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Research Article

Use of Centromeric and Telomeric DNA Probes in In Situ Hybridization for Differentiation of Micronuclei Induced by Lomefloxacin

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Classification of micronuclei induced by lomefloxacin, a difluorinated quinolone bactericidal agent, in mouse bone marrow was performed by fluorescence in situ hybridization using DNA probes for the centromere repeated minor satellite DNA and the telomeric hexamer repeat (5′-TTAGGG-3′). Colchicine and mitomycin C were used as a positive control aneugen and clastogen, respectively, and these compounds produced the expected responses. Single doses of 40, 80, 160, or 320 mg/kg lomefloxacin were given via oral intubations and bone marrow was sampled at 24 and 48 hr after treatment. The micronuclei showed significant increases in both sampling times after doses of 320 mg/kg. A statistically significant increase of micronuclei frequency was also detected for 160 mg/kg lomefloxacin at 48 hr after treatment. The responses were directly correlated with bone-marrow cytotoxicity. Following treatment with 160 and 320 mg/kg lomefloxacin, 48.2 and 50.0% of the induced micronuclei, respectively, showed double labeling with centromeric signals and several telomeric signals, indicating that the induced micronuclei were composed of whole chromosomes. Similarly, 51.8 and 50.0% of the induced micronuclei, respectively, were centromere-negative, demonstrating that lomefloxacin not only induces chromosome loss but also chromosome breakage. The results also showed that chromosomes can be enclosed in a micronucleus before and after centromere separation. Overall, this study provides the first evidence of the potential of lomefloxacin to induce aneugenic effect in mice. However, given the high doses used in this study, the clinical significance of this finding is uncertain. Environ. Mol. Mutagen. 50:394–403, 2009.

Key words: lomefloxacin; micronuclei; FISH; clastogenicity; aneugenicity; genotoxicity

INTRODUCTION

The extensive use (and misuse) of antibiotics to treat infectious diseases has led to increased bacterial resistance, stimulating the development of new chemicals with less bacterial resistance. Among the new developments are quinolones, which include drugs such as nalidixic acid or pipemidic acid, which have the parental carboxy-quinolone chemical structure, and the more potent fluoroquinolones such as lomefloxacin. Because of their broad spectrum activity against both gram-negative and gram-positive pathogens, these antibiotics are frequently used against infections, including those of the genitourinary and upper respiratory tracts, meningitis, and skin infections [Appelbaum and Hunter, 2000]. The primary target of quinolone antibacterial drugs is bacterial DNA gyrase, which is similar to DNA topoisomerase II of eukaryotic cells [Fisher et al., 1989]. The drugs inhibit the resealing of a DNA strand break by the A subunit of DNA gyrase following supercoiling. This inhibition results from saturation of binding sites on bacterial DNA and subsequent structural changes in the DNA. In a photocarcinogenicity study using SKH1 hairless mice exposed to UVA, all the fluoroquinolone antibiotics studied enhanced UVA-induced phototumorigenesis, but only lomefloxacin caused the development of cystic squamous cell carcinomas in the majority of treated animals [Klecak et al., 1997; Mäkiminen et al., 1997]. With respect to the genotoxicity of quinolones, in general, they have been reported to be positive

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for chromosomal aberrations in cultured mammalian cells, but negative for bacterial reverse mutations in *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537, and *Escherichia coli* WP2uvrA [Shimada et al., 1992; Shimada and Itoh, 1996]. Moreover, many quinolones have been confirmed to exhibit mutagenicity in TA102 and in WP2uvrA/pKM101 [Ysern et al., 1990; Gocke, 1991; Mamber et al., 1993; Watanabe et al., 1998; Martínez et al., 2000; Hayasaki et al., 2006].

