Combined Exposure of ELF Magnetic Fields and X-rays Increased Mutant Yields Compared with X-rays Alone in pTN89 Plasmids

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Extremely low frequency magnetic fields/X-rays/Mutation/pTN89 plasmids.

We have examined mutations in the supF gene carried by pTN89 plasmids in Escherichia coli (E. coli) to examine the effects of extremely low frequency magnetic fields (ELFMFs) and/or X-rays to the plasmids. The plasmids were subjected to sham exposure or exposed to an ELFMF (5 mT), with or without X-ray irradiation (10 Gy). For the combined treatments, exposure to the ELFMF was immediately before or after X-ray irradiation. The mutant fractions were 0.94 × 10⁻⁵ for X-rays alone, 1.58 × 10⁻⁵ for an ELFMF followed by X-rays, and 3.64 × 10⁻⁵ for X-rays followed by an ELFMF. Increased mutant fraction was not detected following exposure to a magnetic field alone, or after sham exposure. The mutant fraction for X-rays followed by an ELFMF was significantly higher than those of other treatments. Sequence analysis of the supF mutant plasmids revealed that base substitutions were dominant on exposure to X-rays alone and X-rays plus an ELFMF. Several types of deletions were detected in only the combined treatments, but not with X-rays alone. We could not find any mutant colonies in sham irradiated and an ELFMF alone treatment, but exposure to ELFMFs immediately before or after X-ray irradiation may enhance the mutations. Our results indicate that an ELFMF increases mutation and alters the spectrum of mutations.

INTRODUCTION

There has been increasing concern about electromagnetic fields (EMFs) associated with human health since Wertheimer and Leeper suggested a correlation of EMFs and childhood leukemia. Recently, some epidemiological studies also revealed that extremely low frequency magnetic fields (ELFMFs) had association with risk of childhood leukemia. Davis et al. showed that exposure to 60-Hz magnetic fields may increase breast cancer risk by suppressing the normal nocturnal rise in melatonin which is a powerful antioxidant and radical scavenger. Ishido et al. also stated that magnetic fields suppressed the action of melatonin in magnetic field-sensitive MCF-7 cells. The other report has suggested possible genotoxicity of magnetic fields. However, many investigations have shown that exposure to extremely low frequency electromagnetic fields causes almost no harmful effects to bacterial or mammalian cells. Gurney et al. reviewed that no recent research had emerged to provide reasonable support for a casual role of EMF on brain cancer. Thus, though many experiments have been performed and a possible association between EMFs and some diseases has been proposed, there still have been contradictions. These inconsistencies might be attributable to different cell lines and/or to different methodology and quality of measurement.

Previously, we reported that exposure to power-line frequency magnetic fields increased chromatid-type chromosomal aberrations in mouse m5S cells, and that exposure to a 5 mT ELFMF might induce mutations and enhance X-ray-induced mutations. In addition, we observed that exposure to a 400 mT ELFMF for 2 h increased X-ray-induced mutations in human melanoma MeWo cells, and that exposure to strong magnetic fields at power frequency potentiated X-ray-induced DNA strand breaks in MOS4 cells. Besides, we revealed that a mutation frequency in pTN89 plasmids DNA treated with H₂O₂ was enhanced by the exposure to a 5 mT ELFMF.

In this study, the plasmid DNAs were exposed to ELFMFs

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with or without X-rays and then transformed into *Escherichia coli* (*E. coli*). The plasmids were subsequently extracted and sequence analysis was performed. We suggest that exposure to ELFMs enhance DNA damage after X-ray irradiation, and we observed some differences in the mutational spectrum between treatment with X-rays alone and the combined treatment with X-rays and ELFMs.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

An *E. coli* strain, KS40 (lacZam rslpr gyA r), derived from MBM7070, that harbors pOF105 and carries the gyrA Am and rslpAm genes in the pACYC plasmid, was designated as KS40/pOF105. An *E. coli* strain SY1033 was used to confirm that the plasmids had a mutation in the supF gene.

**Reagents and media**

Luria-Bertani (LB) broth consisted of tryptone, yeast extract and salt which were purchased from Nakalai Tesque Inc., Japan. LB plates were obtained from GIBCO BRL. and phosphate buffer was purchased from TaKaRa Bio Inc., Japan. McConkey medium was obtained from DIFCO. Amoxicillin (Am) and chloramphenicol (Cm) were added to the LB plates at concentrations of 50 μg/ml and 30 μg/ml, respectively. Am and tetracycline (Tet) were added to the McConkey plates at concentrations of 50 μg/ml and 10 μg/ml, respectively. Minimal agar medium, used for supF mutant selection, is composed of 300ml M56 salt with 6ml of 20% glucose, 12ml of 10% casamino acids, 0.1ml of 0.1% thiamin and 300ml of 3% agar. Am (150 μg/ml), Cm (30 μg/ml), nalidixic acid (Nal; 50 μg/ml) and streptomycin (Sm; 100 μg/ml) were added to the medium. Reagents used for extracting DNA were purchased from QIAGEN, and enzymes and reagents for DNA sequencing were obtained from Amersham Biosciences. Isopropyl-b-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) used for detection of mutants were purchased from Wako, Japan. The oligonucleotide 5'-GTATCCAGGGC-CCTT-3' used as the primer for DNA sequencing was purchased from Invitrogen Life Technologies.

