Aneugenic and clastogenic effects of doxorubicin in human lymphocytes

Alok Dhawan, Mahmood A.Kayani1, James M.Parry1,
Elizabeth Parry1 and Diana Anderson2,3

Industrial Toxicology Research Centre, PO Box-80, M.G.Marg,
Lucknow 226 001, Uttar Pradesh, India, 1Centre for Molecular Genetics
and Toxicology, University of Wales, Swansea SA2 8PP, UK and
2Department of Biomedical Sciences, University of Bradford,
Bradford BD7 1DP, UK

Doxorubicin, a benzanthroquinone anticancer agent, was examined for its effect on micronucleus induction in
cultured human lymphocytes. A statistically significant
dose-dependent increase in micronucleus frequency (P <
0.001) in binucleated cells was seen and an increase in the
kinetochore-positive (P < 0.001) and kinetochore-negative
micronuclei (P < 0.001) was observed. An increase was
also observed in the number of necrotic cells, but the fre-
quency of apoptotic cells remained almost constant. This
confirms that doxorubicin is both clastogenic and aneu-
genic.

Introduction

Doxorubicin (adriamycin, CAS no. 25316-40-9) is a benzan-
throquinone drug, which is useful in the treatment of several
types of human malignancies (Fisherman et al., 1996; Misset
et al., 1996). It is cytotoxic and mutagenic in both bacterial and
mammalian systems. One type of interaction with the DNA is
associated with the production of reactive free radicals (Akman
et al., 1992), but its cytotoxic activity has been related to its
interaction with nuclear topoisomerase II (Wassermann, 1996).
Doxorubicin is known to be a cell cycle-specific agent.
Doxorubicin was shown to produce an increase in DNA strand
breakage and in the percentage of abnormal frequencies of
chromosomal damage in the FISH and conventional chromo-
somal aberration assays (Anderson et al., 1997). In vivo and
in vitro studies in mouse and in different cell lines have shown
that doxorubicin increases the frequency of micronuclei
(Boucher et al., 1993; Amara-Mokrane et al., 1996; Jagetia
and Vijayashree, 1996; Duffaud et al., 1998). The present
single study has investigated the effect of low doses of
doxorubicin in human lymphocytes using the micronucleus
assay using kinetochore staining. This is a test procedure
recommended in the Committee on Mutagenicity Guidelines
of the Department of Health, UK, for determining the mutageni-
city of chemicals (Committee on Mutagenicity of Chemicals in

Materials and methods

Chemicals

Micronucleus assay. Doxorubicin hydrochloride (adriamycin, CAS no. 25316-40-9) and dimethyl sulphoxide were purchased from Sigma Chemical Co.
(Poole, UK). Phytohaemagglutinin (PHA) was obtained from Abbott
Laboratories, Maidenhead, UK. RPMI 1640 growth medium and foetal calf
serum were purchased from Gibco BRL (Paisley, UK).

Kinetochore labelling. Primulin stain (ICN, UK) was used at a concentration of
0.5 µg/ml. Commercial anti-kinetochore antibody (Quadratech, Epsom, UK)
and fluorescein isothiocyanate (FITC)-conjugated anti-human antibody (Sigma
Chemical Co.) were diluted 1:1 and 1:13, respectively, in phosphate-buffered
saline (PBS). Bovine serum albumin (BSA) (Sigma Chemical Co.) was used to
remove the unbound antibody. Vectashield Antifade (Vector, Peterborough,
UK) was used to dilute DAPI (Cambio, Cambridge, UK) for staining the nuclei.

Preparation of media

Growth medium (GR10) consisted of final concentrations of 20% heat-
inactivated foetal calf serum (HIFCS) and a 1:1:1 mix of 10 000 IU/ml
(10 000 µg/ml) penicillin/streptomycin, 100 mM sodium pyruvate and 200 mM
L-glutamine, and 1250 IU/ml recombinant interleukin-2 (Chiron, Emeryville,
CA) in RPMI 1640 medium.

