Analysis of radiation-induced micronuclei by FISH using a combination of painting and centromeric DNA probes

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In situ hybridization with whole chromosome painting probes (chromosome 1, 7, 11, 14, 17 and 21) in combination with a human pancentromeric α-satellite probe was used to analyse the presence of specific chromosomal material in micronuclei (MN) induced in human lymphocytes by ionizing radiation. The purpose was to investigate the nature of radiation-induced cytogenetic damage, especially to study whether the fraction of paint-positive MN is proportional to the relative DNA content of the respective chromosomes which might indicate a random breakage of chromosomes. Flow-sorted MN and MN in binucleated cells were analysed with the six chromosome specific painting probes. It was found that the fraction of paint-positive MN increased linearly with the DNA content of the respective chromosomes. About 13% radiation-induced MN in human lymphocytes were found to contain centromeric signals independent of the presence of specific chromosome painting signals. The data obtained on flow-sorted MN and MN in binucleated cells agreed well, indicating that flow-sorted MN can be used for studying their chromosomal content with the FISH technique. If it is assumed that the chromosomal content of MN reflects radiation-induced damage, then these results support a random model of radiation-induced cytogenetic damage in human lymphocytes for the six chromosomes studied.

Introduction

Micronuclei (MN) are small, DNA-containing particles found outside the cell nucleus in the cytoplasm of interphase cells. The frequency of MN in cell cultures or cultured human peripheral lymphocytes can be used as a quantitative measure of both structural and numerical chromosome aberrations induced in cells by ionizing radiation or chemicals. MN can arise fromacentric chromosome or chromatid fragments, from one or several chromosomes, or by various combinations of these events. The relative amount of these events depends on the clastogenic or spindle damaging property of an inducing compound. Recently, fluorescence in situ hybridization (FISH) techniques have been used to study in detail the chromosomal content of MN using centromeric DNA probes (Becker et al., 1990; Miller et al., 1991; Salassidis et al., 1992; Migliore et al., 1993; Nüsse et al., 1996; Huber et al., 1996), combinations of centromeric and telomeric DNA probes (Miller et al., 1992; Miller and Nüsse, 1993; Schriever-Schwemmer and Adler, 1994) and painting probes (Slavotinian et al., 1996). In addition, the loss of certain chromosomes in MN due to malsegregation has been studied by labelling marker chromosomes in transgenic mice with foreign DNA (Boei and Natarajan, 1995).

On the other hand, FISH techniques with chromosome painting probes have, recently, greatly facilitated the scoring of chromosomal aberrations induced by ionizing radiation. A mathematical model has been used to calculate the frequency of aberrations from the frequency of aberrations in a subset of painted chromosomes. Usually, such a model assumes that radiation-induced chromosome breaks are random. Some experimental evidence suggests that a random model of radiation-induced breakage is correct. However, several studies have shown that spontaneous and radiation-induced chromosome breakage may not be random. It has been found that some chromosomes are involved more frequently in radiation-induced aberrations than would be expected on the basis of their length or DNA content (Buckton, 1976, 1983; Bauchinger and Götz, 1979; Lee and Kamra, 1981; Dutrillaux, 1983; Kano and Little, 1986; Barrios et al., 1989, with conventional cytogenetics; Lucas et al., 1992; Knehr et al., 1994, 1996, with the FISH technique).

Recently, we used FISH with whole chromosome painting probes for chromosomes no. 1, 7, 11 and 14 to paint radiation-induced MN in three different human cell lines with differential radio-sensitivity (Slavotinian et al., 1996). The purpose of those earlier experiments was to study whether the fraction of paint-positive MN (MN+) is proportional to the relative DNA content of the respective chromosome. Such a proportionality should indicate a random breakage of the chromosome, if it is assumed that theacentric fragment produced by chromosome breakage will form a MN in an unbiased way. To perform those experiments, the presence of materials from chromosomes no. 1, 7, 11 and 14 was analysed in MN found in binucleated cells (CB technique) or in flow-sorted MN. With both techniques it was found in all three cell lines that chromosome 7 was under-represented in the numbers of signal-positive MN among the chromosomes examined.

