Low Levels of Chromosomal Mutations in Germ Cells Derived from Doxorubicin-treated Stem Spermatogonia in the Mouse

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ABSTRACT

The mutagenic effects of doxorubicin (Adriamycin, ADR) on mouse spermatogenic stem cells were examined by analysis of spermatocyte chromosomes and of dominant lethality transmitted through the spermatogonia. The effects of ADR on mutations, cytotoxicity, and sperm head abnormalities were compared with those of radiation. The cytotoxic effect of 6 Gy of γ-radiation on stem spermatogonia was equivalent to about 4-5 mg ADR/kg. Chromosomal translocations were observed in 0.6% of the spermatocytes of mice treated with ADR (2-6 mg/kg). In contrast, 6 Gy of radiation induced translocations in 11.1% of spermatocytes. No increase in dominant lethality was observed after treatment with ADR at doses up to 6 mg/kg, while the frequency after 6 Gy of radiation was 3.6%. Based on these results, ADR would be expected to be only a weak inducer of balanced chromosomal rearrangements. Because ADR at 4.5 mg/kg was much weaker than 6 Gy of γ-radiation at inducing chromosomal translocations, but just as effective at inducing sperm head abnormalities, the level of sperm head abnormalities is not indicative of balanced chromosomal rearrangements induced in stem spermatogonia by cytotoxic agents.

INTRODUCTION

With increased survival of young adult male cancer patients, their future reproductive potential has become an important clinical concern. Doxorubicin (ADR)-containing regimens are effective in curing a high proportion of cancers, such as testicular cancer (1), Hodgkin's lymphoma (2), and osteosarcoma (3), while permitting recovery of sperm production within 2 years after treatment. However, ADR is mutagenic, and mutations induced in spermatogonia by ADR will be carried by the sperm. Thus, the risks to the development of any children conceived after treatment with ADR must be considered.

Mutations may be of two types: point mutations and chromosomal abnormalities. Most point mutations are recessive and hence would not necessarily appear in the offspring of the patients, but the genetic damage would accumulate in the population. Chromosomal abnormalities, however, present a direct risk to the offspring. The mutations in stem cells are of primary concern to chemotherapy patients since any pregnancies occurring at more than 3 months after treatment result from sperm that were stem spermatogonia at the time of treatment. Although many chemicals are potent at inducing point mutations in stem cells and both chromosomal and point mutations in later germ cell stages, it had been thought that chromosomal mutations produced in stem cells by chemicals would be strongly selected against and lost during spermatogenesis (see Ref. 4 for review).

Several studies have indicated that ADR may be an exception and indeed be a potent chromosomal mutagen towards stem cells in mice. One study reported chromosome breaks in 4.5% and translocations in 1.3% of spermatocytes that were derived from stem spermatogonia exposed to 3 mg/kg ADR (5). Study of dominant lethality in matings several months after treatment of male mice with ADR at 8 mg/kg demonstrated a 22% incidence by an in vitro assay (6). Another possible indicator of mutagenic damage, sperm head abnormalities (7), remains permanently elevated in sperm from mice treated with 6 or 8 mg ADR/kg.

However, there are also indications that the incidence of chromosomal mutations may be low after ADR treatment. In the above study (6) there was little or no significant increase in dominant lethals after a slightly lower ADR dose of 6 mg/kg. Another study of spermatocytes reported a 0.7% incidence of translocations after ADR at 5 mg/kg, and breaks were not mentioned (8). Diploid sperm, which had been induced by 5 mg ADR/kg treatment of differentiating spermatogonia, were not induced by treatment of stem spermatogonia (9).

To resolve some of these differences, we performed a dose-response study of the mutagenicity of ADR toward the stem cell, combining the various endpoints in the same mice. Radiation, a known inducer of transmissible chromosomal aberrations in stem cells (10), was included as a positive control.

MATERIALS AND METHODS

Specific pathogen-free C3H/Kam mice 9 weeks of age at the time of treatment were used.

Mice were injected i.v. with 0, 1, 2, 3, 4, 5, 6, 7, or 8 mg ADR/kg. Each ADR-dose group consisted of 10 animals; the control group consisted of 20. An additional group of 10 mice was treated with 6 Gy of γ-radiation to their testes from a pair of 137Cs sources.

