Micronuclei containing whole chromosomes harbouring the selectable gene do not lead to mutagenesis

Inge Eckert1, William J. Caspary2,3, Michael Nütse4, Melissa Liechty4, Lisa Davis4 and Helga Stopper1

1Department of Toxicology, University of Würzburg, 97074 Würzburg, Germany, 2National Institutes of Health, Research Triangle Park, NC 27709, USA, 3GSF-Flow Cytometry 85764 Neuherberg, Germany and 4Applied Genetics Laboratories Inc., 1335 Gateway Drive, Melbourne, FL 32901, USA

To whom correspondence should be addressed

Loss of heterozygosity is one genetic change observed in many tumours. We do not know whether the loss of chromosomal material through micronucleus formation is a viable mechanism associated with, and possibly leading to, genetic disease. Previously, we treated L5178Y mouse lymphoma cells with four aneugens. Although these aneugens induced micronuclei containing predominantly whole chromosomes, they did not induce mutations at TkI, the selectable gene, under the same non-toxic conditions in which they induced micronuclei. This suggested that the induction of micronuclei containing whole chromosomes was not an early event leading to phenotypically expressed mutations in these cells under the conditions used. However, it is possible that chromosome 11, on which TkI resides, may be under-represented in the micronucleus population. To find out the frequency of induction of micronuclei containing chromosome 11, we applied fluorescence in situ hybridization using a chromosome 11 paint to micronuclei induced by colcemid and vinblastine. We found that the numbers of micronuclei containing chromosome 11 are more than sufficient to be detectable as mutations if these micronuclei lead to viable mutants. We conclude that the formation of micronuclei containing whole chromosomes does not lead to viable, dividing mutants in this system.

Introduction

Micronuclei reside in the cytoplasm of cells and form during the process of cell division. They contain chromatin as whole chromosomes or chromosomal fragments and are surrounded by a membrane. The loss of chromosomal material may mediate human genetic diseases such as cancer, where loss of heterozygosity characterizes tumour suppressor gene inactivation (Weinberg, 1993; Hinds and Weinberg, 1994). Since micronuclei contain whole chromosomes or chromosomal fragments, it is possible that their formation may be a mechanism leading to mutations responsible for human genetic diseases. However, we do not know whether monosomy of a single chromosome mediated by micronucleus formation is a viable mechanism leading to genetic disease.

The induction of micronuclei in mammalian cells is a widely used toxicological endpoint to assess the potential genotoxic effects of environmental chemicals (Fritzenschaf et al., 1993; Matsuoka et al., 1993; Müller et al., 1993; Shelby et al., 1993; Stopper et al., 1993a,b; Stopper et al., 1992; Müller et al., 1995; Zhang et al., 1995). It is also used to biomonitor human populations for exposure to potential toxic chemicals (Fenech, 1993). However, the biological consequences of the formation of micronuclei are unknown.

Our long-range goal is to determine whether the formation of micronuclei has biological consequences that could cause genetic disease in humans. Specifically, we wanted to test the hypothesis that the loss of chromosomal material via the formation of micronuclei can lead to genetic changes resulting in viable cells. As a model for studying the relationship between these two endpoints, we used L5178Y mouse lymphoma cells, which can detect both micronucleus formation and mutation (Stopper et al., 1993a,b, 1995). TkI resides on the distal end of chromosome 11 in these cells (Kozak et al., 1975; Hozier et al., 1991). Karyotype analysis of L5178Y mouse lymphoma cells indicates that the modal chromosome number in the wild-type population is 40 (Hozier et al., 1981; Blazak et al., 1986). Although the G-banded karyotype of these cells differs markedly from the normal mouse karyotype, the two homologous chromosomes 11 appear cytogenetically normal and can be distinguished by heteromorphism at the centromere (Hozier et al., 1981; Blazak et al., 1986).