**Exposure of pTN89 plasmids to ELFMs**

The apparatus for ELFm exposure is described elsewhere. In brief, the ELFm equipment consists of a CO₂ incubator (model BNR-110, Tabai ESPEC) with a built-in magnet constructed using Helmholtz coils (FA System Co., Ltd.) being 250mm in inner diameter, 160mm in distance with 128 turns, a slide regulator (model CM-1320, Matsunaga Seisakusyo) and a thermocontroller (model UC-55N, Tokyo Rikakikai). A 5 mT ELMF at 60 Hz was used and the duration of ELFm exposure was 16 hr. The temperature of the apparatus for ELFm exposure was monitored by thermocouple sensors (model PRb442A/100, Sakaguchi Co., Ltd.). A thermocontroller was used to keep the incubator temperature at 37 ± 0.2°C by circulating warm water. For the control experiments, a conventional incubator in a separate room was used. The inside and outside of the incubators were shielded by silicon steel and Permalloy C, respectively. The measured ELFm in the conventional incubator was < 0.5 μT.

**X-ray irradiation**

The method used for X-ray irradiation has been described previously. In brief, X-ray irradiation was performed using a Hitachi MBR-1520 operating at 150 kV and 20 mA. The dose rate of X-rays was approximately 8 Gy/min.

**Treatment of plasmid pTN89**

pTN89 plasmids at concentration of 50 μg/ml in distilled water were exposed to ELFm alone, irradiated with X-rays alone, and exposed to an ELFm immediately before or after X-ray irradiation at 10 Gy.

**Transfection and supF mutant analysis**

pTN89 plasmids were introduced into 40 μl of KS40/pOF105 by electroporation (Easyjet Optima; EQUIBIO) and the cells were then incubated in 960 μl of SOC solution at 37°C. After 1 hr incubation, cell suspensions were diluted to 1/1000 and samples of 100 μl were plated onto LB plates containing Am and Cm at the concentrations described above. KS40/pOF105 cells transformed with plasmids were centrifuged at 3000 rpm for 15 min and the pellets were plated onto the minimal plates. The total number of transformants was determined by counting the white colony number per plate. The KS40/pOF105 strain is resistant to Sm and Nal if it contains a mutant supF; whereas cells carrying an active supF do not produce colonies on such plates. The minimal plates were incubated at 37°C overnight. Sm- and Nal-resistant colonies were streaked on LB plates containing X-gal and IPTG, and each white colony was collected for incubation in LB broth to amplify the plasmids. The mutant fraction was calculated from the ratio of the supF(-) transformation rate on the minimal plate to the supF transformation rate on the LB plate.

Plasmid DNA was extracted from a putative supF mutant transformant and transfected into *E. coli* SY1033 to confirm that the plasmid DNA contained the supF mutant. Transfected *E. coli* were plated onto McConkey plates and incubated overnight. White colonies indicated the presence of a plasmid DNA with the supF mutant.

**DNA sequencing**

After confirmation of the presence of the supF mutant in the plasmid, the DNA was sequenced by the dideoxy chain termination method, using a Long-Read Tower sequencing machine (Amersham Biosystems).
RESULTS

Mutant fraction

We were unable to detect a mutant plasmid in controls subjected to sham-exposure and in plasmids exposed to an ELF-MF alone. The mutant fractions were less than at least $2.01 \times 10^{-3}$ for sham-exposure and were less than $3.12 \times 10^{-2}$ for ELF-MF treatment alone. Two to 166 colonies (X-rays alone, 11-116; X-rays→ELF, 2-26; ELF→X-rays, 4-12) were found on LB plates, and 1 to 17 colonies (X-rays alone, 1-17; X-rays→ELF, 1-6; ELF→X-rays, 1-9) were found on selective plates. The transformation rate was calculated from the numbers of colonies per 1 µg DNA. These wide ranges of the colony number could be depended on cell competency. The transformation rates were calculated on LB and selective plates. Then, the mutant fractions were determined by the calculation as described above. The mutant fractions of the pTN89 plasmids carrying the supF gene treated with X-rays alone, X-rays followed by an ELF-MF, and an ELF-MF followed by X-rays were $0.94 \times 10^{-5}$, $3.64 \times 10^{-5}$ and $1.58 \times 10^{-5}$, respectively (Fig. 1). The mutant fraction for plasmids irradiated by X-rays and then exposed to an ELF-MF was about 4-fold higher than that for X-ray treatment alone, and 2.3-fold higher than that for an ELF-MF followed by X-ray treatment. There was statistically significant difference ($P < 0.01$) among these treatments, as determined by a Student's-t-test. However, there was no difference in the mutant fraction between the treatment with X-rays alone and that of exposure to an ELF-MF followed by X-rays.