To resolve some of these differences, we performed a dose-response study of the mutagenicity of ADR toward the stem cell, combining the various endpoints in the same mice. Radiation, a known inducer of transmissible chromosomal aberrations in stem cells (10), was included as a positive control.

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2 Address reprint requests should be addressed, at the Department of Experimental Radiotherapy, Box 66, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030.

3 The abbreviations used are: ADR, doxorubicin (Adriamycin); MI, first meiotic division; MII, second meiotic division; SSC, saline sodium citrate buffer.

Since the use of inbred female mice produced a high background of dead embryos, mice in which three or more dead embryos were found in one horn of the uterus were excluded since this was probably a result of maternal rather than sperm-related factors. This criterion excluded 2% of all pregnant females, which was similar for all treatment groups. At the end of the mating period, at least 10 days of sexual rest were allowed for the males to replenish their epididymal sperm reserves. At 32 weeks after injection the mice were killed so that sperm counts, cytogenetic analysis of spermatocytes, and morphological analysis of epididymal sperm could be performed. All mice were injected i.p. with...
40 mg/kg of colchicine 2 h before sacrifice so that MII figures would accumulate (12). Testes were excised and, after the tunica was removed, 40 mg/kg of colchicine 2 h before sacrifice so that MI figures would accumulate (12). From each mouse, 50 MI and MII figures, when available, were analyzed for chromosomal abnormalities according to the criteria previously described (13). Where necessary to distinguish between intact chromatids and chromatid fragments in MII cells, c-banding was performed by incubation in 0.2 N HC1 at room temperature for 15 min, 0.07 N barium hydroxide at 37°C for 6 min, 2 x SSC at 60°C for 1 h and subsequent staining in 10% Giemsa in phosphate buffer for 20 min.

The 1 ml removed for sperm head counts was homogenized with a Polytron homogenizer and then sonicated. Sperm heads were counted in a hemocytometer using phase contrast microscopy. The precision of the sperm counts on these suspensions of cells in sodium citrate was not as high as in previous studies employing hemocytomerization in water.

Suspensions of sperm from the cauda epididymis of the treated mice were stained with eosin Y, smeared onto slides, and scored for head abnormalities according to the criteria of Wyrobek and Bruce (14).

Statistical Analysis. The significance of differences in fertility between mice was determined by nonparametric statistics using the Mann-Whitney U test. The significance of differences in the fraction of embryos that were dead was computed with a x2 analysis using the Fisher’s exact test. Differences in testis weights and sperm head counts between groups of mice were evaluated using Student’s t test. The significance of differences in chromosomal abnormalities between control and treated groups of mice were determined by the Fisher’s exact test.

RESULTS

Nongenetic Effects of Doxorubicin. No animals died as a result of treatment with ADR in the doses used in this study, indicating that general toxicity to the mice should not be a factor in the outcome of these experiments.

Fertility was affected by ADR treatment (Table 1), as evidenced by the significant reduction in the fraction of females

<table>
<thead>
<tr>
<th>Doses of ADR (mg/kg)</th>
<th>Number of males</th>
<th>Number of mated females</th>
<th>Number of females with implants</th>
<th>Fraction of mated females with implants</th>
<th>Total number of implants</th>
<th>Implants per pregnant female</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>359</td>
<td>312</td>
<td>0.87</td>
<td>2744</td>
<td>8.8</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>180</td>
<td>144</td>
<td>0.80</td>
<td>1269</td>
<td>8.5</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>165</td>
<td>126</td>
<td>0.76</td>
<td>1131</td>
<td>9.0</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>180</td>
<td>126</td>
<td>0.56</td>
<td>677</td>
<td>6.8</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>180</td>
<td>1</td>
<td>0.01</td>
<td>6</td>
<td>6.0</td>
</tr>
<tr>
<td>Radiation (6 Gy)</td>
<td>10</td>
<td>179</td>
<td>1</td>
<td>0.73</td>
<td>1129</td>
<td>8.5</td>
</tr>
</tbody>
</table>

* Differs from control at P < 0.0005, values not marked do not differ from control at P •¿-

* Numbers in parentheses, numbers of mice in which aberrations were observed.

* Fraction of cells with aberrations differs from control at P < 0.05.

* The four translocations observed in MI and the three in MII were in the same mouse.

* Fraction of cells with aberrations differs from control at P < 0.01.

