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REVIEW

What do human micronuclei contain?

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As micronuclei (MN) derive from chromosomal fragments and whole chromosomes lagging behind in anaphase, the MN assay can be used to show both clastogenic and aneugenic effects. The distinction between these phenomena is important, since the exposure studied often induces only one type of MN. This particularly concerns the use of MN as a biomarker of genotoxic exposure and effects, where differences in MN frequencies between exposed subjects and referents are expected to be small. A specific analysis of the induced type of MN may considerably improve the sensitivity of detecting the exposure effect. MN harbouring chromosomes can be distinguished from those harbouring acentric fragments by the presence of a centromere. The proportion of centromere-positive MN in human lymphocytes increases with age, which primarily reflects an age-dependent micronucleation of the X and Y chromosomes. The X chromosome especially tends to lag behind in female lymphocyte anaphase, being micronucleated more efficiently than autosomes. There is some evidence for an enhanced prevalence of fragments from chromosome 9 in spontaneous human lymphocyte MN and from chromosomes 1, 9 or 16 in MN induced *in vitro* by some clastogens; the breakage appears to occur in the heterochromatic block of these chromosomes. Although there are indications that centromere identification can improve the detection of clastogenic effects in humans *in vivo*, smokers have not shown an increase in centromere-negative MN in their cultured lymphocytes, although smoking is known to produce chromosomal aberrations. This may suggest that fragment-containing MN and chromosomal aberrations cover partly different phenomena. Understanding the mechanistic origin and contents of MN is essential for the proper use of this cytogenetic end-point in biomarker studies, genotoxicity testing and risk assessment.

Introduction

The analysis of micronuclei (MN) has gained increasing popularity as an *in vitro* genotoxicity test and a biomarker assay for human genotoxic exposure and effect. The main reasons for this development are obvious. In comparison with chromosomal aberrations (CA), the scoring of MN is simpler, requires shorter training and is less time consuming. In principle, the MN assay can be expected to be more sensitive than the CA assay, because of the increased statistical power brought about by the fact that the number of cells analysed can easily be increased to thousands when only a hundred or a few hundred cells are usually scored for CA.

In humans, most MN studies have been conducted using cultured peripheral lymphocytes, which lend themselves well to both genotoxicity testing and biomonitoring. The cytokinesis block assay, based on cytokinesis inhibition by cytochalasin B (Cyt-B), has facilitated MN analysis exclusively in (binucleate) cells that have completed their first *in vitro* division after treatment with the test agent or following culture initiation (Fenech and Morley, 1985; Fenech, 1993, 1997, 1998, 2000). The identification of these cells is important, since MN are formed in cell division, and an accurate estimate on MN frequency can only be obtained from the first post-mitotic interphase after exposure. For biomarker studies, MN formed *in vivo* can also be examined from uncultured (or *in vitro* undivided) lymphocytes and from exfoliated epithelial cells collected, e.g. from buccal, nasal or urothelial mucosa (Moore *et al.*, 1993; Surrallés *et al.*, 1996a, 1997; Albertini *et al.*, 2000; Kirsch-Volders and Fenech, 2001).

The two basic phenomena leading to the formation of MN in mitotic cells are chromosome breakage and dysfunction of the mitotic apparatus. MN are formed from acentric chromosome or chromatid fragments and whole chromosomes or chromatids that lag behind in anaphase and are left outside the daughter nuclei in telophase (Ford *et al.*, 1988; Lindholm *et al.*, 1991; Ford and Corell, 1992; Catalán *et al.*, 2000; Falck *et al.*, 2002). Laggards cannot move to the poles, because they are detached from the mitotic spindle or, as described by Cimini *et al.* (2002) for lagging chromatids, have bipolar (merotelic) orientation. Besides these fundamental mechanisms, some MN may have their origin in fragments derived from broken anaphase bridges (Cornforth and Goodwin, 1991; Saunders *et al.*, 2000) formed due to chromosome rearrangements such as dicentric chromatids, intermingled ring chromosomes or union of sister chromatids.

MN formation is undoubtedly an important mechanism for chromosome loss (Ford *et al.*, 1988), although it is not the only mechanism. Confusingly, chromosome loss is often used as a synonym for (chromosome-containing) MN, as MN are generated by chromosome loss from the nucleus. However, MN are not necessarily lost from the cell. As pointed out by Eastmond and Tucker (1989) and Schuler *et al.* (1997), MN accompany either the daughter nucleus they derive from or the other daughter nucleus. In the former case, neither of the daughter cells is aneuploid, and in the latter case the micronucleated cell has gained a chromosome, while its daughter cell has lost it. Assuming MN are segregated randomly between the daughter cells, the frequency of centromere-containing MN in binucleate cells (where both daughter nuclei are present) gives an indirect estimate of chromosome loss (or gain) due to micronucleation. Although it has been suggested that MN are eliminated from cells (Ford *et al.*, 1988), the fate of the MN (e.g. expulsion, inclusion in nucleus in mitosis, cell death) remains unclear. It should be kept in mind that the segregation of both sister chromatids of a chromosome to the same daughter nucleus (Zijno *et al.*, 1994, 1996a,b,c), which

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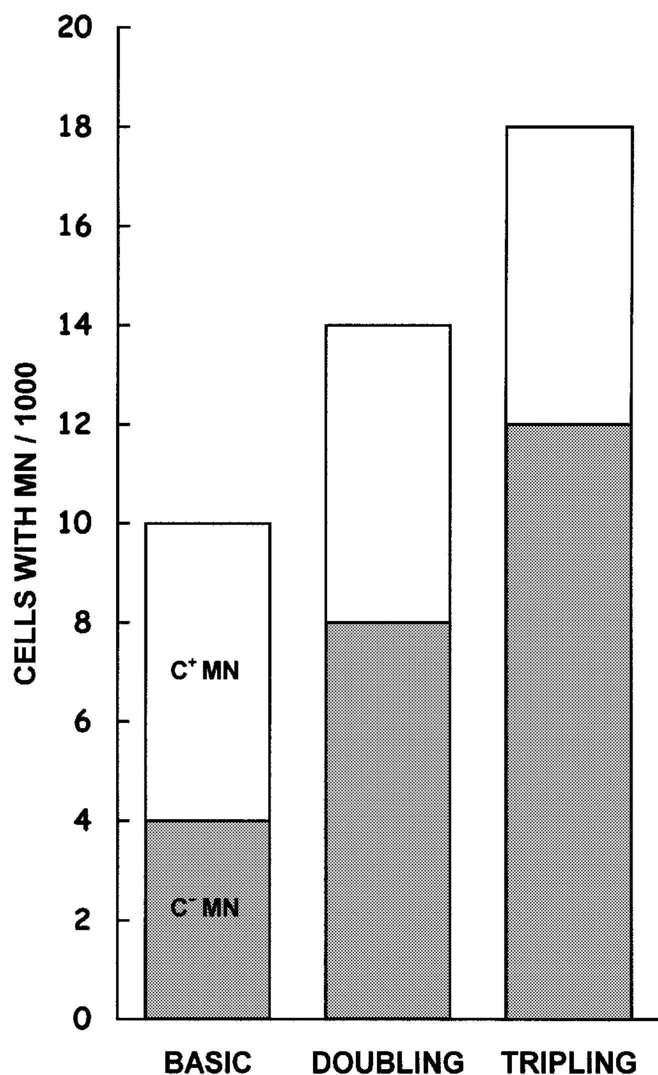

Fig. 1. A theoretical example of how centromere identification in micronuclei (MN) could improve the detection of a clastogenic effect. Before the clastogenic exposure (or in the control group), MN harbouring chromosomal fragments (C– MN) represent 40% of all MN. When their frequency is doubled or tripled, the frequency of all MN would only show a 1.4- or 1.8-fold increase, respectively.

may result from the failure of sister chromatids to separate properly (non-disjunction) or engulfment of a lagging chromatid to the same nucleus as its sister chromatid, is an important source of both chromosome loss and gain and does not result in MN (Schuler *et al.*, 1997). Thus, although micronucleation contributes to chromosome loss, these two phenomena cannot be equated.