Stimulating medium (SR10) consisted of a final concentration of 0.4 µg/ml
phytohaemagglutinin in GR10 medium.

Culture of human lymphocytes

Peripheral blood was obtained from a healthy non-smoking male donor (30
years of age). Lymphocytes were separated using standard procedures as
described previously (Dey et al., 2001) and were then frozen in liquid nitrogen
until the experiments were performed. After removal from the liquid nitrogen
freezer, cell stocks were thawed rapidly in a water bath at 37°C for 5 min. The
thawed cell suspension was transferred to a sterile Universal tube containing
10 ml of wash medium (RPMI 1640 + 10% HIFCS). After gently mixing, the
cells were centrifuged at 500 r.p.m. for 10 min, the supernatant was removed
and the pellet was resuspended. These cells (at a concentration of ~1 × 10^6)
were then transferred to a small tissue culture flask containing 10 ml of
stimulating medium. The flask was gassed with CO2, sealed and placed at an
angle of 45° in an incubator at 37°C. On day 5, cells were disaggregated and
counted to check for growth and prepared for the in vitro micronucleus assay.

Micronucleus assay

The micronucleus assay was performed using the method of Fenech and
Morley (1985) as modified by Eillard and Parry (1993). On day 5 after thawing
the cells, two flasks were set up for each dose and incubated for another 24 h.
Thereafter the cells were treated with doxorubicin (1% v/v, 100 µl/ml culture
) until harvest. At 44 h after setting up the flasks, cytochalasin B was
added to the cultures at a final concentration of 6 µg/ml. Harvesting was
performed at 72 h. Each culture was transferred to a correspondingly labelled
Universal tube and centrifuged at 1500 r.p.m. for 10 min. The supernatant was
discarded and the pellets resuspended in 1–2 ml of RPMI.

Slide preparation

Aliquots of 100–300 µl of lymphocyte suspensions were cytocentrifuged
(Shandon Cytospin, Runcorn, UK) onto microscope slides at 500 r.p.m. for
10 min. The slides were air dried prior to fixation with 90% methanol at −20°C
for 8 min and the slides were kept at −20°C until they were stained for kinetochores
or with Giemsa.

Slides were stained with 5% Giemsa for 3 min and washed with PBS.
Coverslips were mounted using Depex mounting cement.

Kinetochore labelling

Immunofluorescent staining of kinetochores was done using CREST serum
containing anti-kinetochore antibodies as described by Eillard et al. (1991)
with minor modifications.

Slides were removed from the −20°C freezer and rehydrated in PBS at room
temperature. Slides were immersed in a 0.5 µg/ml (in PBS) solution of primulin
for 1 min and then placed in a fresh PBS solution. Slides were removed and
excess PBS around the cytodont (a dot of cells obtained after centrifugation) was
carefully removed with absorbent paper. An aliquot of 50 µl of commercial
anti-kinetochore antibody, diluted 1:1 with PBS, was applied to the cytodont. A
plastic coverslip was lowered gently, to avoid air bubbles, onto the slide. The
slides were placed in a pre-warmed humidified chamber, which was placed in

3To whom correspondence should be addressed. Tel: +44 1274 233569; Fax: +44 1274 309742; Email: d.anderson1@bradford.ac.uk

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487

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an incubator at 37°C for 45 min. Following incubation, any unbound antibody was removed by a series of washes: 3 × 5 min in PBS + 1% BSA, followed by 3 × 5 min in PBS. After a series of washings, excess PBS around the cytodot was removed with absorbent paper. An aliquot of 50 μl of the second antibody, FITC-conjugated goat anti-human IgG, diluted 1:13 with PBS, was added to the slides and the slides were washed as before. After the second wash, the slides were rinsed with distilled water and allowed to dry in the dark. Dry slides were stored at −20°C. Slides were mounted with 25 μl of DAPI/antifade solution (1 μl DAPI + 9 μl dH2O + 990 μl antifade) and visualized using an Olympus BH2-RFL fluorescent microscope with a triple bandpass filter to allow the simultaneous observation of both the kinetochore signals and the DAPI counterstain and single bandpass filters (Olympus) for green and blue spectra, to allow the signals and DAPI to be observed separately.