It has been shown previously that lomefloxacin is a clastogen capable of inducing micronuclei in SKH1 hairless mice [Itoh et al., 2002a], Chinese hamster V79 cells [Kersten et al., 1999; Synder and Cooper, 1999], and HaCaT cells [Zhang et al., 2001] in conjunction with high UVA/UVB irradiation doses. Lomefloxacin can also enhance chromosomal aberrations in Chinese hamster lung cells with light irradiation in vitro [Itoh et al., 2002b]. The photogenotoxicity of lomefloxacin has also been previously demonstrated in different in vitro and in vivo systems by the comet assay as measured by different comet parameters [Brendler-Schwaab et al., 2004; Wirnitzer et al., 2006]. An in vivo genotoxicity study has shown that lomefloxacin induced a statistically significant reduction in mitotic index, and an increase in chromosomal aberrations and percent abnormal metaphases in mouse bone marrow after treatment with 160 mg/kg lomefloxacin [Singh et al., 2003].

The micronucleus (MN) test is used extensively to evaluate irreversible impacts on genome stability, and increased MN frequency predicts the risk of cancer in humans [Bonassi et al., 2007]. Since MN can result from chromosome breakage (clastogenicity) or lagging chromosomes (aneugenicity), the detection of MN has the potential to be used as a screen for numerical chromosomal aberrations induction if methods are included to allow the identification of whole chromosomes inside of MN. Various techniques have been developed to distinguish MN induced by clastogens or by aneugens. MN containing entire chromosomes were first characterized by their large size [Yamamoto and Kikuchi, 1980], by C-banding [Verschaeve et al., 1988; Van Hummelen et al., 1992], or by measurement of DNA content [Vanderkerken et al., 1989; Grawé et al., 1994]. However, the most reliable methods are the ones that mark centromeres (i.e., Immunofluorescent CREST-staining and in situ hybridization techniques).

The CREST method applied to the bone marrow MN test is described in detail by Miller and Adler [1990]. Using CREST antibody along with propidium iodide, Krishna et al. [1992] described a method to differentiate immature erythrocytes, mature erythrocytes, and MN containing centromeres. Fluoroscence in situ hybridization (FISH) with a centromeric DNA probe (minor satellite probe) allows detection of corresponding chromosomal regions on metaphase chromosomes as well as in interphase nuclei and, specifically, individual MN. The probe can be produced in unlimited amounts at any time and preparative alterations rarely affect centromeric DNA [Schriever-Schwemmer and Adler, 1994]. Telomeres represent repetitive tandem arrays of a hexa-nucleotide sequence (TTAGGG) that are highly conserved through evolution and necessary for chromosome stability and DNA replication. Telomere probes show an exclusively telomeric labeling pattern in the mouse [Meyne et al., 1990]. Generally, the telomere probe is used with the aim of obtaining further differentiation of MN chromosomal contents.

The present experiments were designed to obtain more insight into the genotoxic potential of lomefloxacin at different doses and different time intervals. This was achieved through an in vivo assay evaluating the formation of MN that result either from chromosome breakage or a whole chromosome loss. In addition, the origin of the MN was determined with FISH assay using mouse centromeric and telomeric DNA probes simultaneously.

MATERIALS AND METHODS

Animals

Male Swiss albino mice were used at the age of 9–13 weeks and weighed 25–30 g. The animals were obtained from a closed breeding colony at Al-Azhar University. The animals were maintained under proper environmental conditions, i.e., temperature 25–28°C, humidity at ≈50%, with a 12-hr light-dark period. Each treatment group and vehicle control group consisted of five randomly assigned animals. The animals were maintained with food and water ad libitum until being euthanized. All experiments on animals were carried out according to the Guidelines of the Animal Care and Use Committee at Al-Azhar University.

Drugs and Chemicals

Lomefloxacin (Sigma, St. Louis, MO) was dissolved in phosphate-buffered saline (PBS) and administered by a single oral administration at 0.1 ml per 10 g of body weight. The negative control groups received PBS only, while the positive control groups were injected intraperitoneally with either 2 mg/kg mitomycin C (MMC; Sigma) or 2 mg/kg colchicine (COL; Sigma). The doses for the positive controls were chosen by reference to earlier in vivo studies [Schriever-Schwemmer and Adler, 1994; Schriever-Schwemmer et al., 1997; Attia et al., 2003; Attia, 2007]. The doses of lomefloxacin were selected on the basis of their effectiveness in inducing chromosomal alterations in mouse bone marrow [Singh et al., 2003]. All other chemicals and reagents were of highest analytical grade

