification.

Folic acid deficiency-induced fragile site expression and DNA hypomethylation may have also contributed to the promotion of gene amplification and resulted in elimination of this DNA by nuclear budding in our system. For example, amplification of the multi-drug resistance 1 gene in Chinese hamster cells occurs through the induction of fragile sites that determine the initiation and size of amplicons (Coquelle *et al*. 1997), and the induction of hypomethylation by 5-aza-2′deoxycytidine has been reported to enhance *N*-(phosphonylacetyl)-L-aspartate-induced amplification of the CAD gene in Syrian hamster kidney cells (Perry *et al*. 1992).

In summary, the genomic instability phenotype can be readily recognised simply by examining cells for abnormal nuclear morphology indicative of BFB cycles, i.e. MNi, NPB and nuclear blebs. In addition, genomic instability can also be manifested by a high rate of aneuploidy and multipolar mitoses, which are detectable by fluorescence *in situ* hybridisation with chromosome-specific centromere probes or cytologically, respectively. One of the better methods for measuring and observing BFB cycles and non-disjunction and chromosome loss is the CBMN assay. The results with folic acid show quite clearly that micronutrient deficiencies can on their own cause the type of genomic instability observed in cancer. In fact these observations: (a) have provided further impetus for the concept that the RDA should be based on the prevention of genomic instability; (b) highlight the potential of micronutrient concentration as an important modifier not only of spontaneous chromosome abnormality but also chemically induced genome damage. These points have important implications in the relative risk assessment of chemical and radiation exposure depending on micronutrient status.

The concept of recommended dietary allowances for genome stability

Current RDA for vitamins and minerals are based largely on the prevention of diseases of deficiency such as scurvy in the case of vitamin C, anaemia in the case of folic acid and pellagra in the case of niacin. However, these diseases of deficiency are rare in the developed world but degenerative disease and developmental disease are very important. Recently the dietary allowance for folic acid for the prevention of neural tube defects has been revised to more than double the original RDA (Centers for Disease Control, 1992). There is a strong international awareness that it is also necessary to redefine RDA for the prevention of degenerative disease (such as cancer, cardiovascular disease and Alzheimer's disease) and compression of the morbidity phase during old age. Because diseases of development, degenerative disease and ageing itself are partly caused by damage to DNA (Holliday, 1995; Ames, 1998, 1999) it seems logical that our attention would be better focused on defining optimal requirements of key minerals and vitamins for preventing damage to both nuclear and mitochondrial DNA. To date our knowledge on optimal micronutrient levels for genomic stability is scanty and disorganised.

Both *in vitro* and *in vivo* studies with human cells clearly show that folate deficiency, vitamin B_{12} deficiency and elevated plasma homocysteine are associated with expression of chromosomal fragile sites, chromosome breaks, excessive uracil in DNA, MN formation and DNA hypomethylation (Jacky *et al*. 1983; Everson *et al*. 1988; Cravo *et al*. 1994; Blount & Ames, 1995; Blount *et al*. 1997; Duthie & Hawdon, 1998; Fenech *et al*. 1998; Jacob *et al*. 1998; Titenko-Holland *et al*. 1998; Crott *et al*. 2001*a*,*b*). *In vitro* experiments indicate that DNA breaks in human cells are minimised when folic acid concentration in culture medium is greater than 180 nmol/l (80 ng/ml) (Jacky *et al*. 1983; Duthie & Hawdon, 1998). Recently it has been shown that uracil incorporation in human lymphocytes cultured for 8 d is minimised at a folic acid concentration of 120 nmol/l (Crott *et al*. 2001*a*,*b*). Intervention studies in human subjects taking folate and/or vitamin B_{12} supplements show that DNA hypomethylation, chromosome breaks, uracil misincorporation and MN formation are minimised when the plasma concentration of vitamin B_{12} is greater than 300 pmol/l, plasma folate concentration is greater than 34 nmol/l, erythrocyte folate concentration is greater than 700 nmol folate/l and plasma homocysteine is less than 7·5 μmol/l (Everson *et al*. 1988; Cravo *et al*. 1994; Blount & Ames, 1995; Blount *et al*. 1997; Fenech *et al*. 1998; Jacob *et al*. 1998; Titenko-Holland *et al*. 1998). These concentrations are best achieved at intake levels in excess of current RDA, i.e. more than 400 μg folic acid/d and more than 2 μg vitamin B_{12}/d. It is relevant to point out that epidemiological studies on diet and colorectal or breast cancer suggest that intake greater than 400 μg folate/d may be required to minimise cancer risk (Zhang *et al*. 1999; Giovannucci, 2002), yet recent intake data indicate that less than 25 % Americans met this intake level before 1998, when folate fortification became mandatory in the USA