We report here on a continuation of these experiments in human lymphocytes by studying fragments of specific chromosomal origin in MN using chromosome paints for chromosomes no. 1, 7, 11, 14, 17 and 21. In addition, the presence of centromeric regions in MN was analysed using a pancentromeric probe simultaneously with painting probes.

Materials and methods

Isolation, cultivation and irradiation of human lymphocytes

Blood samples were obtained from three healthy donors aged 25–35 years. Lymphocytes were separated using LeucoPrep tubes (Becton Dickinson, Heidelberg, Germany), centrifuged at 1500 g for 25 min at room temperature. After two washes in phosphate-buffered saline (PBS), lymphocytes were resuspended at a concentration of 3 x 10^9/ml in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Berlin, Germany) containing 15% fetal calf serum (Seromed, Berlin, Germany), 2% phytohaemagglutinin A (Wellcome, Burgwedel, Germany) and incubated at 37°C. Lymphocytes were irradiated with 2 Gy 137Cs γ-rays from a HWM-2080 machine (Siemens, Erlangen, Germany) at a dose rate of 1.2 Gy/min.
Preparation of binucleated cells

At 44 h after the start of incubation, 5 μg/ml cytochalasin B (cyt B; Sigma, Deisenhofen, Germany) was added to a 2 ml fraction of the original culture. After 72 h incubation at 37°C, cultures were stopped. Cells were treated with hypotonic solution and fixed in ice-cold 3:1 methanol/acetic acid. Slides were stored in nitrogen gas at -20°C.

Preparation of a suspension of micronuclei and nuclei for flow cytometry and sorting

At 72 h, a suspension of nuclei and MN was prepared as described in detail by Viaggi et al. (1995) using immunomagnetic isolation of the CD2+ lymphocyte subpopulation. MN and nuclei were stained with ethidium bromide (EB) and Hoechst 33258 (HO) according to the procedure described by Schreiber et al. (1992).

Flow cytometry and sorting

Dual laser flow cytometry and sorting was performed using a FACStar+ flow cytometer (Becton Dickinson) equipped with an argon laser (Innova 90; Coherent Radiation, Palo Alto, CA, USA) operating at 488 nm (500 mW) for excitation of the EB and a second laser (Innova 100; Coherent Radiation) adjusted to the UV multilines (351.1-363.8 nm, 300 mW) for the excitation of HO. For further details of the measurement of MN see Viaggi et al. (1995).

Results

The fraction of paint-positive MN is shown in Figure 2 as a function of the DNA content of the chromosomes [DNA content of human chromosomes according to Morton (1991): 8.22 (chromosome 1), 5.34 (chromosome 7), 4.50 (chromosome 11), 3.41 (chromosome 14), 2.87 (chromosome 17) and 1.56 (chromosome 21)]. The results presented in Figure 2 and Table I show that the fraction of paint-positive MN depend on the relative EB and HO fluorescence (1-20% of the DNA content of G1-phase nuclei) were sorted onto clean microscope slides. G1-phase nuclei were also sorted onto another position of the same slide to check for satisfactory hybridization signals. The slides were air-dried, fixed in 100% ice-cold methanol and stored in nitrogen gas at -20°C.

In-situ hybridization

The same in-situ hybridization method was used for both types of slides. The slides were incubated in 100 μg/ml RNase A (Sigma) in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) for 1 h at 37°C, and washed for 2 min in consecutive solutions of 70, 80 and 100% ethanol. The dried slides were denatured with 70% formamide in 2× SSC at 70°C for 2 min and washed in consecutive solutions of ice-cold 70, 80 and 100% ethanol.

