Role of epididymal inflammation in the induction of dominant lethal mutations in Fischer 344 rat sperm by methyl chloride

(activated phagocytes/oxygen radical toxicity/DNA damage/BW 755C)

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ABSTRACT This study assessed the possible relationship between methyl chloride (MeCl)-induced epididymal inflammation and the formation of dominant lethal mutations in sperm of Fischer 344 rats. Groups of 40 males were exposed to MeCl (3000 ppm 6 hr/day for 5 days), with or without concurrent treatment with the anti-inflammation agent 3-amino-1-[m-(trifluoromethyl)phenyl]-2-pyrazoline (BW 755C; 10 mg/kg, i.p. 1 hr pre- and postexposure); BW 755C was shown previously to inhibit MeCl-induced epididymal inflammation. Control groups (n = 20) were either untreated, injected as described above with BW 755C, or injected on the afternoon of day 5 with triethylenglycolamine (0.2 mg/kg), a known dominant lethal mutagen. Each male was mated with one female weekly for 3 weeks; 12-18 days after mating, females were killed to assess dominant lethal parameters. In females bred to MeCl-exposed males, significant increases were observed in postimplantation loss at postexposure week 1 (0.84 dead implants per female vs. 0.29 in untreated controls) and in dead implants/total implants at both week 1 (0.10 vs. 0.04 control) and week 2 (0.24 vs. 0.06 control). These increases were not observed in females bred to males treated with BW 755C during MeCl exposure. Coadministration of BW 755C to males along with MeCl also reduced the percentage of mated females with two or more postimplantation losses from 31% to 8% (week 1) and 30% to 12% (week 2). Therefore, the dominant lethal mutations induced by MeCl appear to be a consequence of its induction of inflammation in the epididymis. These data demonstrate the potential genotoxicity of inflammatory processes in vivo.

Activated phagocytes in vitro can produce a variety of genetic lesions in the DNA of neighboring bacterial and mammalian cells, including mutations, chromosome aberrations, and malignant transformation (1-5). The production of genotoxic lesions by activated inflammatory cells appears to involve the generation of highly reactive oxygen intermediates such as superoxide anions, hydrogen peroxide, and hydroxyl radicals (6, 7). Inflammatory cells can also produce oxidant species such as N-chloroamines, which have half-lives as long as 18 hr and which could accumulate at inflammatory sites or diffuse over long distances before oxidizing target molecules (8). Although production of these oxidative species is likely to be important in the normal bacteriocidal functioning of phagocytic cells (9), it may also lead to adverse biological effects such as killing of normal cells (10) or tumor promotion (11, 12).

The available evidence for the generation of genotoxic products by phagocytic inflammatory cells has come from in vitro experimentation. To our knowledge, there has been no direct demonstration that inflammatory cells can produce biologically adverse genotoxic effects under in vivo condi-

tions. However, an experimental model for the in vivo assessment of the genotoxic potential of inflammatory reactions was suggested by previous work in our laboratory on the reproductive toxicity of the industrial gas methyl chloride (chloromethane, MeCl). Exposure of Fischer 344 (F344) rats to MeCl by inhalation (3000-3500 ppm 6 hr/day for 5-9 days) caused an acute inflammatory response in the cauda epididymis, with subsequent formation of sperm granulomas (13, 14). Dominant lethal mutations were detected in sperm that completed their maturation in this site of active inflammation; these mutations were detected as increased postimplantation losses (dead implants) at week 1 postexposure in females bred to MeCl-exposed males (14). Dominant lethal mutations result from DNA damage in male germ cells sufficient to cause pre- and/or postimplantation embryonic deaths in females bred to genotoxicant-exposed males (15). Postimplantation loss is the more reliable index of genotoxicity, since preimplantation loss can result not only from genotoxicity but also from cytotoxic events that lead to failure of fertilization (16).