**Slide analysis**

The slides were coded prior to analysis. The criteria for MN were as described by Fenech (1993, 1996). Scoring of necrotic and apoptotic cells was done according to Fenech et al. (1999). Giemsa stained slides were analysed using an Olympus BH2 light microscope at 1000× magnification and mono-, bi- and multi-nucleated cells were recorded. Micronuclei were scored in a total of 1000 binucleate cells per culture (2000 cells/dose).

The kinetochore labelled slides were viewed under an Olympus BH2-RFL fluorescence microscope. A total of 100 (where possible) micronuclei were scored, there was a significant decrease (P < 0.001) in the percentage of apoptotic cells (Figure 1).

As shown in Table I, a dose-dependent increase in the mean frequency of binucleated cells containing micronuclei was observed. This increase was 12-, 22- and 27-fold higher in comparison with the negative control and was highly statistically significant. There was a corresponding decrease in the mean percentage of binucleated cells, which was greatly reduced at doses of 0.05 and 0.10 μM.

Kinetochore studies revealed that among the micronuclei counted, there was a significant decrease (P < 0.001) in the proportion of kinetochore-negative (K−) micronuclei after treatment with doxorubicin, with a concomitant significant increase (P < 0.001) in the kinetochore-positive (K+) micronuclei (Table II). However, the frequency of binucleated cells containing either K− or K+ micronuclei showed a significant dose-dependent increase (P < 0.001). At the lowest dose (0.01 μM), an almost 5-fold increase was observed in the K− micronucleated cells, although a 59-fold increase was observed in the K+ micronuclei. At a dose of 0.10 μM, no micronuclei could be scored for kinetochore staining (Table II).

An increase was also observed in the frequency of necrotic cells in a dose-dependent manner and there was a slight decrease in the percentage of apoptotic cells (Figure 1).

**Table I.** The effect of doxorubicin on micronucleus formation in human lymphocytes

<table>
<thead>
<tr>
<th>Dose (μM)</th>
<th>Mono</th>
<th>Bi</th>
<th>Bi+</th>
<th>Total</th>
<th>Bi (%)</th>
<th>Mean Bi (%)</th>
<th>Micronuclei</th>
<th>Total</th>
<th>MNC/Bi (%)</th>
<th>Mean MNC/Bi (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>495</td>
<td>1000</td>
<td>22</td>
<td>1517</td>
<td>65.92</td>
<td>6</td>
<td>6</td>
<td>0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.010</td>
<td>499</td>
<td>1000</td>
<td>17</td>
<td>1516</td>
<td>65.96</td>
<td>5</td>
<td>5</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.025</td>
<td>505</td>
<td>1000</td>
<td>15</td>
<td>1520</td>
<td>65.79</td>
<td>62</td>
<td>62</td>
<td>6.20</td>
<td>6.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>0.050</td>
<td>543</td>
<td>1000</td>
<td>34</td>
<td>1618</td>
<td>64.16</td>
<td>130</td>
<td>130</td>
<td>12.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>511</td>
<td>1000</td>
<td>31</td>
<td>1542</td>
<td>64.85</td>
<td>117</td>
<td>117</td>
<td>11.70</td>
<td>12.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>452</td>
<td>199&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>1654</td>
<td>12.03</td>
<td>32</td>
<td>32</td>
<td>16.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>1452</td>
<td>118&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5</td>
<td>1082</td>
<td>10.91</td>
<td>16</td>
<td>16</td>
<td>13.59</td>
<td>14.84&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2121</td>
<td>53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>2174</td>
<td>2.44</td>
<td>1</td>
<td>1</td>
<td>1.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.010</td>
<td>826</td>
<td>13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>839</td>
<td>1.55</td>
<td>1</td>
<td>1</td>
<td>7.69</td>
<td>4.79</td>
<td></td>
</tr>
</tbody>
</table>

Mono, mononucleated cells; Bi, binucleated cells; Bi+, cells with more than two nuclei; Bi (%), frequency of binucleated cells; MNC/Bi (%), frequency of binucleated cells containing micronuclei.