Experimental Design

Two separate experiments were performed with the conventional MN test. In the first experiment, animals were treated with 40, 80, 160, or 320 mg/kg of lomefloxacin, 2 mg/kg of MMC, 2 mg/kg of COL, or PBS as solvent control I, and bone marrow was sampled 24 hr after treatment. In the second experiment, mice were treated with 40, 80, 160, or 320 mg/kg of lomefloxacin or PBS as solvent control II, and bone marrow was sampled 48 hr after treatment. The animals tolerated the highest dose with some toxic symptoms. The animals became sluggish after treatment but recovered within 2 hr of the treatment.

Slide Preparations

Mice were killed by cervical dislocation at the specified time after treatment with the test chemicals or the solvent and both femurs were removed. The bone marrow cells were collected in fetal calf serum, centrifuged, and the pellet was carefully resuspended in as little supernatant as possible before slide preparation, as previously described [Adler, 1984]. At least four slides were made for each animal and allowed to dry overnight. Two slides per animal were stained for conventional assessment of the MN frequencies. The remaining unstained slides from the animals treated with MMC, COL, and 160 or 320 mg/kg of lomefloxacin (second experiment), as well as from the solvent controls, were used for the FISH analysis with the mouse DNA probes to discriminate MN of clastogenic and aneugenic origin.

Conventional MN Test

For conventional assessment of MN frequencies, the slides were stained with May-Grünwald/Giemsa as previously described [Adler, 1984]. The slides were coded and cover-slipped in Eukitt mounting medium, and then stored at room temperature until scoring.

In Situ Hybridization Analysis

DNA Probes

The murine minor satellite DNA probe, pMKB6 [Wong and Rattner, 1988], a 273-bp fragment that represents approximately two tandem repeats, in plasmid pTZ19U, was a gift from Dr. Vig (Reno, NV, USA). Hexamers of animal telomeric repeats (5′-TTAGGG-3′) were synthesized as 30mer oligonucleotides in both the sense and antisense orientations [Moyzis et al., 1988], and amplified using PCR [Ijdo et al., 1991]. The probes were labeled with the fluorescent dyes Cy3 and FITC using the biotin or digoxygenin (DIG) systems according to the manufacturer's instructions (GIBCO Invitrogen, Carlsbad, CA). The use of biotin and digoxygenin as haptens allowed the use of two DNA probes at the same time due to the different detection systems for the haptens.

Hybridization Technique and Signal Detection

The in situ hybridization technique was performed as described elsewhere [Schriever-Schwemmer and Adler, 1994; Schriever-Schwemmer et al., 1997; Jie and Jia, 2001; Attia et al., 2003; Attia, 2007]. The hybridized probes were detected by streptavidin-Cy3 for the biotin-labeled minor satellite DNA probe and Anti-DIG-FITC for the telomeric DNA probe. The hybridization signals were amplified by two rounds of incubation with biotinylated anti-streptavidin followed by streptavidin-Cy3 and FITC-labeled anti-sheep IgG antibody made in rabbit. The nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; 0.1–0.5 μg/ml in PBS) at room temperature and cover slipped in Vectashield mounting medium. The slides were scored immediately or following storage for several days at 4°C in the dark.

Scoring Criteria

In the conventionally stained slides, the MN-frequency per animal was assessed under light microscope in at least 1000 PCE under blind code. Debris and possible nuclear fragments of ruptured apoptotic nucleated cells were clearly distinguished from true MN by the shape and color of both the PCE and the MN. The double FISH labeling analysis was performed on coded slides. The slides were microscopically examined using a Zeiss Axioplan Fluorescence Microscope with filters for Cy3 (535–610 nm), FITC (480–535), and DAPI (365–397). Records of all signals-containing erythrocytes were taken by digitizing the microscopic image with a CCD camera and the computer program ISIS3 (MetaSystems). The two applied DNA probes are identified by the regular appearance of a red domain for murine minor satellite DNA probe (labeled with Cy3) and a green domain for the telomeric DNA probe (labeled with FITC). Strict scoring criteria were applied to avoid misinterpretation of signal numbers. MN were scored as having two or more domains of the same color if the signals were (a) nearly similar in size and intensity, (b) separated by at least the distance of half the diameter of the domain, and (c) regular in appearance, not diffuse and clearly positioned within the MN. For signal detection, 75 to 150 MN per group were examined for the presence or absence of DNA probes and the numbers of signals per MN were counted.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD) of the means. The analysis parameters were tested for homogeneity of variance and normality and were found to be normally distributed. The data were, therefore, analyzed by employing nonparametric tests, Mann-Whitney *U*-test and χ^2 test [Sachs, 1984]. Results were considered significantly different if the P value was ≤ 0.05.