The ability of the MN assay to detect both clastogenic and aneugenic effects (leading to structural and numerical chromosome alterations, respectively) is an advantage of the MN technique (Kirsch-Volders *et al.*, 1997; Fenech, 2000). The distinction between the two phenomena, by identifying the origin of MN, is important, whether MN analysis is used for genotoxicity testing or for biomonitoring of genotoxic exposure and effect in humans (Thomson and Perry, 1988; Eastmond and Tucker, 1989; Becker *et al.*, 1990; Norppa *et al.*, 1993a,b). The exposure studied often induces only one type of MN, and other types of MN may be uninformative for the assay. Figure 1 shows a theoretical example of a biomarker study on clastogenic exposure. Forty per cent of MN in the subjects represent MN deriving from chromosomal fragments. Unless these MN are specifically identified, the sensitivity of the assay is compromised. Looking at total MN frequency, a doubling of fragment-containing MN only results in a 1.4-fold increase and a tripling only in a 1.8-fold increase.

Centromere and kinetochore identification have successfully been used in numerous *in vitro* and *in vivo* studies to examine the contents of MN. Information on the more detailed ingredients of MN is more limited, but there are clear indications that different chromosomes are micronucleated non-randomly (see below). It is important to realise that we do not yet know enough on the contents of MN. Yet, a thorough understanding of what MN actually stand for is a basic requirement for the correct use of the MN assay. The present paper reviews the current knowledge on the contents of human MN and how this information may influence the use of the MN assay in short-term testing and biomonitoring.

Detection of micronucleus contents

As chromosomes contain a centromere associated with a kinetochore structure, the occurrence of an entire chromosome (or chromatid) in MN can be shown by the presence of centromere-specific DNA sequences (Ford *et al.*, 1988; Becker *et al.*, 1990; Migliore *et al.*, 1993; Norppa *et al.*, 1993b) or centromeric (kinetochore) proteins (Hennig *et al.*, 1988; Thomson and Perry, 1988; Eastmond and Tucker, 1989). MN with acentric fragments are not expected to contain centromeric DNA or kinetochore proteins.

The two main classes of MN can be distinguished from each other by using *in situ* hybridization (ISH) with DNA probes that identify α-satellite DNA in all human chromosomes (Figure 2A and B). α-Satellite DNA, characterized by a diverged 171 bp motif repeated in a tandem fashion, is found in all human centromeres (see Schueler *et al.*, 2001). The pancentromeric DNA probes used for MN characterization have been cloned alphoid probes (Becker *et al.*, 1990; Thierens *et al.*, 1999a), oligonucleotides with an alphoid consensus sequence (Norppa *et al.*, 1993b; Elhajouji *et al.*, 1995; Darroudi *et al.*, 1996), commercially available probes for all human centromeres (Migliore *et al.*, 1993; Titenko-Holland *et al.*, 1994; Doherty *et al.*, 1996) or α-satellite probes prepared by PCR (Huber *et al.,* 1996). Fluorescence *in situ* hybridization (FISH) with digoxygenin- or biotin-labelled DNA probes detected in a fluorescence microscope by immunofluorescence has been used in most studies, but DNA probes directly labelled with a fluorochrome are nowadays available. Immunohistochemical detection by, for example, alkaline phosphatase or peroxidase has been utilized in some papers (Guttenbach *et al.*, 1994; Nardone, 1997; Vral *et al.*, 1997). In principle, centromeric DNA can also be detected by primed *in situ* labelling (PRINS) (Russo *et al.*, 1996; Basso and Russo, 2000), but this technique appears not to have been applied for human MN. Immunofluorescence or immunohistochemistry have also been used for the detection of kinetochore proteins in human MN (Hennig *et al.*, 1988; Thomson and Perry, 1988; Eastmond and Tucker, 1989; Fenech and Morley, 1989). Kinetochore proteins are identified by anti-kinetochore antibodies derived from the serum of scleroderma CREST (Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly and Telangiectasia) patients (Moroi *et al.*, 1980).

Schuler *et al.* (1997) have reviewed the advantages and disadvantages and technical aspects of the CREST and FISH

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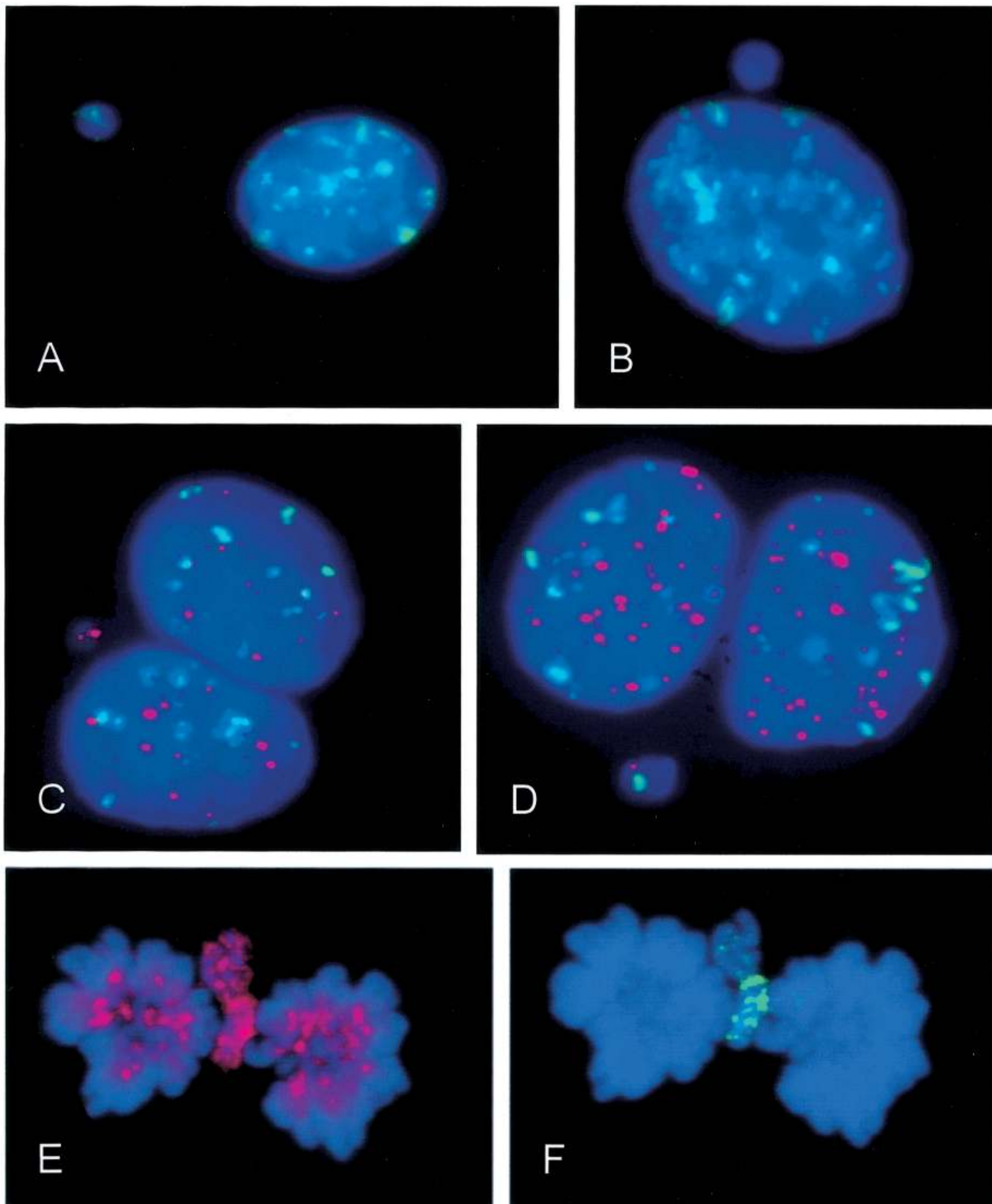