It is generally assumed that chemically induced dominant lethal mutations result from direct interaction of the chemical or its metabolites with DNA. However, several observations suggested that this was not the case for MeCl. First, the pattern of dominant lethality induced by MeCl was not typical of that produced by germ-cell mutagens known to act via a direct, genotoxic mechanism (14). Second, MeCl was known to be an extremely weak direct-acting mutagen, producing genotoxic damage in vitro only at concentrations far in excess of those which produced dominant lethal effects in vivo—e.g., 50,000 ppm for bacteria, >10,000 ppm for human lymphoblasts, and 30,000-50,000 ppm for rat spermatocytes (17-19). Third, MeCl exposure conditions that caused epididymal inflammation and dominant lethality did not induce DNA repair in spermatogenic cells in vivo (18, 19). Fourth, MeCl did not alkylate mammalian DNA after in vivo exposure (20, 21). Therefore, we hypothesized that direct action of MeCl to damage sperm DNA in vivo was unlikely, and that instead, MeCl-induced dominant lethal mutations in sperm resulted from the induction of epididymal inflammation and the concomitant production of genotoxic oxidative metabolites by activated phagocytes.

Previous studies in our laboratory showed that the induction of epididymal inflammation by MeCl in F344 rats was inhibited by concurrent administration of 3-amino-1-[m-(trifluoromethyl)phenyl]-2-pyrazoline (BW 755C) (22, 23).

Abbreviations: MeCl, methyl chloride; BW 755C, 3-amino-1-[m-(trifluoromethyl)phenyl]-2-pyrazoline.
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BW 755C is a potent anti-inflammatory agent by virtue of its dual inhibition of cyclooxygenase and lipooxygenase enzymes, thus preventing both prostaglandin and leukotriene synthesis, respectively (24, 25); both of these classes of arachidonic acid derivatives are known to mediate inflammatory processes (26–28). Therefore, to determine the role of inflammation in the genotoxicity of MeCl to sperm, the present study assessed the dominant lethality of MeCl in rats exposed to MeCl with and without concurrent treatment with BW 755C.

MATERIALS AND METHODS

Animals. Male (200–220 g) and female (170–190 g) Fischer 344 rats (CDF F344/Crl BR) were obtained from Charles River Breeding Laboratories. Upon arrival, animals were placed in quarantine for 2 weeks; during this time it was determined that the rats were free of pathogenic viruses (standard rat virus titer screen; Microbiological Associates). Food and filter-purified tap water (29) were provided ad libitum, except during exposures; lighting was controlled by an automatic timer on a 12-hr light/12-hr dark cycle.

Immediately after quarantine 175 males were housed 1 per cage and premated with 1 female each for a period of 2 weeks. Of the 175 males, 140 breeders were selected and allocated by use of a computerized randomization program into the five weight-matched treatment groups described below.

Exposures and Treatment Groups. Male rats were exposed by inhalation to MeCl 6 hr/day for 5 consecutive days, using previously published methods (23). MeCl concentrations in the exposure chamber were determined hourly; the mean time-weighted concentration of MeCl was 3009 ± 74 ppm (mean ± SD). Chamber temperature and relative humidity ranged from 25°C to 27°C and from 44% to 53%, respectively.

Groups of 40 male rats each were exposed to MeCl, with or without concurrent treatment with the anti-inflammatory agent BW 755C. BW 755C was dissolved in distilled water and injected i.p. in a volume of 0.4 ml; 10 mg of BW 755C per kg of body weight was injected daily both 1 hr pre- and 1 hr postexposure. Control groups (n = 20) were either untreated, or injected as described above with BW 755C, or injected on the afternoon of day 5 with triethylenemelamine (0.2 mg/kg), a known dominant lethal mutagen (30).

Breeding and Data Collection. Beginning on day 2 after exposure, each male was caged with one female weekly for 3 weeks and checked daily for evidence of mating (presence of copulatory plug in the underlying female). Females were removed when bred or after 7 days; killed 12 days after the end of each respective mating week; and, in accordance with normal dominant lethal protocol, examined for pregnancy and the number of live fetuses, early fetal deaths (resorptions), late fetal deaths, and corpora lutea (14). The uteri of animals that were not pregnant, or in which the difference between the number of implants and the number of corpora lutea was more than two, were stained with 10% ammonium sulfide to reveal otherwise undetectable implantation sites. Additional groups of males (n = 8) were used for histopathology. These rats were exposed to MeCl as described above, with or without BW 755C treatment, and killed immediately after the fifth day of exposure. The epididymides were removed, fixed in Bouin’s solution, and processed for examination by light microscopy.