RESULTS

Conventional MN Test

Two separate micronucleus tests were performed with lomefloxacin. The incidence of MNPCE in each treatment group as well as the PCE/normochromatic erythrocytes (NCEs) ratio is shown in Table I. The frequencies of MNPCE in control I of 0.32% (95% confidence limits—CL = 0.15–0.48%) and in control II of 0.3% (CL = 0.1–0.49%) were not significantly different. At 24 hr after treatment with the positive controls MMC and COL, a statistically significant increase in the incidence of MNPCE over the control value was observed [1.12 (CL = 0.88–1.35) and 1.04 (CL = 0.62–1.45) MN/100 PCE, respectively, $P < 0.01$]. In the 24-hr sampling time, 320 mg/kg lomefloxacin significantly increased MNPCE frequencies [0.86 (CL = 0.52–1.19) MN/100 PCE, $P < 0.05$] and the response was directly correlated with bone marrow toxicity, as significant suppression of bone marrow was noted ($P < 0.01$). Lomefloxacin treatment at 40, 80, and 160 mg/kg did not exhibit significant differences in the frequency of MNPCE and there were no significant effects on the % PCE compared to the concurrent solvent control. The aim of the second experiment (48 hr sampling) was to confirm the finding of the first experiment that lomefloxacin induces MNPCE, and decreases % PCE. At 48 hr after 160 and 320 mg/kg, lomefloxacin treatment, the incidences of MNPCE over the control value were significantly increased [0.76 (CL = 0.42–1.09) and 1.06 (CL = 0.68–1.43) MN/100 PCE, respectively]. These were accompanied by significantly greater bone marrow toxicity in both groups ($P < 0.01$). The frequency of MNPCE and the ratio of PCE to total erythrocytes did not indicate genotoxic or cytotoxic effects when the animals were treated with 40 or 80 mg/kg lomefloxacin. In NCEs, the MN frequencies were between 0.01 and 0.036/100 NCE in all groups. Thus, no discrimination between

TABLE I. Frequencies of MNPCE, MNNCE, and PCE in Bone Marrow of Mice after Treatment with Mitomycin C (MMC), Colchicine (COL), and Lomefloxacin (LFLX)

Chemicals (mg/kg)	Time interval (hr)	% MNPCE (mean ± SD)	% MNNCE (mean ± SD)	% PCE (mean ± SD)
First experiment				
Control I	24	0.32 ± 0.13	0.014 ± 0.015	48.0 ± 1.5
MMC (2)	24	1.12 ± 0.19**	0.026 ± 0.027	42.6 ± 2.07*
COL (2)	24	1.04 ± 0.33**	0.022 ± 0.016	43.2 ± 2.16*
LFLX (40)	24	0.34 ± 0.16	0.014 ± 0.011	47.0 ± 2.7
LFLX (80)	24	0.44 ± 0.24	0.010 ± 0.012	46.8 ± 3.1
LFLX (160)	24	0.60 ± 0.28	0.024 ± 0.019	45.8 ± 2.7
LFLX (320)	24	0.86 ± 0.27*	0.036 ± 0.019	39.6 ± 3.7**
Second experiment				
Control II	48	0.30 ± 0.15	0.010 ± 0.012	48.8 ± 1.3
LFLX (40)	48	0.36 ± 0.19	0.016 ± 0.013	47.4 ± 1.6
LFLX (80)	48	0.46 ± 0.16	0.020 ± 0.020	48.2 ± 1.7
LFLX (160)	48	0.76 ± 0.27*	0.030 ± 0.012	40.8 ± 4.3**
LFLX (320)	48	1.06 ± 0.30**	0.030 ± 0.020	39.0 ± 2.5**

MNPCE, micronucleated polychromatic erythrocyte; MNNCE, micronucleated normochromatic erythrocytes.
*$P < 0.05$ and **$P < 0.01$, compared with the concurrent solvent control (Mann-Whitney U-test and χ^2 test).