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Fig. 2. FISH characterization of (A and B) micronuclei in human buccal cells, (C and D) micronuclei in binucleate lymphocytes and (E and F) anaphase laggards in female lymphocyte anaphase. (**A**) A centromere-positive (green signal) micronucleus; (**B**) a centromere-negative micronucleus; (**C**) a micronucleus with telomere signals (red); (**D**) a micronucleus with telomere (red) and centromere (green) signals; (**E**) two entire lagging X chromosomes (red chromosome painting; red signals in the poles represent centromeres); (**F**) one of the X chromosomes is late replicating (green; immunofluorescence detection of 5-bromo-2′-deoxyuridine label). Counterstaining by 4′,6-diamidino-2-phenylindole. Micrographs by (A–D) H.Järventaus and (E and F) J.Catalán and G.C.-M.Falck.

methods in characterizing MN. Fragments with the break point at the centromere may form centromere-positive (C+) MN if the breakage destroys centromere function. True centric fragments with a complete centromere are expected to be rare in MN of non-cancerous cells, as they should normally be attached to the spindle, unless they lack a functional kinetochore. It is also possible that a MN contains both one or more fragments and one or more chromosomes; in this case, the detection of centromeric DNA would only suggest the presence of the latter. In general, MN deviating from the two simple categories (acentric fragment versus chromosome) should in most cases be relatively rare in non-cancerous cells. They would either require an inducer with a very specific mode of action or the simultaneous occurrence of two or several (more or less) independent events whose combined probability should be low.

MN formed from entire chromosomes with a disrupted or

detached kinetochore may result in MN with no kinetochore signal (Schuler *et al.*, 1997). This appears to be a real problem for the use of kinetochore identification in human biomonitoring and studies of spontaneous MN, since a considerable portion of MN harbouring the centromere of the X or Y chromosome seem to be kinetochore-negative (K–) (Hando *et al.*, 1994, 1997; Nath *et al.*, 1995; Tucker *et al.*, 1996). Kinetochore defects may be common for the micronucleation of other chromosomes as well, but even the X chromosome alone is expected to give rise to errors in the assessment of MN contents using kinetochore antibodies, since this single chromosome may account for 70% of MN in binucleate lymphocytes of women (Hando *et al.*, 1994, 1997). In practice, centromere and (with some reservations) kinetochore detection can be expected to be accurate enough in distinguishing the two main types of MN in experimental studies, but it appears that centromeric FISH should be recommended for studies of human exposure and spontaneous MN levels, due to the low prevalence of kinetochore label in MN harbouring entire sex chromosomes.

It is preferable to evaluate the centromere/kinetochore content of MN by calculating the frequencies of signal-positive and signal-negative MN than by looking at their proportions (Fenech and Morley, 1989; Surrallés *et al.*, 1995, 1996a; Falck *et al.*, 1997; Schuler *et al.*, 1997). Proportions do not well take into account the magnitude of the effect and can be misleading if the exposure induces both types of MN or actually decreases the frequency of one type of MN (Falck *et al.*, 1997; Schuler *et al.*, 1997).

Chromosome-specific centromeric DNA probes have been used to identify the presence of specific chromosomes in MN by FISH (Guttenbach *et al.*, 1994; Hando *et al.*, 1994, 1997; Richard *et al.*, 1994; Catalán *et al.*, 1995; Tucker *et al.*, 1996; Acar *et al.*, 2001; Bakou *et al.*, 2002). The use of a pancentromeric probe, an X chromosome-specific centromeric probe and (in men) a Y chromosome-specific centromeric probe allows one to estimate the contribution of acentric fragments, X chromosomes, Y chromosomes and autosomes in MN (Catalán *et al.*, 1995, 1998, 2000; Surrallés *et al.*, 1996a; Falck *et al.*, 2002).

The contribution of all human chromosomes in MN has been studied by chromosome painting (Fauth *et al.*, 1998, 2000; Fauth and Zankl, 1999) and spectral karyotyping (Komae *et al*., 1999; Leach and Jackson-Cook, 2001); coupled with centromeric probes this approach can distinguish between MN harbouring centric and acentric DNA derived from specific chromosomes.

Fragments and chromosomes in spontaneous micronuclei

In various studies of spontaneous MN in cultured human lymphocytes, the average proportion of whole chromosomes in MN has usually varied between 30 and 80%, as measured by CREST antibody or FISH (Fenech and Morley, 1989; Yager *et al.*, 1990; Norppa *et al.*, 1993b; Scarpato *et al.*, 1996; Surrallés *et al*., 1996a,b; Migliore *et al.*, 1997, 1999a,b; Calvert *et al*., 1998; Carere *et al.*, 1998; Davies *et al.*, 1998; Thierens *et al.*, 1999a, 2000; Maffei *et al.*, 2000; Leach and Jackson-Cook, 2001; Bakou *et al.*, 2002).

In exfoliated buccal, nasal and urothelial cells of non-smoking volunteers 23–42 years of age, the proportion of C+ MN was 56–57% (Moore *et al.*, 1993; Titenko-Holland *et al.*, 1994). In buccal cells of male benzene-exposed workers and controls (aged 19–61 years, mean 41 years, 45% smokers), C+ MN constituted 35% of identifiable MN (Surrallés *et al.*, 1997). The same prevalence (35%) of C+ MN was observed in nasal cells of non-smoking male stainless steel production workers aged 34–62 years (Huvinen *et al.*, 2002). A considerably lower level (6%) of C+ MN in buccal cells of hyperthyroidism and thyroid cancer patients before radiation therapy was reported by Ramírez *et al.* (1999a).

The prevalence and frequency of kinetchore-positive (K+) MN (Fenech and Morley, 1989; Hando *et al.*, 1994, 1997; Odagiri and Uchida, 1998) and C+ MN (Catalán *et al.*, 1995; Scarpato *et al.*, 1996; Ramírez *et al.*, 1997; Thierens *et al.*, 1999a, 2000; Bakou *et al.*, 2002) in human lymphocytes increases with age. Thierens *et al.* (2000) estimated that 80% of the effect of age on MN frequencies is due to C+ MN; women had higher frequencies of C+ MN than men. Also, Scarpato *et al.* (1996) noticed a clearer age-dependent increase in lymphocyte C+ MN in women than in men.

The effect of age on fragment-containing MN would appear to be less clear. An increase in K– MN with age was observed in cultured binucleate lymphocytes of male subjects (Nath *et al.*, 1995) and in a pooled group of males and Turner syndrome patients (Hando *et al*., 1997). Also, Odagiri and Uchida (1998) reported that the age-dependent increase in lymphocyte MN frequency concerns both K+ and K– MN. In addition, they observed that serum vitamin C level was associated with an increased frequency of cultured lymphocytes with K+ MN and that serum folic acid level was negatively related to K+ MN after age adjustment. As the majority of MN harbouring X or Y chromosomes, the two chromosomes primarily responsible for the age effect on MN, appear to be devoid of kinetochore signals (Hando *et al.*, 1994, 1997; Nath *et al.*, 1995; Tucker *et al.*, 1996), it is probable that kinetochore identification is not accurate enough in discriminating the two main groups of MN. Using FISH on binucleated lymphocytes, Bakou *et al.* (2002) reported a 1.7-fold higher frequency of centromere-negative (C–) MN in four women 47–50 years of age as compared with four women 22–26 years of age, but Thierens *et al.* (1999a), who studied 215 subjects, did not observe a significant age effect on C– MN.