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(Ames & Wakimoto, 2002). The most recent data (analysed from CSIRO 1999 National Nutrition Survey; S Record, unpublished data) for Australians indicate that more than 85 % had intake levels below 400 μg folate/d in 1998 and in Holland more than 50 % of the population were below this optimum intake before 1998 (Konings *et al*. 2001). Dietary intakes above the current RDA may be particularly important in those with extreme defects in the absorption and metabolism of these vitamins, for which ageing is a contributing factor. The above findings suggest that both controlled *in vitro* experiments and placebo-controlled *in vivo* interventions are informative in determining optimal micronutrient intake for prevention of genomic instability.

Our current stage of knowledge on the role of micronutrients in maintenance of genomic stability has been recently reviewed in a special issue of *Mutation Research* (Fenech & Ferguson, 2001). These reviews identify the current gaps in our knowledge and provide the basic information for appropriate design of both *in vitro* studies with normal human cells (Fig. 7) and placebo-controlled intervention trials (Fig. 8). These studies are needed to define optimal tissue concentration and determine RDA for genomic stability. In the future, clinical trials with a wide array of complementary DNA damage end-points would be necessary including measures of mitochondrial DNA deletions and point mutations, nuclear microdeletions and point mutations, telomere shortening, balanced chromosomal translocations, chromosome non-disjunction or aneuploidy, MN formation, single- and double-strand breaks in DNA, DNA adducts and microsatellite instability. It is clear that this objective requires multiple expertise. That there is a need for an international collaborative effort to establish RDA for genomic stability is evident.

Nutritional genomics of genome stability

Perhaps a more useful approach would take into consideration the genotype of individuals with a focus on common genetic polymorphisms that alter the bioavailability of specific micronutrients, their metabolism and the affinity of key enzymes involved in DNA metabolism for their micronutrient cofactor. Supplementation of the diet with appropriate minerals and vitamins could, in some cases, help overcome inherited metabolic blocks in key DNA maintenance pathways (Ames *et al*. 2002). The latter is expected to be particularly effective when a mutation (polymorphism) in a gene decreases the binding affinity for its cofactor resulting in a lower reaction rate. The interaction between genotype and diet in modulating risk is emerging as an exciting area of research as regards micronutrient effects on DNA. This is illustrated by recent research on the common mutations in the methylene tetrahydrofolate reductase (MTHFR) gene and other genes in the folate–methionine cycle with regard to developmental defects and cancer risk (Skibola *et al*. 1999, 2002; Brody *et al*. 2002). The product of the MTHFR gene determines the availability of folate for the synthesis of thymidylic acid from deoxyuridylic acid and is predicted to minimise uracil misincorporation into DNA whilst making less methylfolate available for synthesis of *S*-adenosyl methionine, the common methyl donor (Ames, 1999; Fenech, 2001). Epidemiological studies have suggested that