TABLE II. Results of Double FISH Labeling of Mitomycin C (MMC), Colchicine (COL), and Lomefloxacin (LFLX)-Induced MN with the Telomere and the Minor Satellite DNA Probe

		In situ hybridization analysis of MN			
Chemicals (mg/kg)	Time interval (hr)	No. of MN scored	MN without any signal	MN with telomere signals only	MN with telomere and minor satellite signals
Controls	24 and 48	75	22 (29.3%)	21 (28.0%)	32 (42.7%)
MMC (2)	24	105	18 (17.2%)	69 (65.6%)	18 (17.2%)
COL (2)	24	85	08 (9.4%)	12 (14.1%)	65 (76.5%)
LFLX (160)	48	135	29 (21.4%)	41 (30.4%)	65 (48.2%)
LFLX (320)	48	150	29 (19.4%)	46 (30.6%)	75 (50.0%)

MN induced in PCE and NCE was required for the fluorescent analysis of MN with in situ hybridization since the NCE only contributed minimally to the total number of MN.

In Situ Hybridization Analysis

Before hybridization of MN, the specificity of the two DNA probes and the hybridization procedure were evaluated using mouse bone marrow cells at metaphase [Adler, 1984; Schriever-Schwemmer and Adler, 1994; Jie and Jia, 2001]. Metaphase examination indicated an excellent labeling efficiency for centromeres and an acceptable ~94% efficiency for telomeres (data not shown). The results for double labeling with minor satellite and telomere probes in both NCE and PCE are shown in Table II. In the solvent control groups, 22 (29.3%) of the 75 MN analyzed had no signal, 21 MN (28%) showed telomere signals, and 32 (42.7%) MN were double-labeled with the telomere and the minor satellite probe. The telomere-positive MN without a minor satellite signal represents MN containing an acentric fragment with pericentric heterochromatin. All MN with minor satellite signals also had telomeric signals and these MN contained entire chromatids or entire chromosomes. After treatment with the positive control clastogen MMC, 18 (17.2%) of the 105 MN analyzed had no signal, 69 MN (65.6%) showed telomere signals, and 18 (17.2%) MN were double-labeled with the telomere and the minor satellite probe, confirming the predominantly clastogenic effects of MMC. After treatment of male mice with the positive control aneugen COL, 8 out of 85 (9.4%) MN showed no signal, 12 (14.1%) MN were telomere-positive, and 65 MN (76.5%) were telomere- as well as minor satellite-positive, reflecting the expected aneugenic effects of COL. After treatment with 160 mg/kg of lomefloxacin, 29 (21.4%) out of 135 analyzed MN showed no signal, 41 (30.4%) MN were telomere-positive, and 65 (48.2%) MN were minor satellite- as well as telomere-positive. With 320 mg/kg of lomefloxacin, 29 (19.4%) of the 150 analyzed MN had no signal, 46 MN (30.6%) showed minor satellite signals, and 75 MN (50.0%) were double-labeled with the telomere and the minor satellite probe.