Biotinylated whole chromosome paints (Angewandte Gentechnologie Systeme GmbH, Heidelberg, Germany) and the digoxigenin-labelled human centromeric α-satellite (Apphgene, Heidelberg, Germany) probe were denatured in a 70°C water bath for 10 and 5 min respectively. The painting probes were preannealed at 37°C for 30 min-2.5 h according to the manufacturer’s instructions. Hybridization for human centromeric probe was performed for 4 h. Then the whole chromosome painting probe was added to the same slide and the hybridization was carried out overnight at 37°C in a humidified chamber.

Post-hybridization washing was performed in 2× SSC for 5 min at 72°C. The biotinylated probes were detected by incubating the slides with successive layers of avidin–fluorescein isothiocyanate (FITC; 4 μg/ml in 4× SSC and 5% skimmed milk, w/v; Angewandte Gentechnologie Systeme GmbH), biotinylated anti-avidin (0.5 μg/ml in 4× SSC and 5% skimmed milk, w/v; Angewandte Gentechnologie Systeme GmbH) and avidin–FITC. Detection of centromeric signals was performed by means of anti-digoxigenin mouse immunoglobulin (Ig)G 1 (ng/μl in 4× SSC and 5% skimmed milk, w/v; Boehringer-Mannheim, Mannheim, Germany), anti-mouse-lg-digoxigenin 1 (ng/μl in 4× SSC and 5% skimmed milk, w/v; Boehringer-Mannheim) and anti-digoxigenin-AMCA conjugate (50 μg/ml in 4× SSC and 5% skimmed milk, w/v; Boehringer-Mannheim). The cells were counterstained with propidium iodide (0.6 μg/ml antifade). Slides with satisfactory signals were scored with the ×100 objective of a fluorescence microscope (Zeiss) with filters for FITC (green) and AMCA (blue). A total of 2000 and 500 MN were scored from the slides prepared by flow cytometry and the CB technique respectively.

Statistical analysis

The relationship between the fraction of MN (y) with painting signals and the relative DNA content (x) of involved chromosomes was analysed by maximum-likelihood regression analysis, using the linear model y = c + bx with c = 0 for DNA proportionality assumption. Goodness of fit was tested with the Pearson χ² value. The difference between regression curves, derived from the flow sorting method or from the CB technique, was analysed by the variance ratio test, also called the F test.

Results

Figure 1 shows a flow cytometric measurement of MN and nuclei in suspension to indicate the region where MN were sorted for FISH analysis. With the technique developed by Viaggi et al. (1995) a nearly pure population of MN with a very low amount of unspecific particles could be sorted on slides. Thus, MN stained with painting and centromeric probes could be scored easily for the simultaneous occurrence of centromeric and paint signals due to the low amount of artefacts, an impressive advantage compared with the expected few paint-positive MN in binucleated cells.

Table I shows the results of the measurement of the fraction of centromere-positive and paint-positive MN for the six chromosome-specific paints studied (chromosomes no. 1, 7, 11, 14, 17, 21). In all, 2000 sorted MN per chromosome paint were scored for this analysis. The data obtained from the scoring of slides prepared from lymphocytes of three donors were pooled. It was found that 12-14% of all MN scored showed centromeric signals. The overall mean for centromere-positive MN in total MN was 13.6 ± 3% (10.7% with one signal, 2.9% with two signals). Similar values were obtained when centromeric signals were scored in MN with paint signals only. Table I also shows the results from scoring of MN in binucleated cells. Only 500 MN per chromosome paint could be scored for this type of analysis. The comparatively lower number was due to the rare occurrence of binucleated cells on the slides containing MN. It should be mentioned that no significant differences of the fraction of paint-positive MN were found in MN prepared from the three donors studied.