Spectrum of mutations produced in the supF gene

Table 1 shows the distribution of the mutational classes for each treatment. In every treatment, the majority of changes were single base pair substitutions, except for double base pair substitutions that occurred at the position of 105 and 109 in plasmids irradiated with X-rays alone. Other mutations that occurred in the supF gene were an insertion, deletions and a single base frameshifts. An insertion sequence was found only in plasmids irradiated with X-rays followed by exposure to an ELF-MF. In this case, a cytosine was inserted between positions 94 and 95 (not shown in Fig. 2). Deletions were not detected in plasmids irradiated by X-rays alone.

Both types of transitions and four types of transversions were identified. Base substitutions at G:C sites occurred more frequently than at A:T sites under all treatment conditions (Table 2). Deletions and frameshifts are shown in Table 3. Treatment with X-rays alone did not induce a deletion, while the X-ray/ELF-MF combination treatments caused several types of deletions.

Base substitutions

Figure 2 shows the site distribution of base substitution

![Figure 1](http:ll.irr,jstagc.jst.go.jp)

**Table 1.** Distribution of the supF mutants by class.

<table>
<thead>
<tr>
<th>Class of change</th>
<th>Number detected (% of total mutants examined)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X-rays alone</td>
</tr>
<tr>
<td>Base substitution</td>
<td>49 (94)</td>
</tr>
<tr>
<td></td>
<td>one base change 47</td>
</tr>
<tr>
<td></td>
<td>two base change 1</td>
</tr>
<tr>
<td>Insertion</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Deletion</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Frameshift</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
</tr>
</tbody>
</table>

Fig. 2. Locations of base substitutions in the supF gene in pTN89 plasmids treated with X-rays alone, X-rays followed by an ELFMF, and an ELFMF followed by X-rays.

Table 2. Type of base substitution.

<table>
<thead>
<tr>
<th>Type of mutations</th>
<th>Number detected (% of total base substitution examined)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X-rays alone</td>
</tr>
<tr>
<td>Transition</td>
<td></td>
</tr>
<tr>
<td>G:C → A:T</td>
<td>5 (10)</td>
</tr>
<tr>
<td>A:T → G:C</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Transversion</td>
<td></td>
</tr>
<tr>
<td>G:C → T:A</td>
<td>20 (41)</td>
</tr>
<tr>
<td>G:C → C:G</td>
<td>22 (45)</td>
</tr>
<tr>
<td>A:T → T:A</td>
<td>1 (2)</td>
</tr>
<tr>
<td>A:T → C:G</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Mutagenesis in the supF gene of the pTN89 plasmids. There were some differences in site distribution among the three treatments. Although the mutated sites were generally different, several mutational hot spots were apparent (positions 133, 156, 159, 160, 168 and 169). A:T → G:C base substitution occurred at position 95 in the supF gene (not shown in Fig. 2).
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Table 3
Deletion and frameshift found among supF mutants.

<table>
<thead>
<tr>
<th>Class of change</th>
<th>Sequence change (Number detected)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X-rays alone</td>
</tr>
<tr>
<td>Deletion</td>
<td>not detected</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Frameshift</td>
<td>172-176 (3)</td>
</tr>
<tr>
<td>TCCCCCA</td>
<td>140 (1)-T</td>
</tr>
<tr>
<td></td>
<td>164 (1)-G</td>
</tr>
<tr>
<td>TCCCCCA</td>
<td>172-176 (3)</td>
</tr>
<tr>
<td></td>
<td>TCCCCCA</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The numbers described 60-105, for example, represent the range deleted from the position of 60 to 105 sequencing.

\textsuperscript{b}The numbers described 95-, for example, represent that we could not detect the following sequences from the number of described before.

**DISCUSSION**

There have been contradictory reports on whether there is a correlation between exposure to electromagnetic fields and human health. Since epidemiological studies indicated a correlation between exposure to ELFMFs and an increased risk of childhood leukemia and numerous other cancers, many studies have been undertaken to investigate this further. In general, it has been concluded that the lower, non-ionizing energy associated with an ELFMF is not sufficient to cause direct damage to DNA but doubts persist regarding this. In in vitro studies, the contradictory results might have been caused by the use of different cell lines, and/or the use of different methodology for exposure. Therefore, we chose a simple system to detect the effects of ELFMFs on plasmid DNAs. Previously, we detected an enhancement of mutant fraction by a 5 mT ELFMF-exposure using the same system, in which combined treatment of ELFMFs and H2O2 induced mutation about 2.5 times compared with the treatment of H2O2 alone.