In uncultured T lymphocytes of women, the proportion of C+ MN was higher (71.6%) than in cultured cells (53.3–55.2%) (Surrallés *et al*., 1996a). This particularly reflected an increase in C– MN in the cultures. In comparison with the uncultured T cells, the frequency of C– MN per 1000 nuclei was 2.3–2.9 times higher in the cultured cells. The increase in C– MN by cell culture probably reflected the expression, by the *in vitro* mitosis, of damage accumulated *in vivo* in the quiescent lymphocytes, i.e. conversion of pre-existing DNA lesions into chromatid breaks and appearance of *in vivo* chromosome type breaks.

Several studies on human lymphocytes have shown that MN frequency is increased with increasing culture time, with and without Cyt-B (see Falck *et al.*, 1997). This effect appears to be mainly due to C+ MN (Falck *et al.*, 1997; Sgura *et al.*, 1997). The reason for the finding is not clear, but it could reflect differential growth rate of subpopulations of lymphocytes with different baseline MN frequencies, prolonged cell cycle of damaged cells, deteriorating culture conditions or higher X micronucleation at longer culture times (Richard *et al.*, 1994; Falck *et al.*, 1997). In cultures without Cyt-B, the culture time-dependent increase in C+ MN could just be due to the increase in mitotic activity. Prolonged incubation with Cyt-B can lead

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to the formation of binucleate cells that have actually divided twice in the presence of Cyt-B; such cells are expected to have a high MN frequency (Lindholm *et al.*, 1991; Norppa *et al.*, 1993b; Surrallés *et al.*, 1994; Zijno *et al.*, 1994). The second division in the presence of Cyt-B (division of a binucleate cell) is grossly irregular, resulting in high frequencies of lagging chromosomes in anaphase and formation of C+ and K+ MN, mostly containing autosomes (Lindholm *et al.*, 1991; Norppa *et al.*, 1993b; Falck *et al.*, 2002).

Binucleate human lymphocytes produced by Cyt-B were found to have a higher proportion of C+ and K+ MN than cells cultured without Cyt-B, which reflected decreased frequencies of C– and K– MN per 1000 nuclei in binucleate cells (Norppa *et al.*, 1993b; Surrallés *et al.*, 1996a; Falck *et al.*, 1997, 2002; Catalán *et al.*, 1998). The diminished micronucleation of fragments was suggested to be due to reduced distance between the poles in cytokinesis-blocked cells, leading to increased engulfment of laggards into the daughter nuclei (Norppa *et al.*, 1993b; Surrallés *et al.*, 1996a; Falck *et al.*, 1997; Minissi *et al.*, 1999). Measurements in human lung fibroblasts subsequently showed that the pole-to-pole distance is, indeed, decreased in the presence of Cyt-B (Cimini *et al.*, 1999). In female lymphocytes, the frequency of lagging acentric fragments in bipolar anaphases was lower in the presence of Cyt-B than in its absence (Falck *et al.*, 2002). Presumably, less acentric fragments are left outside the poles due to the shortened pole-to-pole distance. The short distance between the poles and absence of a contractile ring also appears to result in an increase in C-anaphases where sister chromatids have not travelled apart enough to give rise to two daughter nuclei but result in a polyploid restitution nucleus (Minissi *et al.*, 1999); this seems to occur for heavily impaired anaphases of colchicine-treated lymphocytes, but may also concern spontaneously damaged anaphases.

Apparently healthy subjects with exceptionally high MN frequencies are occasionally observed in human studies. Thierens *et al.* (1999a) reported a high frequency of C+ MN (53/1000 binucleate cells) in lymphocytes of a 50-year-old non-smoking man. No apparent reason for this finding was indicated. In another study (Thierens *et al.*, 2000), two female radiological workers with similarly high frequencies of C+ MN were encountered, many of the micronucleated binucleate cells carrying two or more MN. Most of the MN turned out to contain the X chromosome.

Some diseases have been associated with an increase in specific types of MN in cultured lymphocytes. Systemic lupus erythematosus patients and systemic sclerosis patients with topoisomerase I antibodies (but not antibodies against centromeric proteins) showed an increase in C– MN, while systemic sclerosis patients with centromeric antibodies (but not antibodies against topoisomerase I) had an elevation in C+ MN (Migliore *et al.*, 1999b). Parkinson's disease patients, who appear to have elevated oxidative stress and DNA damage, showed an increase in binucleate lymphocytes with C– MN (Migliore *et al.*, 2002). Both C+ and C– MN were increased in patients suffering from mitochondrial encephalomyopathies, possibly reflecting the consequences of increased radical production caused by altered mitochondrial respiratory chain (Naccarati *et al.*, 2000).

Fragments in MN can be of the chromatid type (single) or chromosome type (double), but it is not known whether micronucleation occurs equally for both types of fragments. In metaphase, chromatid fragments are attached to the homologous area of the sister chromatid while acentric chromosome fragments are apart from their chromosome of origin. It might be envisaged that acentric chromatid fragments are not always separated from the sister chromatid in anaphase, in which case they would form MN less efficiently than acentric chromosome fragments. Some information on the involvement of both types of fragments in MN may be obtained by simultaneous identification of telomeres and centromeres in MN (Figure 2C and D). Preliminary FISH data on binucleate lymphocytes of four human donors (Norppa *et al.*, unpublished results) indicated that 60% of C– MN contain one telomere signal, suggesting no marked reduction of chromatid type break micronucleation. In general, acentric fragments lagging in female lymphocyte anaphase seemed to be micronucleated quite efficiently, with 41–65% of them apparently ending up in MN (Falck *et al.*, 2002).

The contribution of centric fragments to human MN is poorly known. Such MN may be frequent in cells with genomic instability. In HeLa cells, the expression of human T cell leukaemia virus type I (HTLV-I) Tax oncoprotein increased the proportion of C+ MN without telomeres or with free 3′-OH ends (examined by terminal transferase-mediated *in situ* addition of digoxygenin-dUTP) from ~20 to ~37% but decreased the proportion of C– MN with telomere signals or without free 3′-OH ends (Majone and Jeang, 2000). The authors concluded that Tax interferes with protective cellular mechanisms that stabilize DNA breaks by adding telomeric caps.

Specific chromosomes in spontaneous micronuclei

If the micronucleation of each human chromosome is random, each single chromosome should appear in 1/46 (2.17%) of chromosome-containing MN (assuming only one chromosome in a MN) and chromosome-specific DNA should be found in acentric fragment-containing MN corresponding to the length of each chromosome. FISH studies have, however, shown that different human chromosomes are non-randomly involved in MN. In cultured human lymphocytes, the increase in C+ MN with ageing has primarily been attributed to an age-dependent micronucleation of the X and Y chromosomes (Guttenbach *et al.*, 1994; Hando *et al.*, 1994; Richard *et al.*, 1994; Catalán *et al.*, 1995, 1998; Surrallés *et al.*, 1996a; Zijno *et al.*, 1996a,b; Bakou *et al.*, 2002). These findings are in line with the well-known age-dependent loss of X and Y chromosomes (see Stone and Sandberg, 1995; Catalán *et al.*, 1998).

X chromosome

Richard *et al.* (1994) observed by centromeric FISH that the proportion of X chromosome-positive (X+) MN in cultured lymphocytes of five female donors was dependent on age, ranging from 7.4% in the youngest (27 years) to 20.7% in the oldest (80 years); the frequency of X+ MN varied from 0.1 to 1.3 per 1000 cells in the same persons. The X chromosome was clearly over-represented in the MN, while chromosomes 11 and 22, detected (by chromosome painting and with a non-centromeric cosmid probe, respectively) in the oldest of the donors, were very rare. Guttenbach *et al.* (1994) found that the proportion of X+ MN among all lymphocyte MN was 20% in five older women (aged 71–84 years) and 8% in five girls (aged 1–10 years). As the frequency of total MN was more than 4 times higher in the older age group, there appeared to be an almost 10-fold age difference in the frequency of X+ MN. In six women over 50 years of age, the proportion of

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X+ MN among all MN was 24.0%, while it was 14.0% in six women below 30 years of age (Catalán *et al.*, 1995); an age dependency was also observed for the proportion of autosome-containing MN (28 versus 20%).