At this point, 1 ml was removed for sperm counts (see below). The remaining cells were subjected to hypotonic treatment, fixation, preparation of slides, and Giemsa staining according to standard methods (12). From each mouse, 50 MI and MII figures, when available, were analyzed for chromosomal abnormalities according to the criteria previously described (13). Where necessary to distinguish between intact chromatids and chromatid fragments in MII cells, c-banding was performed by incubation in 0.2 N HC1 at room temperature for 15 min, 0.07 N barium hydroxide at 37°C for 6 min, 2 x SSC at 60°C for 1 h and subsequent staining in 10% Giemsa in phosphate buffer for 20 min.
with implants at 6 and 8 mg/kg. In the dose groups below 6 mg/kg or in controls there were no changes in the fraction of fertile matings with time. In the mice receiving 6 mg/kg the fractions of fertile matings increased from 0.38 in the first six matings to 0.68 in matings 7–12 and to 0.58 in matings 13–18, but remained significantly below control levels. The reduced level of fertility in the male mice receiving 6 mg/kg was also manifest in a significantly reduced number of implants per pregnant female; this reduction did not occur after lower doses or 6 Gy of radiation.

The irreversible effect of ADR on the spermatogenic function of the testis is seen in the testis weights and sperm head counts presented in Fig. 1. Testis weights were reduced in a dose-dependent manner, dropping to 29% of control values at 8 mg/kg. The first dose that gave a significant ($P < 0.05$) reduction from control was 3 mg/kg. Irradiation produced a decrease in testis weight equivalent to that by an ADR dose of about 4 mg/kg. The reduction in sperm production was more marked, dropping to about 7% of control at 8 mg/kg. Again the decrease due to irradiation was equivalent to that observed with ADR at 4–5 mg/kg.

Chromosomal Aberrations in Spermatocytes. Both primary spermatocytes in diakinesis/metaphase I and secondary spermatocytes in metaphase II were investigated for chromosome aberrations. Results are given in Table 2. While no translocation was observed in the untreated mice, a significant number were seen in the irradiated mice. The overall frequency of translocations was 11% and the ratio of ring to chain quadrivalents was 1:1. In the ADR-treated groups, seven translocations were observed in 1238 primary spermatocytes analyzed, leading to an overall percentage of 0.6%, which is still much lower than the translocation rate induced by radiation. With the exception of one translocation in the 4 mg/kg dose group, which formed two unequal bivalents, all other translocations formed chain quadrivalents (Fig. 2a). The possibility that ADR reduces chiasma frequency was investigated by determining the frequencies of autosomal and X-Y univalencies. Similar frequencies occurred in the control, ADR-treated, and irradiated mice (data not shown), suggesting that ADR does not affect chiasma formation. Furthermore, ADR did not elevate the frequency of breaks in MI (Table 2).

In secondary spermatocytes (MI), we examined the frequencies of cells with translocations, hyperhaploidy, and breakage that included gaps, breaks, and fragments. These frequencies did not differ significantly between the control, ADR-treated, and irradiated mice, except that mice treated with 5 mg/kg of ADR had a higher frequency of breakage than the controls ($P < 0.05$). Interestingly, in the mouse (in the 6 mg/kg dose group) in which quadrivalents of the chain-of-four configuration were observed in four primary spermatocytes, Robertsonian (centromeric fusion) chromosomes (one of which is shown in Fig. 2b) were observed in three secondary spermatocytes. It is expected that a Robertsonian translocation would appear as a chain-of-four in MI.

Dominant Lethal Effects. Any increase in the frequency of embryonic death as a result of ADR treatment would indicate dominant lethality, which is usually a result of chromosomal abnormalities carried by the gametes. Data on embryonic lethality are shown in Table 3. The fraction of dead embryos was not significantly different from control ($P > 0.10$) at any dose of ADR. When the points were fitted by a straight line (Fig. 3) using a linear trend for proportions (15) a slope of 0.0004 ± 0.0023 (not significantly different from zero) was obtained. Thus, no postimplantation-dominant lethal effects produced by ADR could be detected in this study. A dose of 6 Gy of ionizing radiation, included as a positive control, significantly ($P = 0.03$) increased the proportion of dead embryos to 0.197 from the value of 0.167 observed in controls. This corresponds to a frequency of induction of dominant lethals of 3.6%.