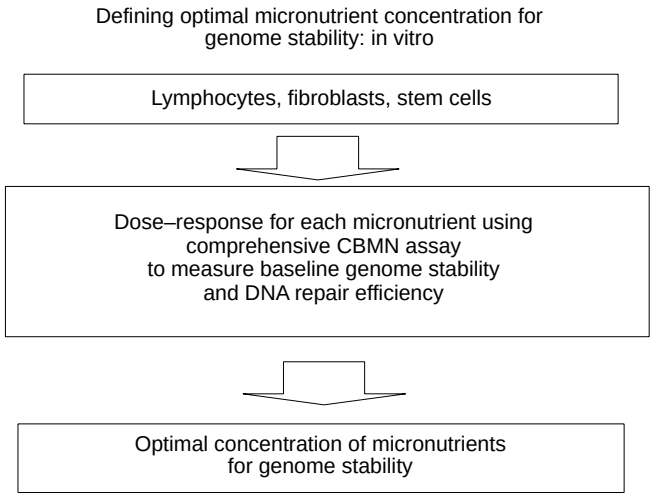

Fig. 7. Schematic diagram of *in vitro* studies that should be performed to determine optimal micronutrient concentration for genome stability. The cells should be cultured for a minimum of 8 d in chemically defined culture medium (ideally without serum which varies greatly in composition and may contain traces of the micronutrient under investigation even after dialysis or chelation). A culture time greater than 6 d is required to observe the effects of micronutrient deficiency *in vitro*. A dose–response study is essential to define not only the optimal concentration range for genome stability but also to determine the concentration when excess of the micronutrient induces cytotoxicity. CBMN assay, cytokinesis-blocked micronucleus assay.

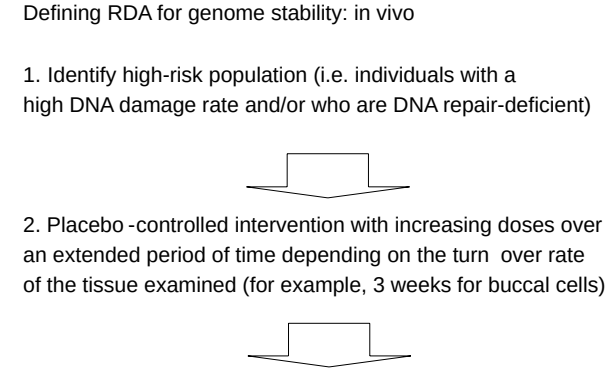

Fig. 8. Schematic diagram for intervention studies required to define the optimal dietary intake of a particular micronutrient for genome stability. Ideally more than one cell type is sampled for analysis to verify that an optimum is achieved for more than one cell type. A combination of the micronucleus assay with other molecular and cytogenetic biomarkers of genome stability such as telomere shortening, DNA oxidation, DNA hypomethylation and aneuploidy of specific chromosomes (for example, chromosomes 17 and 21) and/or chromosomal regions (for example, loss of the p arm of chromosome 17 which includes the p53 gene) associated with increased cancer risk are recommended. RDA, recommended dietary allowances.

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individuals with this genotype may be protected against colorectal cancer and acute lymphocytic leukaemia (Chen *et al*. 1999; Skibola *et al*. 1999). Recent results from our laboratory have shown that there are important significant interactions between the MTHFR C677T polymorphism, its cofactor riboflavin and folic acid with regard to chromosomal instability (M Kimura, K Umegaki, M Higuchi, P Thomas and M Fenech, unpublished results). This is illustrated by: (a) the reduction in nuclear bud frequency in T677T homozygotes relative to C677C homozygotes; (b) the observation that high riboflavin concentration increases nuclear bud frequency under low folic acid conditions probably by increasing MTHFR activity, which diverts folate away from deoxythymidine triphosphate synthesis, increasing the odds for uracil incorporation into DNA synthesis, the generation of BFB cycles and subsequent gene amplification and nuclear bud formation (Fig. 9). Other common polymorphisms, such as the manganese superoxide dismutase alanine to valine change in the –9 position, which disables transport of this enzyme to the mitochondrion where it is normally located (Ambrasone *et al*. 1999), increases susceptibility to oxidative stress and breast cancer risk. Individuals with this mutation appear to benefit more than controls from a higher intake of fruit and vegetables and/or vitamin C in terms of protection against breast cancer (Ambrosone *et al*. 1999). In the past, considerable attention has been given to gene–environment interactions as they relate to mutagen or carcinogen exposure and genotoxic and/or cancer risk. Fig. 10 illustrates the concept that perhaps the gene–diet interaction as it relates to efficacy of DNA repair and DNA metabolism and micronutrient deficiency may be equally important in determining genomic stability and its consequent impact on fertility, development, cancer risk and the rate of ageing (Ames, 1998, 1999; Chen *et al*. 1999; Skibola *et al*. 1999; Trkova *et al*. 2000).