The fraction of paint-positive MN is shown in Figure 2 as a function of the DNA content of the chromosomes [DNA content of human chromosomes according to Morton (1991): 8.22 (chromosome 1), 5.34 (chromosome 7), 4.50 (chromosome 11), 3.41 (chromosome 14), 2.87 (chromosome 17) and 1.56 (chromosome 21)]. The results presented in Figure 2 and Table I show that the fraction of paint-positive MN depend on the DNA content of the chromosomes. Assuming DNA proportionality, nearly identical slopes b = 0.98 ± 0.04×10⁻³/DNA% and b = 0.99 ± 0.08×10⁻³/DNA% were calculated for data from flow sorting and from the CB technique respectively. However, the goodness-of-fit for paint positive MN detected by flow sorting was insufficient (χ² = 24.0, df = 5, P < 0.001), indicating deviations from DNA proportionality. For data from the CB technique, the fit was sufficient (χ² = 1.73, df = 5, P > 0.05). Assuming an intercept c in the regression
The solid line shows the trend calculated by binomial regression. Content DNA% of the respective chromosomes. Triangles = results obtained with flow sorted MN; squares = results obtained with MN in binucleated cells. The solid line shows the trend calculated by binomial regression.

Table I. Fraction of signal-positive MN in slides prepared by flow sorting or CB technique

<table>
<thead>
<tr>
<th>Chromosome no.</th>
<th>MN scored</th>
<th>With centromeric signal</th>
<th>Group A</th>
<th>Group B</th>
<th>1</th>
<th>2</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow-sorted slides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2000</td>
<td>1606 (0.80)</td>
<td>260 (0.13)</td>
<td>210</td>
<td>50</td>
<td></td>
<td>134 (0.067)</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>2000</td>
<td>1617 (0.81)</td>
<td>275 (0.14)</td>
<td>215</td>
<td>60</td>
<td></td>
<td>108 (0.054)</td>
<td>14</td>
</tr>
<tr>
<td>11</td>
<td>2000</td>
<td>1626 (0.81)</td>
<td>279 (0.14)</td>
<td>216</td>
<td>60</td>
<td></td>
<td>85 (0.043)</td>
<td>12</td>
</tr>
<tr>
<td>14</td>
<td>2000</td>
<td>1615 (0.81)</td>
<td>286 (0.14)</td>
<td>227</td>
<td>59</td>
<td></td>
<td>69 (0.035)</td>
<td>10</td>
</tr>
<tr>
<td>17</td>
<td>2000</td>
<td>1651 (0.83)</td>
<td>287 (0.14)</td>
<td>229</td>
<td>58</td>
<td></td>
<td>62 (0.031)</td>
<td>8</td>
</tr>
<tr>
<td>21</td>
<td>2000</td>
<td>1703 (0.85)</td>
<td>243 (0.12)</td>
<td>188</td>
<td>55</td>
<td></td>
<td>54 (0.027)</td>
<td>8</td>
</tr>
<tr>
<td>Slides prepared with the CB technique</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>500</td>
<td>410 (0.82)</td>
<td>50 (0.10)</td>
<td>42</td>
<td>8</td>
<td></td>
<td>40 (0.08)</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>500</td>
<td>424 (0.85)</td>
<td>51 (0.10)</td>
<td>40</td>
<td>9</td>
<td></td>
<td>25 (0.05)</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>500</td>
<td>431 (0.86)</td>
<td>49 (0.10)</td>
<td>40</td>
<td>9</td>
<td></td>
<td>20 (0.04)</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>500</td>
<td>432 (0.86)</td>
<td>48 (0.10)</td>
<td>38</td>
<td>10</td>
<td></td>
<td>20 (0.04)</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>500</td>
<td>429 (0.86)</td>
<td>58 (0.12)</td>
<td>46</td>
<td>12</td>
<td></td>
<td>13 (0.026)</td>
<td>1</td>
</tr>
<tr>
<td>21</td>
<td>500</td>
<td>439 (0.88)</td>
<td>51 (0.10)</td>
<td>39</td>
<td>12</td>
<td></td>
<td>10 (0.02)</td>
<td>0</td>
</tr>
</tbody>
</table>

Group A = MN without any signal; group B = MN with centromeric signals; group C = MN with painting signal for the specific chromosome; group D = MN with painting signal for the specific chromosome plus centromeric signal.