<sup>a</sup>There were no cells with more than one micronucleus.

<sup>b</sup>P < 0.001 when compared with the controls.

<sup>c</sup>Total number of binucleates that could be counted on the slide.

**Table II.** Classification of micronuclei by CREST staining

<table>
<thead>
<tr>
<th>Dose (μM)</th>
<th>K+ MN</th>
<th>K− MN</th>
<th>Total MN</th>
<th>Proportion</th>
<th>%MNBn&lt;sup&gt;a&lt;/sup&gt;</th>
<th>%MNBn&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>72</td>
<td>81</td>
<td>0.12</td>
<td>0.88</td>
<td>0.55</td>
</tr>
<tr>
<td>0.010</td>
<td>54</td>
<td>32</td>
<td>86</td>
<td>0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.55&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.025</td>
<td>54</td>
<td>28</td>
<td>82</td>
<td>0.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.050</td>
<td>26</td>
<td>12</td>
<td>38</td>
<td>0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.84&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.100</td>
<td>No MN found; impossible to score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

%MNBn<sup>a</sup>, frequency of binucleated cells containing K+ micronuclei; %MNBn<sup>b</sup>, frequency of binucleated cells containing K− micronuclei. %MNBn<sup>a</sup> and %MNBn<sup>b</sup> were calculated by multiplying %MNBn by the proportion of MN K+ or K−.

<sup>a</sup>P < 0.001 when compared with the controls.

**Statistics**

The χ² test was used to compare the treated cultures with controls.

**Results**

A pilot experiment was conducted to assess the dose–response relationship of doxorubicin. Doses above a concentration of 0.1 μM showed no binucleate cells (data not shown), hence the maximum dose taken in this study was 0.1 μM.

**Discussion**

The results have shown that doxorubicin produces a dose-dependent increase in micronucleus formation in human lymphocytes in vitro and an increase in kinetochore-positive and kinetochore-negative stained micronuclei, indicating the
induction of both aneugenicity and clastogenicity. An increase in micronucleus formation in human lymphocytes, over a similar dose range, was also observed by Migliore et al. (1987). The results of clastogenicity confirm the findings of previous studies (Migliore et al., 1987; Jagetia and Vijayashree, 1996). This study also confirms the aneugenic effect of doxorubicin in vitro reported by Aly et al. (1999), where in lymphocytes from healthy individuals and cancer patients, an increase in the trisomy of chromosomes 7 and 17, using FISH DNA-specific probes, was observed.

The micronucleus index reduction at the 0.10 μM dose compared with the lower doses could be due to the fact that higher necrosis was observed in this dose range. Doxorubicin binds to DNA by intercalation and its antitumour activity has been associated with the production of protein-concealed DNA strand breaks as a result of DNA topoisomerase II poisoning. It is thought that interference of the DNA–topoisomerase complex with replication or transcription may induce the lethal event (Wassermann, 1996). It may also be due to the response of the cells to the genotoxin causing necrosis or mitotic slippage, as discussed by Kirsch-Volders and Fenech (2001). The necrosis observed at higher doses of doxorubicin suggests that while apoptosis has an important role in the elimination of cells with DNA damage, the majority of cells were eliminated by necrosis in lymphocyte cultures. This has also been reported in an earlier study where hydrogen peroxide was used as the DNA-damaging agent (Fenech et al., 1999). This is an important parameter in understanding the toxicity of a compound and has been extensively reviewed (Fenech, 2000; Kirch-Volders and Fenech, 2001).

In an earlier study by Anderson et al. (1997), dose-dependent clastogenicity was observed over a similar dose range to the present study, when measured using FISH and conventional chromosomal analysis. This previous observation corresponds to the findings of the present study and probably explains the observed dose-dependent increase in micronucleus formation. This single study, using kinetochore staining, has additionally shown that along with clastogenicity, doxorubicin also exerts aneugenic effects in human lymphocytes under in vitro conditions.

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References


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