Sperm Head Abnormalities. ADR increased the incidence of abnormalities in the shape of the sperm head above the control level of about 3% (Fig. 4). Significant increases ($P < 0.001$) were seen at doses of 5 mg/kg or more. Irradiation with 6 Gy also produced a significant increase in sperm head abnormalities, which was comparable to 4–5 mg ADR/kg.
The frequency of induced translocations in spermatocytes (uncorrected for clustering) of 0.6% is in agreement with 0.7% found by van Buul (8), and although it is lower than the 1.3% frequency observed by Au and Hsu (5), the difference is not statistically significant. Combining all of the experiments, we obtain a highly significant incidence of 0.6% of translocations after ADR treatment. However, this incidence is much lower than that produced by a 6-Gy dose of radiation, which produces roughly equal stem cell killing to that of the ADR doses used in these experiments.

There are differences in the effects of ADR on other types of chromosomal alterations between the current study and previous reports. We failed to observe increases in breaks and fragments in the chromosomes of the ADR-treated mice. It is possible that the injection of colchicine prior to harvesting the tissue may have caused the chromosomes to become slightly more condensed, which might make some breaks less visible.

Translocations in spermatocytes correlate with dominant lethal effects. For irradiation of mouse testicular stem cells, the incidence of chromosomal translocations is three to four times the resulting frequency of dominant lethals observed after mating (10; and present result). Thus, we only expect an increase of 0.2% in the frequency of dominant lethality after ADR treatment. Given the limits of error of the dominant lethal assay, it is not surprising that we were unable to detect any.

The failure to find dominant lethals in vivo is consistent with a recent report showing no dominant lethality in hybrid mice treated with doses up to 12 mg/kg of ADR (17). These results appear to contrast with the conclusions from an in vitro dominant lethal assay (6), where there was a significant induction in dominant lethals at 8 mg/kg. The infertility of the mice receiving 8 mg/kg in this study and the use of hybrid mice in the other in vivo dominant lethal study precludes a direct comparison with the in vitro study. The studies agree in that no dominant lethal mutations are detectable by either method at doses of 6 mg/kg. Also, most of the dominant lethals observed by the in vitro techniques occurred at the early preimplantation stages, which cannot be measured in this in vivo assay. The loss of embryos in early preimplantation development in the previous study raises a question as to whether this apparent dominant lethality could have been a result of severely damaged sperm that can, upon fertilization, induce eggs to begin abortive gynogenetic development with only the haploid female pronucleus (18).

The use of head shape abnormalities as a measure of mutation-induced chromosomal damage in sperm has been tested in this study. Although the level of sperm head abnormalities produced by 6 Gy of radiation was comparable to that produced by 4 to 5 mg ADR/kg, 6 Gy produced an 18-fold higher incidence of balanced chromosomal rearrangements than did ADR at a median dose of 4 mg/kg. Previously we had shown that doses of busulfan of 16 to 20 mg/kg produced a similar increase in the incidence of sperm head abnormalities but no significant increase in dominant lethality (19) or balanced chromosomal translocations in stem cells (20). Thus the incidence of sperm head abnormalities was not an indicator of the relative effectiveness of ADR, busulfan, and radiation in inducing this type of chromosomal abnormality. This result is consistent with an earlier report that in most cases sperm from mice heterozygous for reciprocal and Robertsonian translocations have an abnormal chromosome complement but normally shaped heads.

### Table 4 Incidence of chromosomal translocations in spermatocytes (MI) produced from stem spermatogonia treated with doxorubicin (ADR)

<table>
<thead>
<tr>
<th>Study</th>
<th>Translocations/Cells examined</th>
<th>ADR-treated</th>
<th>Level of induced translocations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>0/389</td>
<td>7/1230*</td>
<td>0.6*</td>
</tr>
<tr>
<td>Au and Hsu (5)</td>
<td>(0/2354)*</td>
<td>7/1230</td>
<td>0.6*</td>
</tr>
<tr>
<td>van Buul (8)</td>
<td>(0/2354)*</td>
<td>4/1001*</td>
<td>0.4*</td>
</tr>
<tr>
<td>All three studies</td>
<td>0/200</td>
<td>4/300*</td>
<td>1.3*</td>
</tr>
<tr>
<td></td>
<td>2/2200</td>
<td>7/1000*</td>
<td>0.7*</td>
</tr>
<tr>
<td></td>
<td>2/2789</td>
<td>18/2530</td>
<td>0.6*</td>
</tr>
<tr>
<td></td>
<td>2/2789</td>
<td>15/2301*</td>
<td>0.6*</td>
</tr>
</tbody>
</table>