Previously we have investigated the treatment of L5178Y mouse lymphoma cells with four known aneugens: colcemid, diethylstilboestrol (DES), griseofulvin or vinblastine to discover whether these micronuclei are intermediates in the process leading to mutation. We established that these compounds induced micronuclei containing predominantly whole chromosomes in L5178Y mouse lymphoma cells under non-toxic conditions and therefore demonstrated their ability to potentially induce monosomy. However, these compounds did not induce mutations at TkI in these cells under these same non-toxic conditions as they induced micronuclei (Stopper et al., 1994). This suggested that the induction of micronuclei containing whole chromosomes was not an early event leading to phenotypically expressed mutations in these cells. However, we did not rule out the possibility that the numbers of micronuclei containing chromosome 11 were insufficient to produce enough mutants to be detected by the mutation assay.

To examine the possibility that chromosome 11 is under-represented in the micronucleus population, we performed in situ hybridization experiments using a chromosome 11-specific probe for the mouse (whole chromosome painting). The purpose was to determine whether the frequency of chromosome 11 in vinblastine- and colcemid-induced micronuclei was high enough to meet the threshold for detecting mutation in these cells. Whole chromosome painting of micronuclei is a technique that, so far, has not been used for the analysis of chemically-induced micronuclei, although it has been used on radiation-induced micronuclei (Slavotinek et al., 1996; Fimognari et al., 1997). This manuscript reports the results of this study.

Materials and methods

Cell culture

Mouse L5178Y cells, clone 3.7.2c (Clive, 1972), were cultured in suspension in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with...
95 U/ml penicillin, 95 μg/ml streptomycin, 0.25 mg/ml l-glutamine, 107 μg/ml sodium pyruvate and 10% heat-inactivated horse serum (Sigma Chemie GmbH, Deisenhofen, Germany). Cell cultures were grown in a humidified atmosphere with 5% CO₂ in air at 37°C.

**Induction and isolation of micronuclei.** Exponentially growing mouse L5178Y cells which have a doubling time of ~10 h were treated for 4 h. The vehicle control was 1% dimethyl sulphoxide (DMSO). After removing the chemicals by centrifugation (140 g) and medium replacement, the cells were incubated for 5 h (expression time). Isolation of micronuclei and nuclei in suspension was performed according to the method published earlier (Miller and Nisse, 1993; Nisse et al., 1996). Briefly, ~1×10⁶ cells that had been treated were centrifuged (140 g) and treated with 1 ml of solution 1 (584 mg/l NaCl, 1000 mg/l sodium citrate, 25 mg/l ethidium bromide, 10 mg/l RNase and 0.3 ml/l Nonidet P-40). Then, after ~1 h, 1 ml of solution 2 (1.5% citric acid, 0.25 M sucrose and 40 mg/l ethidium bromide) was added. Sorting of micronuclei on glass slides was performed using the FACStar plus cell sorter (Becton-Dickinson, Mountain View, CA, USA). For details see Nisse et al. (1996). The slides were then dried briefly at room temperature and the micronuclei were fixed in methanol (~20°C) for hybridization.

**In situ hybridization.** This was performed using biotinylated mouse chromosome 11-specific paint that we prepared by chromosomal microdissection and amplification (Liechty et al., 1995). Briefly, fixation was performed in methanol (~20°C). Slides were aged for at least 3 days and up to 2 months. The painting probe was prepared by placing it at 37°C for 5 min, 75–80°C for 5 min and 37°C for 2 h. The slides were denatured at 72°C for 2 min in formamide solution (70% in 2X sodium chloride/sodium citrate (SSC), pH 7.0). After dehydration in an ethanol-series (70, 85, 90, 100% on ice, 2 min each), slides were air-dried. Next, slides were put on a slide warmer at 42°C and 10 μl of chromosome painting probe was added for overnight incubation in a humid chamber at 37°C. Slides were then washed for 3×5 min in formamide wash solution (55% in 2X SSC, pH 7.0) at 45°C, 3×5 min in 1X SSC at 45°C and then at least 5 min in 4X SSC at room temperature. Detection and signal amplification was performed using materials and protocol of Clontech for fluorescent isothiocyanate (FITC) detection of biotinylated DNA. If required, additional amplification steps were performed until the staining quality was good. A counterstain of whole DNA was achieved with bisbenzimide 33258 (5 μg/ml, 5 min). In the final step, a second counterstain of whole DNA was achieved with staining Vectashield Mounting Medium (Oncor Co., Heidelberg, Germany) containing propidium iodide. The isolated micronuclei suspension contains some cellular debris of similar size. To avoid counting of cellular fragments only those particles were counted as micronuclei that were round or oval and were between about 1/20 and 1/4 of the size of a nucleus. Micronuclei were examined for chromosome 11 FISH signals and the percentage of signal-positive micronuclei was determined. As a control, nuclei were deposited on the same slide and treated with the FISH probe. Numbers were added from several different slides from one experiment to achieve total numbers of scored micronuclei.