Conclusion: a paradigm shift in disease prevention based on the diagnosis and nutritional treatment of genome damage

The technological advances described earlier (pp. 111–116) have opened up a new opportunity in disease prevention based on the concepts that: (a) excessive genome instability, a fundamental cause of disease, is often an indication of micronutrient deficiency and is therefore preventable; (b) accurate diagnosis of genome instability using DNA damage biomarkers that are sensitive to micronutrient deficiency is technically feasible; (c) it should be possible to optimise nutritional status and verify efficacy by diagnosis of a reduction in genome damage rate after intervention. Given the emerging evidence that the dietary requirement of an individual may depend on their inherited genes, we can anticipate: (a) important scientific developments in the understanding of the relationships between dietary requirement and genetic background to optimise genome stability; (b) that the accumulated knowledge on dietary requirements for specific genotypes will be used to guide decisions by the practitioners of this novel preventive medicine in what might be called ‘Genome Health Clinics’. In other words, one can envisage that instead of diagnosing and treating diseases caused by genome damage, health and medical practitioners will be trained to diagnose and nutritionally prevent the initiating cause, i.e. genome instability itself. This also opens up the possibility for the massive numbers of health-conscious consumers to be able to assess directly the effect of their dietary and nutritional supplement choices on their genome and that of their children. The conceptual framework of the diagnostics and databases required to implement this complementary preventive medicine approach is illustrated in Fig. 11.

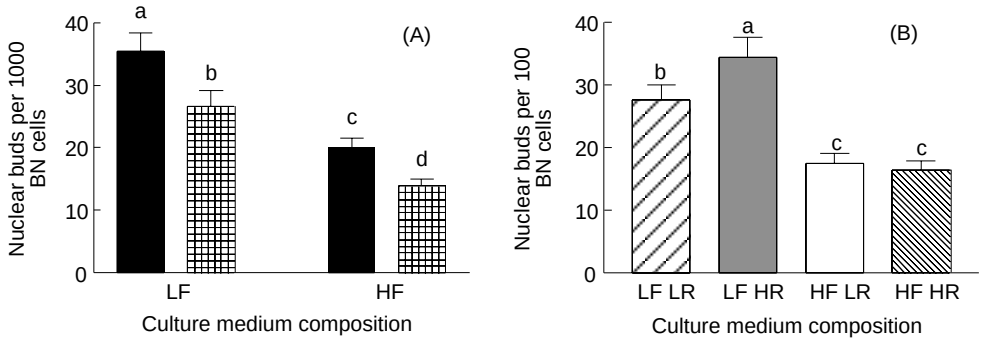

Fig. 9. Nutritional genomics of genome stability illustrated by results from a recent gene amplification experiment from our laboratory using long-term cultured lymphocytes from individuals with either the C677C (■) or the T677T (▦) genotype for the methylenetetrahydrofolate reductase (MTHFR) gene. (A), The results of this study show a significant reduction of nuclear bud formation under both low folate (LF; 12 nmol folic acid/l) and high folate (HF; 120 nmol folic acid/l) conditions for the T677T genotype relative to C677C and a much reduced nuclear bud frequency in the HF medium relative to the LF medium. Values are means, with standard errors of the mean represented by vertical bars. Two-way ANOVA results were: effect of folate concentration, $P < 0{\cdot}0001$; effect of genotype, $P = 0{\cdot}006$. (B), Interaction of folic acid and riboflavin in determining genome stability ($P < 0{\cdot}0001$ by ANOVA). Specifically the results show that when folic concentration is low (12 nmol/l), a high concentration of riboflavin (500 nmol/l), an essential cofactor for MTHFR, increases genome instability (in this case gene amplification). This effect could be due to an increase in MTHFR activity which may divert folate away from thymidylate synthesis and therefore cause uracil incorporation in DNA which leads to chromosome breakage and gene amplification by the breakage–fusion–bridge cycle mechanism (Fenech, 2002). Values are means, with standard errors of the mean represented by vertical bars. ^{a,b,c,d} Mean values with unlike superscript letters were significantly different ($P < 0{\cdot}05$; Tukey’s *post-hoc* test). BN, binucleated; LF LR, 12 nmol folic acid and 0 nmol riboflavin/l; LF HR, 12 nmol folic acid and 500 nmol riboflavin/l; HF LR, 120 nmol folic acid and 0 nmol riboflavin/l; HF HR, 120 nmol folic acid and 500 nmol riboflavin/l. (M Kimura, K Umegaki, M Higuchi, P Thomas and M Fenech, unpublished results.)