Statistical Analysis. Results were analyzed according to recently published recommendations for statistical analysis of dominant-lethal data (16); to achieve desirable distributional properties prior to conduct of parametric testing procedures, a data-clustering scheme was employed which has been extensively evaluated for its applicability to dominant-lethal data (31).

Specifically, data on the number of mated females that became pregnant were analyzed by a one-tailed Fisher’s exact test with Bonferroni correction (32). All other data were evaluated by parametric analysis after randomly grouping pregnant females into clusters of three to achieve normal distributions and homogeneous variances for each clustered variable (31). After square root or arcsine transformation, the clustered data were analyzed by a one-tailed Student’s t test with Bonferroni correction. Probability values less than 0.05 were considered to indicate significant differences.

RESULTS

The kinetics of spermatogenesis were used to calculate that sperm in the ejaculate at weeks 1 and 2 postexposure were in the epididymis at the time of MeCl exposure, whereas those ejaculated at week 3 were in the testis as late-stage spermatids (33, 34). It is not likely that sperm which were in the vas deferens at the time of MeCl exposure were sampled during mating, even during week 1, since there was a 2.5-day gap between the end of exposure and the first possible mating period (see Materials and Methods). Consistent with a previous report from our laboratory (14), MeCl produced dominant lethal mutations only in sperm located in the epididymis at the time of exposure (Table 1). These mutations were detected as significant increases in the number of dead implants per pregnant female at week 1 and in the ratio of dead implants to total implants at weeks 1 and 2; these parameters were increased 2.5- to 4-fold in MeCl-exposed rats compared to untreated controls. In contrast, by week 3 postexposure the number of dominant lethal mutations detected in ejaculated sperm of the MeCl group had declined to control levels (Table 1). Consistent with previous results (14), the positive control triethylenemelamine caused significant increases in postimplantation loss during weeks 1–3 postexposure and in postimplantation loss at weeks 2 and 3 (Table 1).

MeCl-induced formation of dominant lethal mutations in sperm was inhibited by the anti-inflammatory agent BW 755C. BW 755C prevented MeCl-induced increases in the number of dead implants per pregnant female and in the ratio of dead implants to total implants at weeks 1 and 2 (Table 1). BW 755C also reduced the percentage of pregnant females with one or more (Fig. 1A) or two or more (Fig. 1B) postimplantation losses at weeks 1 and 2.

Light microscopy was used to confirm that the induction of epididymal inflammation by MeCl was inhibited by BW 755C. Epididymides from rats treated with BW 755C during MeCl exposure showed only a slight increase in the number of interstitial neutrophils and mononuclear inflammatory cells relative to nonexposed controls. In contrast, rats in the MeCl group showed widespread infiltration of neutrophils and macrophages into the interstitial tissue of the cauda epididymis, as well as frequent appearance of inflammatory cells inside the epididymal tubules, in close association with sperm. Previously published photomicrographs have documented this association of inflammatory cells with sperm of MeCl-exposed rats (13).

DISCUSSION

The results in this report suggest that inflammatory sites in vivo are potential sources of genotoxic damage to neighboring cells. Specifically, epididymal inflammation appears to be an important element in the formation of dominant lethal mutations in sperm of MeCl-exposed rats. In the present study,
significant increases in dominant lethal mutations (dead implants) were observed only at weeks 1 and 2 after MeCl exposure (Table 1); i.e., only in sperm located in the epididymis (and therefore in a site of active inflammation) at the time of exposure. Furthermore, inhibition of MeCl-induced epididymal inflammation with the anti-inflammatory agent BW 755C prevented the induction of dominant lethal mutations in sperm (Table 1, Fig. 1). It is not likely that BW 755C prevented the dominant lethal mutations by a mechanism independent of its anti-inflammatory properties. Previous work in our laboratory has shown that BW 755C does not alter the overall pathway of MeCl metabolism or the distribution to various organs (including epididymis) of radioactivity derived from [14C]MeCl (23).