**Results**

We prepared metaphases of L5178Y mouse lymphoma cells and painted these preparations with a chromosome 11 probe (Figure 1). In these pictures, there are no more than two signals indicating the presence of the painted homologous chromosomes and attesting to the fact that these paints are highly specific. The slides (Figure 1) also showed high sensitivity with negligible background signal.

We treated L5178Y mouse lymphoma cells with colcemid at 0.1 μg/ml or with vinblastine at 0.025 μg/ml for 4 h. After 5 h (within the first cell cycle duration after treatment) the micronuclei were separated from the nuclei by a flow sorting technique. The flow sorting technique separates the micronuclei from the rest of the cell and only the chromatin material from the micronuclei was deposited onto the slide. The advantage of this approach is that many micronuclei are found on one slide.

We found the representation of chromosome 11 is 10% (Table I). When the data is resolved by test chemical, colcemid and vinblastine induce micronuclei with 10.7 and 9.2% of these micronuclei containing chromosome 11.

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**Discussion**

In this manuscript we ask whether the numbers of micronuclei containing chromosome 11 are sufficient to produce enough mutants to meet the threshold level of sensitivity for mutant detection in L5178Y mouse lymphoma cells. The results show that 10% of the micronuclei contained chromosome 11, which is more than sufficient to meet the sensitivity requirements of the assay.

In our previous paper, we reported that 0.1 μg/ml colcemid induced 60 micronuclei per 2000 cells and 0.025 μg/ml vinblastine induced 50 micronuclei per 2000 cells (Stopper et al., 1994). Untreated cultures showed micronucleus frequencies of ~4 per 2000 cells and, therefore, nearly all the micronuclei in the treated cultures are induced by the chemical treatment. We also reported that >90% of these micronuclei contain kinetochores indicating that these micronuclei contain primarily whole chromosomes.

We used these same doses for our fluorescence in situ hybridization (FISH) analysis. Based on these results, ~2.5% of the cells (50–60 micronuclei per 2000 cells) produced micronuclei at the doses used. Mutation fractions are expressed in terms of mutations per 10⁶ viable cells. Thus, of 1×10⁶ viable cells, 25×10⁵ contain micronuclei. The percentage of micronuclei that contains chromosome 11 is 10%. The TkI⁺ allele would reside in half; 5% of 25×10⁵ viable cells is ~1250 mutants which is more than sufficient to be detectable as mutations if cells containing these micronuclei lead to viable mutants. Since we see no TkI⁻ mutations in these cells under the same non-toxic conditions in which micronuclei are formed, we conclude that the formation of micronuclei containing whole chromosome 11 does not lead to viable TkI⁻ mutants.

We used the in situ protocol of the assay that accurately detects the mutation fraction by capturing a greater proportion of the slowly growing colonies than do conventional protocols (Rudd et al., 1990; Spencer and Caspary, 1994; Spencer et al., 1994). In this procedure, cells were immobilized immediately after treatment and the proportion of slowly growing mutants to the total population remained constant during the cloning phase of the assay. This procedure led to a mutation rate that was 50-fold greater than that found with the conventional protocol because slowly growing mutants were not diluted out.
and each mutation led to one mutant colony (Rudd et al., 1990). We found that there was less variability in the results when compared with conventional protocols since the small colonies were not diluted out. However, this protocol, as all other protocols for this assay, will not detect cytostatic mutants (mutants that are metabolically viable but do not divide).