Much higher proportions of X+ MN have been observed in binucleate lymphocytes produced by Cyt-B. Hando *et al.* (1994) observed an X signal in 72.2% of all MN in a group of female donors aged 0–77 years, indicating that X micronucleation is far from being random. About half of the X+ MN contained more than one signal. Interestingly, the majority of both K+ (77.3%) and K– MN (64.9%) contained an X signal; 36.9% (290/785) of the X+ MN did not appear to have a kinetochore, indicating that many micronucleated X chromosomes tend to have faulty kinetochores. The frequency of X+ MN was shown to be age dependent and no X+ MN were observed in newborns, who also otherwise exhibited a low MN frequency (Hando *et al.*, 1994). In cultured binucleate lymphocytes of 12 women (25–56 years of age), X+ MN accounted, on average, for 62.3% of all MN and their frequency was 10.5 per 1000 binucleate cells (Zijno *et al.*, 1996a,b); an age dependency was observed for the frequency but not for the proportion of X+ MN, suggesting that other types of MN also contribute to the age-dependent MN increase. Bakou *et al.* (2002) observed that binucleate lymphocytes of two women 47 and 49 years of age showed clearly higher frequencies of X+ MN and MN with autosomes than lymphocytes from two younger women 22 and 23 years of age.

In uncultured T lymphocytes of five women aged 47–60 years, the proportion of X+ MN of all MN was 28.5%, corresponding to 39.6% of C+ MN (9 times higher than expected by chance), which indicated that the X chromosome is also over-represented in MN *in vivo* (Surrallés *et al.*, 1996a). The percentage of X+ MN did not change much on culturing the T lymphocytes for 72 h (without Cyt-B), although the frequency of X+ MN was increased from 2.6 to 3.9 per 1000 nuclei. However, in binucleate cells produced by Cyt-B, the proportion of X+ MN was 42% of all MN (79.8% of C+ MN). This high percentage was explained by an increase in the frequency of X+ MN (per 1000 nuclei) and reduction (to almost $^1/_4$ of the frequency in cultures without Cyt-B) of the calculated frequency of MN harbouring autosomes. An increase in the proportion and frequency of X+ MN in binucleate cells and a decrease in the frequency of autosome- or fragment-containing MN was also observed in other studies comparing MN contents with and without Cyt-B (Falck *et al.*, 1997, 2002; Catalán *et al.*, 1998). The clearly higher percentages of X+ MN reported in series where Cyt-B was used as compared with those where Cyt-B was not used (Hando *et al.*, 1994; Tucker *et al.*, 1996; Zijno *et al.*, 1996a,b; Hando *et al.*, 1997; Catalán *et al.*, 1998; Bakou *et al.*, 2002) also support the view that Cyt-B favours a high prevalence of the X chromosome in MN.

In anaphase lymphocytes of a 62-year-old woman, the X chromosome frequently lagged behind, constituting 26 (without Cyt-B) and 12% (with Cyt-B) of all lagging chromosomes (Catalán *et al.*, 2000; Falck *et al.*, 2002). X laggards more often contained both sister chromatids (Figure 2E and F) and were more distally located than autosome laggards (Catalán *et al.*, 2000). The high proportion of the X+ MN in binucleate cells appeared to be due to the fact that all X laggards were micronucleated in the presence of Cyt-B, while this efficiency was 'only' 49% without Cyt-B (Falck *et al.*, 2002). It may be that X laggards, detached from the spindle due to faulty kinetochores, are not forced from the periphery nearer the daughter nuclei in cytokinesis-blocked cells which are not elongated and lack the central spindle and contractile ring.

The high loss and micronucleation of the X chromosome in women has been suggested to be primarily due to the inactive X (see Surrallés *et al.*, 1996b, 1999; Tucker *et al.*, 1996; Catalán *et al.*, 2000). The inactive X chromosome was observed to show a higher age-dependent telomere shortening than the active homologue or all chromosomes in metaphases of cultured lymphocytes from newborn, middle-aged and elderly females (Surrallés *et al.*, 1999); the accelerated shortening may affect the segregation of the inactive X chromosome. Tucker *et al.* (1996) observed that the inactive X is more often involved in female MN than the active homologue. They studied the cytokinesis-blocked lymphocytes of two healthy females who had a translocation between chromosome 9 and the active X homologue. The active X in MN was identified by the presence of both chromosome X and 9 paints. The X chromosome was found in 44.2% of all MN (in 52.3% of all MN in a karyotypically normal woman studied in parallel) and 83.3% of the X+ MN contained the inactive X but no chromosome 9 signal. Kinetochore label was absent from 73.8% (59/80) of MN harbouring the untranslocated (inactive) X and from 56.3% (9/16) of MN harbouring the translocated (active) X.

However, Surrallés *et al.* (1996b), who identified the inactive X chromosome through histone H4 underacetylation, observed no significant differences in the micronucleation of the active and inactive X chromosomes, suggesting that both homologues are preferentially micronucleated. The X chromosome was highly over-represented (15.5–68.1% of all MN, average 24.4%) among binucleate lymphocyte MN of the two older (57 and 60 years) and two younger (24 and 27 years) women studied, and the older women had 2.5 and 3.4 times higher frequencies of MN with (respectively) active and inactive X than the younger women. When the late replicating inactive X in female lymphocyte anaphase (without Cyt-B) was labelled with bromodeoxyuridine (Figure 2F), the inactive and active X chromosomes seemed to lag behind in equal proportions (Catalán *et al.*, 2000).

Although the role of inactive X as the source of the high X+ MN frequencies remains unclear, it is obvious that the phenomenon is not entirely explained by the inactive homologue. This is because the X chromosome is also over-represented in lymphocyte MN of men, who have only one (active) X chromosome (Hando *et al.*, 1997; Catalán *et al.*, 1998; Carere *et al.*, 1999). In a combined group of 43 males (0–79 years of age) and seven Turner syndrome (45,X) patients (11–39 years), the X chromosome was found in 6.6% of all MN, which was higher than would be expected by chance, but still only 1/10 of the percentage found in females of similar age range (Hando *et al.*, 1997). A total of 68.2% of the X+ MN did not show kinetochore signals, again suggesting a high prevalence of kinetochore defect in the micronucleated X chromosomes. The frequency of X+ MN in the pooled group of males and 45,X females was age dependent. The results primarily reflected increased X micronucleation in the males studied; conclusions could not be drawn for the Turner syndrome patients, as only the oldest one of them (the others were ~20 years old or younger) had X+ MN among the MN found in 2000 cells scored per individual (Hando *et al.*, 1997).

In another study (Catalán *et al.*, 1998), the X chromosome was included in 7.0 (without Cyt-B) and 15.2% (with Cyt-B)

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of all lymphocyte MN in five men >50 years of age and in 4.8 and 6.2% of all MN (respectively) in five men <30 years of age, showing over-representation among C+ MN in both age groups. Four women (aged 26–58 years), studied for comparison, had much higher rates of X micronucleation than the men, with 26.0 (without Cyt-B) and 41.8% (with Cyt-B) of their MN being X+. The sex difference in the frequency of MN was mainly due to the X chromosome. In 24 male subjects (age range 31–62 years), the frequency of chromosome X in binucleate lymphocytes was 2.33 per 1000 cells, much higher than that of chromosomes 7, 11 and 18 (0.20, 0.16 and 0.08, respectively) (Carere *et al.*, 1999). Correlation between age and the frequency of X+ MN was almost statistically significant ($P = 0.06$).