The data in this report and in a previous report from our laboratory (14) indicate that MeCl is not a particularly strong dominant lethal mutagen; i.e., MeCl induces a 2- to 4-fold increase in the number of dominant lethal mutations relative to nonexposed controls, vs. 2- to 15-fold increases for the positive control triethylenemelamine. The relatively small dominant lethal effect of MeCl, combined with control levels of postimplantation loss that fluctuate 2- to 3-fold from one week to the next (present report; ref. 14), makes it difficult to consistently demonstrate statistical significance for MeCl-induced increases in postimplantation death. For example, in the present report the increase in dead implants per female from 0.53 (control) to 1.13 (MeCl-exposed) at week 2 after MeCl treatment was not significant at the 0.05 probability level (Table 1). Nevertheless, both the magnitude and the timing of the postimplantation loss induced by MeCl have been reproducibly observed in our laboratory in three separate dominant lethal studies (present report and ref. 14).

Furthermore, a consistent pattern of MeCl-induced increases in dominant lethality is seen when not just one, but a battery of dominant lethal indices are examined: dead implants per female, dead implants/total implants, females with one or more postimplantation losses, and females with two or more postimplantation losses. One or more of these indices was significantly elevated in the MeCl-exposed group at both weeks 1 and 2 after MeCl exposure. These data provide

### Table 1. BW 755C inhibition of dominant lethal mutations in sperm of MeCl-exposed F344 rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mated females</th>
<th>% mated females pregnant</th>
<th>Corpora lutea</th>
<th>Total implants</th>
<th>Preimplantation loss</th>
<th>Postimplantation loss (dead implants)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. per pregnant female</td>
<td>No. per pregnant female</td>
<td>No. per pregnant female</td>
<td>No. per pregnant female</td>
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<tr>
<td>Week 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>18</td>
<td>94</td>
<td>167</td>
<td>153</td>
<td>9.0</td>
<td>0.82</td>
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<tr>
<td>BW 755C control</td>
<td>16</td>
<td>88</td>
<td>142</td>
<td>124</td>
<td>8.9</td>
<td>1.29</td>
</tr>
<tr>
<td>MeCl</td>
<td>38</td>
<td>84</td>
<td>310</td>
<td>258</td>
<td>8.1</td>
<td>1.62</td>
</tr>
<tr>
<td>MeCl + BW 755C</td>
<td>33</td>
<td>79</td>
<td>247</td>
<td>185</td>
<td>7.1</td>
<td>2.38</td>
</tr>
<tr>
<td>Triethylenemelamine</td>
<td>20</td>
<td>95</td>
<td>179</td>
<td>141</td>
<td>7.4</td>
<td>2.00</td>
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<tr>
<td>Control</td>
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<td>100</td>
<td>163</td>
<td>127</td>
<td>7.5</td>
<td>2.12</td>
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<tr>
<td>BW 755C control</td>
<td>12</td>
<td>92</td>
<td>104</td>
<td>92</td>
<td>8.4</td>
<td>1.09</td>
</tr>
<tr>
<td>MeCl</td>
<td>36</td>
<td>64*</td>
<td>227</td>
<td>117</td>
<td>5.1*</td>
<td>4.78*</td>
</tr>
<tr>
<td>MeCl + BW 755C</td>
<td>28</td>
<td>61*</td>
<td>159</td>
<td>82</td>
<td>4.8*</td>
<td>4.53*</td>
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<tr>
<td>Triethylenemelamine</td>
<td>17</td>
<td>94</td>
<td>155</td>
<td>82</td>
<td>5.1*</td>
<td>4.56*</td>
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<tr>
<td>Control</td>
<td>20</td>
<td>75</td>
<td>145</td>
<td>127</td>
<td>8.5</td>
<td>1.20</td>
</tr>
<tr>
<td>BW 755C control</td>
<td>18</td>
<td>83</td>
<td>148</td>
<td>139</td>
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<tr>
<td>MeCl</td>
<td>35</td>
<td>49</td>
<td>156</td>
<td>74</td>
<td>4.4*</td>
<td>4.87*</td>
</tr>
<tr>
<td>MeCl + BW 755C</td>
<td>32</td>
<td>75</td>
<td>232</td>
<td>111</td>
<td>4.6*</td>
<td>5.04*</td>
</tr>
<tr>
<td>Triethylenemelamine</td>
<td>18</td>
<td>78</td>
<td>136</td>
<td>47</td>
<td>3.4*</td>
<td>6.36*</td>
</tr>
</tbody>
</table>