In this study, there was also statistically significant difference in the mutant fraction between the treatment with X-rays alone or that of exposure to an ELFMF followed by X-rays and that of exposure to an ELFMF followed by X-rays (Fig. 1). This suggests that post-X-ray irradiation to an ELFMF may enhance the mutant fraction in X-ray-treated plasmids.

Deletions did not occur in plasmids treated with X-rays alone (Table 1). X-rays are well known to induce deletion in cultured cells, however, Frey et al. reported that in gamma-irradiated phage DNA with 40 Gy was induced one double strand break per 100 nucleotides. This corresponds to our result. However, combination treatments of X-rays and an ELFMF induced several types of deletions. This may imply that exposure to an ELFMF causes some enhancement of DNA damage when the exposure is done immediately before or after X-irradiation. Previously, we reported a statistically significant increase in the frequency of chromosomal aberrations in cells exposed to X-rays followed by an ELFMF and that post-X-ray irradiation to a high density ELFMF at more than 50 mT may potentiate X-ray-induced DNA strand breaks. The present results are consistent with those observations, indicating that post-X-ray irradiation to an ELFMF could enhance DNA damage induced by X-rays though there are some reports to deny the combined effect of ELF and ionizing radiation.

As shown in Table 2, base substitutions occurred predom-

Inantly at G:C sites, although the kind of base substitutions differed somewhat with different treatments. There are many reports in the literature that show G:C site transversions induced by ionizing radiation and chemical agents.\(^{30-34}\) Kasai et al.\(^{35}\) reported that X-ray irradiation induced 8-hydroxyguanine formation in aqueous solution, and further suggested that this reaction proceeded via the formation of hydroxyl radicals. Fischer-Nielsen et al.\(^{36}\) have also suggested that reactive oxygen species (ROS) induce 8-hydroxy-2'-deoxyguanosine formation. The 8-hydroxyguanine induces base substitution via G:C→T:A transversion\(^{37}\) and, hence, the G:C→T:A transversions observed in this study might have been induced by 8-hydroxyguanine and/or ROS. Although we did not perform experiments to find evidence that antioxidants could suppress the mutant yield. There are reports in support of the mention. For example, Wolf et al.\(^{38}\) reported that the effects of 50-Hz ELF electromagnetic fields on cell proliferation and DNA damage were prevented by pretreatment of cells with an antioxidant like α-tocopherol, suggesting that redox reactions were involved. Lai and Singh\(^{39}\) have also suggested that free radicals might play a role in magnetic field-induced DNA damage. Therefore, exposure to an ELF MF might affect the production of free radicals or enhance the activity of hydroxyl radicals produced by X-rays. The mechanism of the combination with ELF MFs and X-rays is not proved however, we propose that ELF MFs have some effects to produce free radical or to damage to DNA directly. Some reports showed that weak magnetic fields affected free radical recombination.\(^{30-42}\)

There were significantly different deletions induced by the combined treatment of X-rays and an ELF MF (Table 3). Three short-sequence deletions and three long-sequence deletions occurred in plasmids irradiated by X-rays followed by ELF MF exposure. On the other hand, a deletion was not found in plasmids irradiated by X-rays alone. Eight long-sequence deletions were also detected in plasmids exposed to an ELF MF followed by X-ray treatment. Although ELF MF treatment before X-ray irradiation had no effect on mutant fraction, the deletion frequency was increased. These observations indicate that ELF MF exposure could modify DNA damage after X-ray irradiation, and an ELF MF-exposure before X-ray irradiation has some effects to DNA damage, though there was no difference in mutant fraction.

As shown in Fig. 2, base substitutions in every treatment appeared in similar positions, described as hotspots, except that a base substitution at position 160 was not detected in plasmids irradiated by X-rays alone and at position 156 was not detected in plasmids irradiated by X-rays followed by an ELF MF-exposure. The hotspot data from Nunoshiba et al.\(^{43}\) which were observed in the MutM MutY strain, were similar to our results. This also may suggest that the 8-hydroxyguanine formation were enhanced by an ELF MF itself.

In the present study, we can conclude that an ELF MF at 5 mT can enhance and modify DNA damage induced by X-ray irradiation and it implies that an ELF MF enhance the reactive oxygen activity.

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**REFERENCES**

Combined Effects of ELF-MFs and X-rays on Mutation in Plasmids

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41: 293–302.


Escherichia coli mutant deficient in Mn- and Fe- superoxide dismutases and Fur, a repressor for iron-uptake systems. DNA Repair. 30: 411–418.

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