Table II. Results of maximum-likelihood regression analysis $y = c + bf$ for linear DNA dependence of MN with painting signals

<table>
<thead>
<tr>
<th>Method</th>
<th>$c = \pm SD \times 10^{-2}$</th>
<th>$b = \pm SD \times (10^{-2})$</th>
<th>$\chi^2$</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fit without intercept c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flow sorting</td>
<td>-</td>
<td>0.98 ± 0.04</td>
<td>24.0*</td>
<td>5</td>
</tr>
<tr>
<td>CB technique</td>
<td>-</td>
<td>0.99 ± 0.08</td>
<td>1.73</td>
<td>5</td>
</tr>
<tr>
<td>Fit with intercept c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flow sorting</td>
<td>1.5 ± 0.4</td>
<td>0.64 ± 0.09</td>
<td>2.07</td>
<td>4</td>
</tr>
<tr>
<td>CB technique</td>
<td>0.5 ± 0.7</td>
<td>0.87 ± 0.2</td>
<td>1.06</td>
<td>4</td>
</tr>
<tr>
<td>Pooled data</td>
<td>1.3 ± 0.3</td>
<td>0.69 ± 0.08</td>
<td>4.63</td>
<td>10</td>
</tr>
</tbody>
</table>

*P < 0.001

Discussion

Whole chromosome painting probes were used here to paint radiation-induced MN in irradiated human lymphocytes prepared by flow sorting and the CB technique. The aim of these experiments was to further investigate radiation-induced cytogenetic damage expressed as MN after the first division. In irradiated human lymphocytes, the measured paint-positive fraction of MN was found to be proportional to the relative DNA content of the six chromosomes investigated (chromosomes no. 1, 7, 11, 14, 17 and 21).

Binomial regression analysis of the data presented here, however, showed a slight excess of MN with painting signals from smaller chromosomes relative to those from larger chromosomes. This result was striking for data from flow sorting, where the results from both methods were not significantly different from zero, the difference between the results from the two methods was not significant (F test, $P > 0.05$) and the linear model could commonly be fitted with a significant intercept, $c = 1.3 \pm 0.3 \times 10^{-2}$, and $b = 0.69 \pm 0.08 \times 10^{-2}$ ($\chi^2 = 4.63, df = 10, P > 0.05$).

Fig. 2. Fraction of paint-positive MN as a function of the relative DNA content DNA% of the respective chromosomes. Triangles = results obtained with flow sorted MN; squares = results obtained with MN in binucleated cells. The solid line shows the trend calculated by binomial regression.
earlier study. For example, without chromosome 7, the result is $c = 2.4 \pm 0.52 \times 10^{-3}$, $b = 0.56 \pm 0.10 \times 10^{-3}$/DNA%. Without two extremely low frequencies of MN with painting signals from chromosome 1, $c$ remains significantly positive $(1.4 \pm 0.6 \times 10^{-3})$. A tendency of excess in small chromosomes found by analysis of radiation-induced exchange aberrations was also demonstrated by Knehr et al. (1994, 1996).

If it is assumed that the fraction of paint-positive MN is an accurate reflection of radiation-induced cytogenetic damage for the chromosomes studied, then these experiments support a random model of radiation-induced breakage because these chromosomes in human lymphocytes. These results are in contrast to the data published by us recently (Slavotinek et al., 1996) using the same technique of FISH on sorted MN and on MN in binucleated cells. These MN were induced by ionizing radiation in three human cell lines including an established cell line from an individual with ataxia telangiectasia showing a higher radio-sensitivity. It was found in all three cell lines that the fraction of MN containing DNA from chromosome no. 7 was substantially lower than expected from DNA proportionality within the selected group of four chromosomes tested (no. 1, 7, 11, 14). These results were obtained with both techniques (analysis of flow-sorted MN or of MN in binucleated cells). Our new data in human lymphocytes involving chromosome 7 showed no deviation from a linear DNA dependency. MN frequencies observed with both methods (5.4 and 5.0% paint-positive MN) were higher than the highest frequency (4.6%) found by Slavotinek et al. (1996). Since it was not found in lymphocytes from healthy donors, it is conceivable that the lower fraction of MN containing material from chromosome 7 is a peculiarity of these cell lines, perhaps caused by the cell transformation when establishing these cell lines.