*Both early and late fetal deaths were included in the calculation of dead implants (postimplantation loss). Preimplantation loss was calculated from the corpora lutea count minus total implants. For each week, treatment groups significantly different (P < 0.05) from the corresponding untreated control group are indicated by an asterisk; statistical analyses employed are described in Materials and Methods. Values given for dead implants per total implants (column 12) were calculated from individual animal values and not from the group means given in columns 7 and 11.*
convincing evidence that the induction of dominant lethal mutations by MeCl is a biologically significant effect. In addition to the diminution of MeCl-induced dominant lethal effects by BW 755C, further support for the conclusion that MeCl-induced dominant lethal mutations in sperm are related to epididymal inflammation is provided by the inability of MeCl to directly damage germ-cell DNA during exposure conditions (3500 ppm 6 hr/day for up to 9 days) similar to those used in the present report (18, 19). Even at concentrations approaching the maximum tolerated by F344 rats (15,000 ppm for 3 hr) MeCl does not induce DNA repair in vivo in spermatocytes or early spermatids, as assessed by unscheduled DNA synthesis (UDS) (18, 19). In contrast, the dominant lethal mutations studied thus far that are known to act directly or via their metabolites to damage germ-cell DNA are also inducers of germ cell UDS (35, 36). The only conditions under which MeCl has been demonstrated to directly damage germ-cell DNA have been in vitro at concentrations at least 10 times greater than those used in the present study (18, 19).

An additional line of evidence providing indirect support for the hypothesis that MeCl dominant lethality is mediated by inflammation is that alkylation of cellular macromolecules is not detected after in vivo exposure to MeCl (20, 21); instead, association of MeCl and/or its metabolites with macromolecules such as DNA and protein appears to result primarily from metabolic incorporation (20). Therefore, as possible mechanisms of dominant lethality, alkylation of sperm DNA or alkylation of sperm protein [the apparent cause of dominant lethal mutations in mice exposed to ethyl or methyl methanesulfonate (37, 38)] are not likely applicable to MeCl.

Although BW 755C was effective against the postimplantation losses induced by MeCl exposure, it did not prevent MeCl-induced increases in preimplantation loss at weeks 2 and 3 postexposure (Table 1). This was reflected in the persistent decreases in fertility and total implants per female observed at weeks 2 and 3 in both the MeCl and MeCl-plus-BW 755C treatment groups (Table 1). The preimplantation loss induced by MeCl is thought to result from cytotoxic (rather than genotoxic) effects of the exposure, which lead to failure of fertilization (39, 40). Recent results from our laboratory have suggested that MeCl-induced preimplantation loss at weeks 2 and 3 might be explained by cytotoxic effects of MeCl on the testis rather than on the epididymis; these testicular effects of MeCl do not appear to be markedly inhibited by BW 755C (unpublished data). This explanation is consistent with the fact that week-3 ejaculates would have contained sperm located in the testis at the time of MeCl exposure. Even at week 2, it is likely that ejaculates contained some sperm exposed while in the testis, as well as those exposed in the epididymis, based both on epididymal transection times for sperm in actively mating rats (33, 41) and the mixing between spermatozoa of different generations that is known to occur in the distal cauda epididymis (42).

We conclude that the dominant lethal effects of MeCl are a probable consequence of its induction of epididymal inflammation, rather than a direct effect of MeCl or its proposed metabolites. Our results illustrate the importance of using appropriate exposure concentrations to determine the actual basis for genotoxic effects elicited by chemical exposure. Also, our data extend to in vivo systems the substantial amount of evidence obtained from in vitro models that demonstrates the potential genotoxicity of activated inflammatory cells.

The dominant lethal mutations formed in male germ cells as the apparent result of MeCl-induced epididymal inflammation are important in terms of fetal loss. However, these mutations are not inherited and therefore do not contribute to the genetic load of altered DNA. Nevertheless, many mutagens produce both lethal and nonlethal chromosomal effects; thus, the production of dominant lethal mutations could be accompanied by the production of nonlethal, heritable mutations in otherwise normal cells (16). Determination of the full spectrum of genotoxic effects that can be elicited by in vivo inflammatory reactions may have important implications in the risk assessment of toxicants that induce inflammatory responses.

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