The high micronucleation of the only (active) X in men suggests that the X chromosome has a general tendency to be micronucleated, regardless of the inactive X. Kinetochore defects may be behind this phenomenon (Hando *et al.*, 1994, 1997; Tucker *et al.*, 1996). The reason for the extremely high X micronucleation in women is still, however, unknown, although the inactive X remains an attractive explanation.

Y chromosome

Guttenbach *et al.* (1994) observed that the proportion of Y+ MN in cultured lymphocytes was 14% of all MN in five boys 0.5–4 years of age and 20% in five older men 73–91 years of age, the older men showing a higher mean frequency of Y+ MN (1.0/1000 cells) than the children (0.3/1000 cells). An over-representation of Y in MN was also seen by Nath *et al.* (1995) in binucleate lymphocytes of 35 adult men (22–79 years of age) and 18 newborns, 13.5% of all MN being Y+. A total of 87.8% of the Y+ MN did not show kinetochore signals, indicating that kinetochore damage is an important cause of Y micronucleation. The frequency of Y+ MN showed age dependency. The newborns had a very low MN frequency and no Y+ MN among the 17 MN characterized from them. In the study of Catalán *et al.* (1998), five men above 50 years of age were observed to have the Y chromosome in 10% of MN of their cultured lymphocytes (with and without Cyt-B), indicating a clear over-representation (18.1–23.6% of C+ MN) of this chromosome. The frequency of Y+ MN was 8–11 times higher in these men than in a group of five younger men aged below 30 years, who showed expected rates of Y micronucleation.

Autosomes

The involvement of other chromosomes than X and Y in human MN is poorly known. In anaphases of female lymphocytes, autosomes comprised the majority of laggards (Catalán *et al.*, 2000; Falck *et al.*, 2002). However, autosomes seemed to form MN clearly less efficiently than the X chromosome or acentric fragments, at a rate of only 11 (without Cyt-B) and 8% (with Cyt-B) (Falck *et al.*, 2002). They were probably engulfed in the nuclei due to their proximal location (Catalán *et al.*, 2000) or possibly because they were only delayed in their anaphase movement (Falck *et al.*, 2002) or still had some connection to the spindle (perhaps merotelic orientation; Cimini *et al.*, 2002).

There is some evidence for a preferential inclusion of DNA from chromosome 9 in human lymphocyte MN. Tucker *et al.* (1996) studied binucleate lymphocytes of two t(X;9) females and observed that 16.5% of the X– MN were positive for a chromosome 9 paint signal, which appeared to be higher than expected. Kinetochore signal was absent from 85% of the chromosome 9-positive (9+) MN. In a karyotypically normal woman, studied for comparison, 9.5% of X– MN showed chromosome 9 paint, but this finding was only based on two 9+ MN (Tucker *et al.*, 1996). Fauth *et al.* (1998, 2000) observed by chromosome painting that besides X (11.5% of all MN in two female and one male donor) and Y (11.0% of all MN in a male donor), DNA from chromosome 9 was also over-represented (9.8% of all MN) in lymphocyte MN of two female and one male donor. This effect was suggested to be due to the large heterochromatic block of chromosome 9. Chromosomes 12 and 19 were not found in any MN (Fauth *et al.*, 1998, 2000). The painting technique did not reveal whether the findings concerned an entire chromosome 9 or a fragment. As MN with chromosome 9 could also be highly induced by clastogen treatment (Fauth *et al.*, 1998, 2000; Fauth and Zankl, 1999), fragments were probably involved. It may be of interest in this context that patients with the ICF (immunodeficiency, centromeric heterochromatin instability and facial anomalies) syndrome, who show under-condensation of the heterochromatic blocks of chromosomes 1, 9 and 16 in a portion of their PHA-stimulated lymphocytes, also have a high frequency of micronucleated lymphocytes, and chromosome 1 and (to a lesser extent) chromosomes 9 and 16 appear to be over-represented in the MN (Sawyer *et al.*, 1995; Stacey *et al.*, 1995).

Chromosome 9 over-representation in MN was not, however, observed by Leach and Jackson-Cook (2001), who used spectral karyotyping (SKY) technology to probe the contents of lymphocyte MN in three female donors. Besides a general high contribution of the X chromosome in MN, two of the donors showed a high prevalence (22.2 and 13.1%) of MN with DNA from chromosome 19. Pancentromeric FISH showed a single C+ signal in most of the MN, but these data were not reported for MN harbouring individual chromosomes. When the accuracy of the SKY data was checked by using chromosome-specific DNA probes for 11 chromosomes, the assignments agreed in 71.1% of cases (detailed data not shown). Thus, the SKY technique, developed for metaphase chromosomes, appeared to require some refinements for a more accurate application to interphase nuclei.

Migliore *et al.* (1995) observed that 33% of lymphocyte MN of a male donor hybridized with a β-satellite DNA probe identifying group D (13, 14 and 15) and group G autosomes (21 and 22). This suggested that acrocentric chromosomes could be responsible for the majority of spontaneous chromosome-containing MN. However, when the occurrence of acrocentric chromosomes in MN was further investigated by simultaneous use of the β- and α-satellite probes from cultured lymphocytes of 20 donors, acrocentric chromosomes were found among C+ MN at a rate only slightly higher than the expected value (Scarpato *et al.*, 1996). The micronucleation of acrocentric chromosomes was not age or sex dependent. Richard *et al.* (1994) did not find chromosome 22 in any of the 53 MN they examined from an 80-year-old donor.

Although the non-disjunction of chromosome 21 in cultured lymphocytes was reported to be increased by age, there was no age or sex effect on chromosome 21 micronucleation in healthy persons (Shi *et al.*, 2000). Migliore *et al.* (1997) observed that Alzheimer's disease patients have an increased frequency of C+ MN and suggested that the disease may involve microtubule impairment. When the MN were further characterized with centromeric DNA probes for chromosomes 13 and 21 coupled with a single cosmid for region 21q22.2, the patients had, in comparison with controls, a higher frequency of

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MN with chromosome 21 signals (Migliore *et al.*, 1999a). This led the authors to propose that mosaicism for 21 trisomy could underlie the dementia phenotype of Alzheimer's disease.

Peace *et al.* (1999) described a healthy woman who had exceptionally high frequencies of chromosomal aberrations (12%) and MN (119 per 1000 cells) in her lymphocytes. Chromosome painting revealed that more than half of the MN contained chromosome 2. The reason for the aberrations and the high micronucleation of chromosome 2 was left unclear.

Obviously, data on preferential micronucleus formation by other than sex chromosomes are presently conflicting. The micronucleation of fragments from chromosomes with large heterochromatic blocks, especially chromosome 9, is an interesting possibility that has to be checked in further studies. If it turns out to be true, the causes and mechanisms of formation of such MN and their possible influence on the interpretation and conduct of the MN assay would have to be worked out.

MN content after *in vitro* treatment of human cells

In genotoxicity testing it is crucial to understand whether an MN inducer acts via a clastogenic or an aneuploidogenic (or both) mechanism. The mode of action will influence the interpretation of the positive test result and the use of the data in risk assessment. While both structural and numerical chromosome alterations occur in carcinogenesis, there is still much more information on carcinogen-induced chromosome breakage and clastogenic carcinogens than carcinogen-induced numerical chromosome abnormalities and aneugenic carcinogens. Evidence is presently limited in favour of agents purely modulating chromosome number, such as spindle poisons, cytokinesis inhibitors or disrupters of the kinetochore or centrosome, being (complete) carcinogens. As both numerical and structural chromosome aberrations are involved in birth defects and miscarriages, testing for both clastogenic and aneugenic activity is, naturally, highly justified.