Several authors have reported on involvement of chromosome no. 7 in exchange aberrations. The results are contradictory; sometimes chromosome 7 has been found to be more frequently involved in radiation-induced aberrations (Lee and Kamra, 1981; Barrios et al., 1989). However, Knehr et al. (1994) found that chromosome 7 showed the lowest involvement in exchange aberrations leading to dicentric chromosomes compared to chromosome X, 8, 9, 12 and 14. Our data on chromosome-specific paints in MN show that chromosome 7 is neither under- nor over-represented, the probability of its appearance as a fragment in MN depends only on its DNA content. However, it is still not known if the incorporation of chromosomal material into radiation-induced MN is an unbiased process, because MN are formed by extrusion of chromosomal material from the cell nucleus and are, therefore, a secondary rather than a primary effect.

MN are formed from chromosome aberrations (chromosome or chromatid breaks, asymmetrical chromatid and chromosome type exchanges) that result inacentric fragments. It is well known that whole chromosomes that do not segregate correctly due to radiation-induced spindle damage can also form MN. To check for this effect, we have, therefore, simultaneously stained the centromeric region of chromosomes in MN with a centromeric DNA probe that stains the centromeres of all human chromosomes. The data presented in Table I show that about 13.6 ± 3% of all MN analysed had centromeric signals (10.7% with one signal and 2.9% with two signals). This value is very similar to the fraction of centromere-positive MN in various cell lines published by other authors (Miller et al., 1992; Salassidis et al., 1992) or in human lymphocytes treated with the same dose of 2 Gy (Huber et al., 1996). In addition, Table I shows that 13–15% of the micronuclei with chromosome-specific paint signals showed centromeric signals. This fraction did not depend on the chromosomal DNA content. That means that, at least for the six human chromosomes studied with painting probes, their probability of being included into a MN does not depend on their DNA content. This is similar to results found after treatment of mouse 3T3-cells or Chinese hamster cells with the tear gas CS (2-chlorobenzylidene malonitrile). MN induced by this chemical were found mainly to be produced by whole chromatids, furthermore, all chromosomes had the same probability of being included in a MN (Nüss et al., 1992, 1996). In contrast to these results it has been shown that MN induced by 5-azacytidine mainly contained the chromosomes no. 1, 9, 15, 16 and Y (Guttenbach and Schmid, 1994) and that MN induced by vanadium salts preferentially contained acrocentric chromosomes (Migliore et al., 1995).

The use of flow sorting to enrich MN for FISH analysis greatly increases the number of MN available on a single slide compared to the conventional technique of scoring MN in binucleated cells. Although a certain amount of the sorted MN can be lost during the FISH procedure (usually 10–50% of the MN), there are enough sorted MN on a slide for a relatively fast FISH analysis. Flow sorting of MN for FISH analysis with a synthetic pancentromeric oligonucleotide was also successfully applied by Elhajouji et al. (1995) to study thresholds of chemically-induced aneuploidy in vitro using the MN test in human lymphocytes. If it is ascertained that only a small fraction of unspcific debris is found in the sorted MN which is possible in human lymphocytes using the magnetic separation technique developed by Viaggi et al. (1995) then a fast microscopic analysis of FISH-labelled MN can be obtained. Micronuclei can also be sorted for FISH analysis according to their relative DNA content as shown by us recently (Nüss et al., 1996). With this technique it is possible to quantitatively understand and simulate the shape of the DNA distributions of MN induced by ionizing radiation or chemicals.

However, as discussed by Slavotinek et al. (1996), with the CB technique MN could be seen and scored in relation to BN cells increasing the confidence of the scorer. In addition, the adjacent nuclei can be checked for satisfactory hybridization signals and the loss of certain chromosomes in nuclei can be seen directly as demonstrated by Boei et al. (1995). The main disadvantage of the CB technique is the fact that the numbers of scorable BN cells and scorable MN are usually low and the slides are time consuming to score.

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