There are four possible reasons for the refractivity of the mutagenic response of L5178Y cells to the aneugens under the test conditions used even though these compounds induce micronuclei in these cells.

The first possibility is that the mutation assay cannot detect the frequency of mutants formed by the fraction of cells with induced micronuclei. The results shown in this paper rule out this possibility.

A second possibility is that all micronuclei or just those containing chromosome 11 re integrate into the nucleus of the cell reincarnating a wild-type cell that does not possess a mutant phenotype. However, although reintegration of micronuclei is theoretically possible, it would require the micronucleus to be in cycle with the main nucleus to be successful; otherwise, the result may be premature chromosome condensation in the micronucleus and subsequent destruction of that DNA (Obe et al., 1975). Furthermore, most or all micronuclei would have to be reintegrated to explain our findings. Thus, we consider this explanation unlikely. However, if it were possible, it implies that the formation of micronuclei with whole chromosomes has no long-term biological consequences.

The third possibility is that cells with micronuclei containing whole chromosome 11 are cytostatic. However, cytostatic mutants, if they exist, may not have any biological consequences precisely because they do not divide.

The fourth possibility is that cells with micronuclei containing whole chromosomes 11 die. This perhaps is the most likely possibility. Although we have no direct evidence of this, we examined many mutants from this cell line for chromosomal lesions. Although we have found mutants containing two chromosomes containing the Tkla allele (the inactive allele), we have not found any that contain only one chromosome 11 (M.C. Liechty, unpublished results). There has been one report of a viable cell line containing only one chromosome 11 (Evans et al., 1986). However, painting of this cell line indicates that fragments of chromosome 11 have been distributed throughout the genome (M.C. Liechty, unpublished results). Thus monosomy, if it occurs, probably leads to an unstable state and requires reduplication for the cell to remain viable.

We anticipate that the results found here for colcemid and vinblastine also apply to DES and griseofulvin, the other two compounds examined in our previous paper (Stopper et al., 1994), which induced micronuclei but no mutation under the conditions used.

A recent publication reported that several laboratories found DES to be mutagenic in L5178Y cells in the presence of S9 (Sofuni et al., 1996). The mutagenicity of DES confirms our previous results showing that DES is mutagenic in the presence of S9 (Mitchell et al., 1988; Myhr and Caspary, 1988). However, we were unable to demonstrate mutagenic activity in the absence of S9. The conditions described in this paper are in the absence of exogenously added S9. Since we do not know whether DES produces micronuclei containing whole chromosomes when S9 is present, other mechanisms may be responsible for its mutagenicity.

This same paper also reports the mutagenic activity of griseofulvin (Sofuni et al., 1996). However, this mutagenicity occurred under conditions of substance precipitation. Although we used a comparable dose range, microscopic inspection did not reveal any precipitation <150 μg/ml where we saw micronucleus formation. Since no detailed description of experimental procedures is given by Sofuni et al. (1996), the reason for the different precipitation behaviour is not known. However, when a compound precipitates, the compound can be carried with the cells beyond the 4 h treatment time, effectively increasing the dose to which the cells are exposed. This potentially allows for increases in metabolism of the compound by enzymes that exist in these cells at low levels. Thus, the precipitated compound may have exerted non-aneugenic effects. Under our treatment conditions we found >87% of the induced micronuclei to be kinetochore positive, which indicates that the micronuclei contain whole chromosomes (Stopper et al., 1994).

In our previous paper (Stopper et al., 1994) we asked whether micronuclei containing whole chromosomes are precursors to mutations. Our conclusion was negative, i.e. under conditions where micronuclei containing whole chromosomes are produced, mutations were not observed. However, we were unable to rule out the possibility that the chromosome on which mutation is detected was underrepresented under the conditions where we induced micronuclei. The present paper clearly shows that the number of induced micronuclei containing the chromosome harbouring the selectable gene is sufficient.

It should be noted that the data from this and our previous paper (Stopper et al., 1994) does not rule out the possibility that aneugens may induce mutations under some conditions. However, these data do indicate that loss of one of the two chromosomes 11 through micronucleus formation during the first mitosis after substance treatment does not lead to viable, dividing mutants in this system.
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