During the last 12 years, numerous studies have applied kinetochore or centromere detection to assess the mechanism of action of agents tested for MN induction in human cell cultures (see, for example, Hennig *et al.*, 1988; Eastmond and Tucker, 1989; Fenech and Morley, 1989; Becker *et al.*, 1990; Yager *et al.*, 1990; Robertson *et al.*, 1991; Rudd *et al.*, 1991; Weissenborn and Streffer, 1991; Antoccia *et al.*, 1993; Crofton-Sleigh *et al.*, 1993; Migliore *et al.*, 1993, 1996, 1997, 1998, 1999c,d; Slavotinek *et al.*, 1993; Stopper *et al.*, 1993; Fenech *et al.*, 1994; Kolachana and Smith, 1994; Silva *et al.*, 1994; Surrallés *et al.*, 1995; Van Hummelen *et al.*, 1995; Huber *et al.*, 1996; Kirsch-Volders *et al.*, 1996; Parry *et al.*, 1996; Dopp *et al.*, 1997; Fimognari *et al.*, 1997, 1999, 2001; Schuler *et al.*, 1997; Sgura *et al.*, 1997; Vlachodimitropoulos *et al.*, 1997; Vlastos and Stephanou, 1998; Digue *et al.*, 1999; Gonzáles-Cid *et al.*, 1999; Murg *et al.*, 1999a,b; Andrianopoulos *et al.*, 2000; Buckvic *et al.*, 2000; Nesti *et al.*, 2000; Laffon *et al.*, 2001; Bakou *et al.*, 2002; Decordier *et al.*, 2002). The overall outcome from such exercises has been very promising. Clastogens have preferentially produced K– or C– MN, while aneugens have induced K+ or C+ MN. As pointed out by Schuler *et al.* (1995), there are relatively few genotoxic agents that induce solely one type of MN. For instance, several clastogens also appear to induce some K+ or C+ MN, although the mechanisms behind this apparent aneugenic activity are not known in detail (see, for example, Eastmond and Tucker, 1989; Slavotinek *et al.*, 1993; Schuler *et al.*, 1997; Vlachodimitropoulos *et al.*, 1997; Vral *et al.*, 1997; Murg *et al.*, 1999a; Ramírez *et al.*, 1999b; Touil *et al.*, 2000; Ponsa *et al.*, 2001).

For some agents assayed *in vitro*, there is evidence for a chromosome-specific effect. Migliore *et al.* (1995) used a DNA probe identifying the β-satellite region (pericentric heterochromatin) in cultured male lymphocytes to show that vanadium compounds preferentially induce the micronucleation of acrocentric group D and G chromosomes. Although sodium orthovanadate also specifically induced loss and gain of the X chromosome rather than chromosome 2, there were no clear differences in the occurrence of the two chromosomes in MN (Migliore *et al.*, 1999d). The X chromosome and chromosome 2 also did not differ from each other for MN induction by griseofulvin, but estramustine favoured the micronucleation of chromosome 2 instead of the X chromosome (Migliore *et al.*, 1999d). The X chromosome did not seem to be over-represented in lymphocyte MN induced by vinblastine or colchicine (Parry *et al.*, 1996). Colchicine was, however, reported to induce mostly C-band-positive K+ MN, indicating preferential micronucleation of acrocentric chromosomes (Caria *et al*., 1996). In another study with colchicine (Wuttke *et al.*, 1997), chromosome 7 paint signal was observed 1.5 times more frequently than would be expected on the basis of random distribution of chromosomes; chromosome 2 seemed to be micronucleated at the expected rate. With diethylstilbesterol, another aneugen, material from chromosomes 14, 19 and 21 was significantly more frequently micronucleated in comparison with MN from control cultures (Fauth *et al.*, 2000). Bentley *et al.* (2000) used chromosome-specific centromeric probes (1, 8, 11, 18, X and 17) to study aneuploidy events in female lymphocytes treated with benomyl and carbendazim. Although the X chromosome was slightly more sensitive than the other chromosomes to non-disjunction induction by both chemicals, no differences between the six chromosomes were reported with respect to micronucleus induction. 1,2,4-Benzenetriol, tested in cultured human lymphocytes, was observed to induce more MN with the chromosome 8 centromere than the chromosome 7 centromere (Chung *et al.*, 2002).

The clearest case of chromosome-specific MN induction concerns some clastogens, particularly base analogues preferentially inducing MN harbouring DNA from chromosomes that contain large heterochromatic blocks. 5-Azacytidine is known to induce C– and K– MN and a specific dose-dependent under-condensation of the heterochromatic regions in chromosomes 1, 9, 15, 16 and Y (Guttenbach and Schmid, 1994; Cimini *et al.*, 1996). After an *in vitro* treatment with 5-azacytidine, a significant fraction of male lymphocyte MN showed hybridizations with these chromosomes, but not with chromosomes 11, 17 and X (Guttenbach and Schmid, 1994). Similar results were obtained by Fauth *et al.* (1998), who observed by chromosome painting that an *in vitro* treatment of cultured human lymphocytes with 5-azacytidine resulted in the micronucleation of DNA from chromosomes 1, 9 and 16. It was suggested that under-methylation of the large heterochromatic region in these chromosomes may be associated with their micronucleation. As 5-azacytidine cannot be methylated, its incorporation in DNA leads to changes in the extent and pattern of cytosine methylation, which may influence the conformation of DNA and impair the binding of DNA-interacting proteins such as topoisomerase II and the kinetochore complex (Stopper, 1997). Smith *et al.* (1998) studied the micronucleation of chromosome 16 in human lymphoblas-

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toid TK6 cells by 2,6-diaminopurine, another nucleotide analogue known to enhance the under-condensation of the paracentromeric heterochromatin in chromosomes 1, 9 and 16. Chromosome 16 classical satellite DNA (together with pancentromeric DNA) was preferentially found in the induced MN. The MN were likely to harbour a fragment resulting from breakage within the centromeric area of chromosome 16. Likewise, DNA from chromosomes 1 and 9 was over-represented in human lymphocyte MN after an *in vitro* treatment with idoxuridine, which induces a specific decondensation at chromosome 9q12 and (less so) at 1q12 (Fauth and Zankl, 1999). Similar findings were obtained in studies with mitomycin C, which also induces under-condensation of chromosomes 1 and 9; 62–69% of all MN in mitomycin C-treated lymphocytes contained DNA from chromosome 9 (Fauth *et al.*, 2000). The MN induced by mitomycin C were devoid of the α-satellite of chromosome 9, suggesting that most of the breaks were induced in the pericentromeric heterochromatin (Kusakabe *et al.*, 1999).

It is unclear how the under-condensed chromosome regions would break and form MN. Smith *et al.* (1998) suggested that the breakage results from torsional strain generated by movement of the chromatids towards opposite poles during anaphase. 5-Azacytidine-treated mouse L5178Y cells examined by ultravital UV microscopy showed normal metaphase arrangements, but chromatid separation in anaphase was disturbed with the formation of thin chromatin bridges which were occasionally ruptured, generating MN (Stopper *et al.*, 1993; Stopper, 1997).

When chromosome painting (chromosomes 1, 7, 11 and 14) was used to characterize MN induced by γ-radiation in various human cell lines, MN with DNA from chromosome 7 appeared to be under-represented (Slavotinek *et al.*, 1996). In human diploid skin fibroblasts, the incorporation of DNA from chromosomes 2 and 7 in MN after γ-irradiation was significantly greater than expected by chromosome size, while smaller chromosomes (11 and 16) were micronucleated as expected (Walker *et al.*, 1996). In cultured human lymphocytes, painting of chromosomes 1, 7, 11, 14, 17 and 21 suggested that DNA from these chromosomes is found in γ-ray-induced MN according to the DNA content of each chromosome, supporting a random model for radiation-induced chromosome damage (Fimognari *et al.*, 1997). Wuttke *et al.* (1997) could not see any difference between the expected and observed inclusion of chromosome 2 and 7 material (detected by chromosome painting) in human lymphocyte MN after X-ray treatment. Thus, there does not presently seem to be consistent evidence for a preferential micronucleation of DNA from certain chromosomes after an *in vitro* treatment with ionizing radiation.