* Analyzed at 32 weeks after treatment with 2 to 6 mg ADR/kg.
* Including data on additional controls from our laboratory.
* Corrected for possible clustering at 6 mg ADR/kg by assuming that at this dose four spermatocytes came from each surviving stem cell.
* Analyzed at 70–120 days after treatment with 3 mg ADR/kg. There was no trend in the incidence of translocations with increasing postinjection interval.
* Analyzed at 5 to 9 months after treatment with 5 mg ADR/kg.
* ADR-treated not significantly different from controls, $P = 0.15$.
* ADR-treated significantly different from controls at $P < 0.001$.
* ADR-treated significantly different from controls at $P < 0.01$.

**DISCUSSION**

ADR affects the fertility of mice by killing stem spermatogonia. For example, nine of the 10 mice treated with 8 mg ADR/kg were totally sterile for 30 weeks (212 days) after treatment. This effect appears to be slightly stronger than that reported earlier (6, 16), but this degree of quantitative variability is not unusual in our experiments with ADR done at different periods of time.

The data on chromosomal mutations induced by ADR in spermatogonial stem cells are analyzed in Table 4. When all of the ADR doses are combined, the increase in translocations in the treated group is not significantly different from the control ($P = 0.15$), partly because of the smaller numbers of cells scored in the control group. However, inclusion of controls from other studies (0 translocations/1965 cells) done using the same techniques and strain of mouse, *J. C. Liang, study in progress.* raises the level of significance of the difference between the ADR treated and controls. There is, however, one additional correction to the number of independent mutations that were scored in the ADR-treated mice. Since the four translocations observed after 6 mg ADR/kg were in the same mouse and of the same type, it is probable that they were from the same stem cell. To correct for this possible clustering due to repopulation by a small number of surviving stem cells after high doses of cytotoxic agents, we divided both the numbers of translocations and numbers of spermatocytes by 4.

The frequency of induced translocations in spermatocytes (uncorrected for clustering) of 0.6% is in agreement with 0.7% found by van Buul (8), and although it is lower than the 1.3% frequency observed by Au and Hsu (5), the difference is not statistically significant. Combining all of the experiments, we obtain a highly significant incidence of 0.6% of translocations after ADR treatment. However, this incidence is much lower than that produced by a 6-Gy dose of radiation, which produces roughly equal stem cell killing to that of the ADR doses used in these experiments.

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![Graph showing percentage of sperm heads that were abnormal](image-url)
Whether the increase in sperm head abnormalities in ADR-treated mice was a result of physiological (nongenetic) damage to the testes or point mutations in germ cells was still open to question; we can only conclude that sperm head abnormalities do not correlate with the induction of the forms of chromosomal damage (i.e., balanced chromosomal rearrangements) that are consistent with development of the stem cells to spermatid cells to spermatozoa.

Since ADR does produce a low but significant number of translocations in exposed mouse stem cells, there may be some genetic risk to patients treated with ADR. Possible methods of extrapolating these doses and results to man include the following: (a) on the basis of administered dose, (b) on the basis of cytotoxic effects on stem spermatogonia, and (c) on the basis of dose to produce comparable clastogenic effects in somatic cells (22). Extrapolating the effects to men treated with 800 mg ADR/m² (3) on the basis of administered dose per body surface area (23) results in an incidence of translocations of 35%. Alternatively, extrapolation of dose on a body weight basis yields an incidence of translocation of 3% in humans. If, however, doses were compared on the basis of their cytotoxic effects on stem spermatogonia, the doses used in mouse can be expected to induce translocations in only 0.6% of cells. Although chromosomal abnormalities are produced in mouse bone marrow cells by ADR (5) and ADR treatment of humans also results in chromosomal abnormalities (24), the doses to humans were not quantified and results were not presented in a manner appropriate for direct comparison with the murine data.

The only method now available for direct measurement of the chromosomal damage in the germ cells of the treated patients is by karyotypic analysis following heterospecific fertilization of zona-free hamster ova with human sperm (25, 26). This procedure is difficult and, at present, it is only being applied to patients receiving combination chemotherapy, so the specific mutagenic risk of ADR cannot be sorted out. It is therefore important to determine the most appropriate methods for interspecies extrapolation of the genetic results obtained on rodents to humans.

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REFERENCES

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