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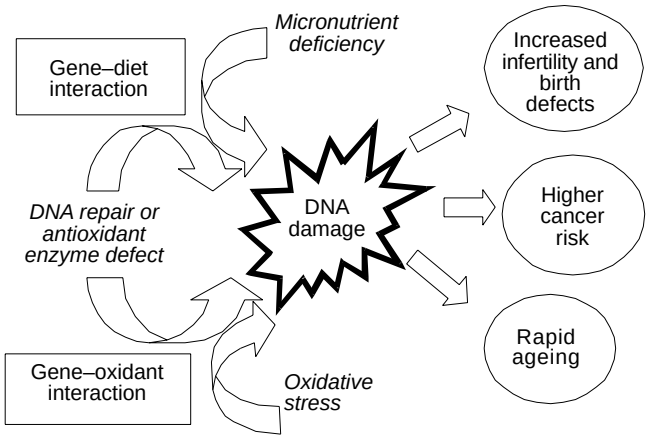

Fig. 10. The concepts of gene–diet and gene–toxin (for example, oxidative stress) interaction and their impact on genome damage and consequent health outcomes.

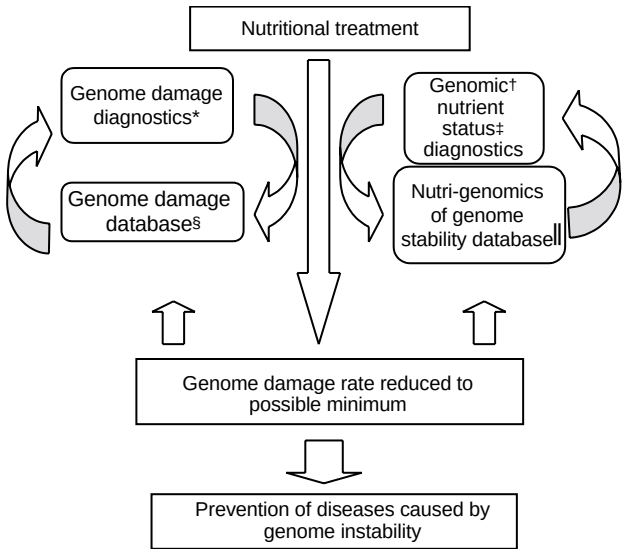

Fig. 11. Conceptual framework of the diagnostics and databases that will be required to implement the novel complementary preventive medicine discipline of nutritional treatment of genome instability, i.e. 'Genome Health Clinics'. * Genome damage diagnostics may include generic chromosomal damage biomarkers (for example, micronucleus assay) as well as genetic anomalies specific to unique disease states (for example, chromosome 17 and 21 aneuploidy or deletions of unique genes such as p53). † Genomics assays would include single nucleotide polymorphisms with reduced affinity of cofactors involved in genome stability maintenance as well as assessment of gene silencing and expression. ‡ Micronutrients essential for genome stability would be measured (for example, folate, Zn etc.). § The genome damage database would include 'normal range' and 'optimal range' values and be used to determine whether an individual's genome damage rate is suboptimal or optimal. || The nutri-genomics database would accrue data on the genome stability response to micronutrient treatment in relation to genetic background and be developed as an expert system to guide future diagnostics and treatments.

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