Effect of *in vivo* exposure on micronucleus contents

As results on C+ or K+ MN in humans exposed to environmental agents started to appear only recently (Titenko-Holland *et al.*, 1996, 1997; Surrallés *et al.*, 1997), there is as yet little information on which to judge whether this approach could really improve the sensitivity of the MN assay. In principle, this should be the case, since the exposure studied is usually expected to induce only one type of MN. As exposure effects in biomonitoring studies are expected to be small in comparison with the situation *in vitro*, a specific analysis of the right type of MN might considerably increase the sensitivity of detecting the exposure effect (Norppa *et al.*, 1993a,b).

Studies of *in vitro* irradiation of human G_0 lymphocytes with low doses of ^{60}Co γ-rays suggested that the centromere MN assay has a detection limit of 0.1–0.2 Gy, considered to be the only assay that can combine high sensitivity with a reasonable scoring time for biomonitoring of large populations (Thierens *et al.*, 1999b). Some data exist to support this expectation. A 16.6-fold increase was seen in buccal cell MN in a patient after 6500 rad photon radiation to the head and neck; all MN induced were C– (Moore *et al.*, 1996). MN frequency returned back to baseline 3 weeks after the therapy. Thyroid cancer patients treated with radioactive iodine (^{131}I) showed increased frequencies of lymphocytes with C– MN, with some increase also in C+ MN (Ramírez *et al.*, 1997). In nuclear power plant workers with radiation doses below the yearly limit of 20 mSv, no statistically significant differences in C– MN yields associated with the radiation dose could be demonstrated (Thierens *et al.*, 1999a). However, a linear regression fit to individual MN data indicated (although with a low correlation coefficient of 0.10) an increase in C– MN (but not C+ MN) by 0.025 MN/mSv, corresponding to 0.5 MN/year at the maximum tolerable dose. Likewise, uranium miners had a significantly lower proportion of C+ MN (62.1%) than unexposed referents (74.6%), although no statistically significant difference was seen in the overall MN frequency (Kryscio *et al.*, 2001). Chang *et al.* (1999) observed a decrease in the ratio C+ MN:C– MN in lymphocytes of subjects with chronic low dose exposure to γ-radiation from ^{60}Co-contaminated steel in radioactive buildings; the frequency of MN appeared to be increased also for C+ MN. *In vitro*, γ-rays also induced some increase in C+ MN, although the great majority of the effect was observed in C– MN (Thierens *et al.*, 1999b). Thus, it was surprising that an increase in MN frequencies in lymphocytes of hospital workers occupationally exposed to X- and γ-rays was observed to be exclusively associated with an increase in C+ MN (Thierens *et al.*, 2000).

As regards chemical exposure, Titenko-Holland *et al.* (1996) observed a 9- and 2-fold increase in C– MN in buccal and nasal cells, respectively, of mortuary science students after exposure to formaldehyde. The effect on nasal cells could not be seen from total MN frequencies. C+ MN were increased >2-fold in buccal cells, but showed no change in nasal cells. The frequency of C+ MN in cultured binucleate lymphocytes was observed to be 2.4 times higher in alcoholics than control persons, which was suggested to indicate an aneugenic effect of alcohol (Maffei *et al.*, 2000). A group of subjects exposed to high levels of inorganic arsenic in drinking water showed, in comparison with matched controls, a 1.8-fold frequency of MN in exfoliated bladder cells, both C– and C+ MN being apparently affected (Moore *et al.*, 1996). The frequencies of both types of MN also correlated with urinary arsenic and its metabolites. Atenolol, a β-adrenergic blocker antihypertensive drug, was reported to increase C+ MN (6.8-fold, as compared with untreated controls) but also C– MN (2.7-fold) in cultured lymphocytes of a small group of patients (Télez *et al.*, 2000).

One of the puzzling questions in the use of the MN assay in human biomarker studies has been the fact that an effect of smoking on MN frequency is not usually seen (Norppa *et al.*, 1993a). In large-scale studies, smoking has been demonstrated to induce chromosomal aberrations, but no effect of smoking on MN frequency has been observed (Galloway *et al.*, 1986; Milillo *et al.*, 1996; Surrallés and Natarajan, 1997; Barale *et al.*, 1998). If these findings reflect the fact that chromosomal fragments represent, depending on the age of the donors, only

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a fraction of the MN, an increase in MN in smokers might be seen by a specific look at C– or K– MN. However, Thierens *et al.* (1999, 2000) observed no effect of smoking on the frequency of C– or C+ MN in studies of nuclear power plant workers, hospital workers and their controls. Nath *et al.* (1995) did not observe any difference in the frequency of K– MN in binucleate lymphocytes of smokers and non-smokers. These findings would suggest that fragments identified in MN by the lack of centromeric label are not representative of fragments induced by smoking or breakage observed in metaphase cells in smokers. One of the explanations could be that smoking appears to be primarily associated with chromatid type breaks (see Scarpato *et al.*, 1997), a subfraction of breaks whose contribution to C– MN in smokers is not known. It could also be speculated that MN with fragments include products of spontaneous breakage events at heterochromatin that are expressed only at anaphase but not yet in metaphase, making it difficult to show the clastogenic effect of smoking by MN analysis.

Conclusions

The contents of MN in human cells have mostly been characterized in cultured lymphocytes. Identification of centromere DNA and kinetochore proteins in MN has indicated that MN consist of acentric chromosomal fragments and entire chromosomes and that the well-known age-dependent increase in lymphocyte MN frequency primarily reflects an increase in the latter type of MN.

A closer look at the chromosomal contents of the spontaneous MN in human lymphocytes has revealed that the age effect is mostly due to excessive micronucleation of the X and Y chromosomes. The X chromosome, over-represented in MN in both females and males, is particularly prevalent in MN of women and may be responsible for the majority of MN in elderly women, especially when binucleate cells produced by Cyt-B are examined.

The high prevalence of the X chromosome in binucleate lymphocyte MN appears to reflect the lower micronucleation of fragments and autosomes and exaggerated micronucleation of the X chromosome in Cyt-B-treated anaphase/telophases which do not elongate or form a contractile ring. Regarding fragments in spontaneous MN, there is suggestive evidence that DNA from chromosome 9 would be included in lymphocyte MN at a higher rate than expected. This might reflect increased fragility of the pericentromeric heterochromatin in chromosome 9, possibly resulting in chromosomal breakage in anaphase. Preferential breakage at the heterochromatic block in chromosomes 1, 9 or 16 has been described in treatment of human cells with some clastogenic agents *in vitro*.

The identification of whole chromosomes in MN has proved to be a useful tool in identifying whether a test agent acts via a clastogenic or aneugenic mechanism *in vitro*. Discrimination between acentric fragment-containing (without centromere) and chromosome-containing (with centromere) MN can be expected to be even more important in studies of human genotoxic effects *in vivo*, and for some clastogenic exposures there is evidence to support this view. However, the centromere MN assay has not been able to shown an increase in C– MN in lymphocytes of smokers, although smoking is known to produce chromosomal aberrations. This may suggest that fragment-containing MN and chromosomal aberrations cover partly different phenomena. Many questions on MN content in lymphocytes and especially in other human tissues used in biomarker studies remain open. The further characterization of MN contents is crucial for the understanding and correct use of the MN assay in genetic toxicology.

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