The distribution of the signal frequencies in MN that were positive only with the telomere probe is shown in Table III. Of the 21 MN analyzed from the solvent-control groups, one signal per MN was observed in 13 (61.9%) MN, two signals were seen in 4 (19.0%) MN,

TABLE III. Distribution of the Telomere Frequencies Among MN after Treatment with Mitomycin C (MMC), Colchicine (COL), and Lomefloxacin (LFLX)

Chemicals (mg/kg)	MN with telomere signals only	One telomere signal	Two telomere signals	Three telomere signals	Four telomere signals
Controls	21	13 (61.9%)	4 (19.0%)	3 (14.3%)	1 (4.8%)
MMC (2)	69	14 (20.3%)	15 (21.7%)	19 (27.5%)	21 (30.5%)
COL (2)	12	6 (50.0%)	3 (25.0%)	1 (8.3%)	2 (16.7%)
LFLX (160)	41	16 (39.0%)	14 (34.1%)	5 (12.2%)	6 (14.7%)
LFLX (320)	46	17 (31.0%)	15 (26.8%)	9 (8.5%)	5 (5.7%)

three signals were seen in 3 (14.3%) MN, and four signals were seen in 1 (4.8%) MN. Of the 69 telomere-positive MN analyzed after treatment with the positive control clastogen MMC, 14 (20.3%) MN had one signal, 15 (21.7%) MN contained two signals, 19 (27.5%) MN had three signals, and 21 (30.5%) MN had four signals. Twelve MN from the group treated with the positive control aneugen COL were analyzed with the telomere probe; of these, 6 (50.0%) MN had one signal, 3 (25.0%) MN contained two signals, 1 (8.3%) MN had one signal, and 2 (16.7%) MN had four signals. Of the 41 MN analyzed following treatment of mice with the 160 mg/kg lomefloxacin, one telomere probe signal per MN was observed in 16 (39.0%) MN, two signals were seen in 14 (34.1%) MN, three signals were seen in 5 (12.2%) MN, and four signals were seen in 6 (14.7%) MN. Finally, of the 46 telomere-positive MN from the lomefloxacin 320 mg/kg group, 17 (31.1%) MN had one signal, 15 (26.8%) MN contained two signals, 9 (8.5%) MN had three signals, and 5 (5.7%) MN had four signals.

The distribution of the signal frequencies in the MN that were double-labeled with both the minor satellite and the telomere probes is shown in Table IV. In the solvent-control groups, 14 (43.7%) out of 32 double-labeled MN had one minor satellite and one or two telomere signals, 7 MN (21.9%) showed one minor satellite and three or four telomere signals, 6 MN (18.8%) showed two minor satellite and one or two telomere signals, and 5 MN (15.6%) showed two minor satellite and three or four telomere signals. After treatment with 2 mg/kg MMC, only 4 (22.2%) out of 18 double-labeled MN had one minor satellite and one or two telomere signals, 6 MN (33.4%) showed one minor satellite and three or four telomere signals, 3 MN (16.6%) showed two minor satellite and one or two telomere signals, and 5 MN (27.8%) showed two minor satellite and three or four telomere signals. With 2 mg/kg COL, 14 (21.6%) out of 65 double-labeled MN had one minor satellite and one or two telomere signals, 9 MN (13.8%) showed one minor satellite and three or four telomere signals, 19 MN (29.3%) showed two minor satellite and one or two telomere signals, and 23 MN (35.3%) showed two minor satellite and three or four telomere signals. After treatment with 160 mg/kg of lomefloxacin, 23 (35.3%) of 65 double-labeled MN had one minor satellite and one or two telomere signals, 13 MN (20.0%) showed one minor satellite and three or four telomere signals, 14 MN (21.6%) showed two minor satellite and one or two telomere signals, and 15 MN (23.1%) showed two minor satellite and three or four telomere signals. In the 320 mg/kg lomefloxacin treated group, 25 (33.3%) of 75 double-labeled MN had one minor satellite and one or two telomere signals, 16 MN (21.3%) showed one minor satellite and three or four telomere signals, 13 MN (17.4%) showed two minor satellite and one or two telomere signals, and 21 MN (28.0%) showed two minor satellite and three or four telomere signals.

DISCUSSION

The conventional bone marrow MN test complemented by FISH with the mouse centromeric and telomeric DNA probes was used to investigate the mechanisms of induction of MN in mice treated with lomefloxacin. This assessment is particularly important for chemical clastogens, which may also have aneugenic potential. Such chemicals may interact, on the chromosomal level, with the respective targets for chromosomal missegregation. To determine the efficiency of the method two model mutagens, MMC and COL, known to have clastogenic and aneugenic actions, respectively, were used. The incidence of MNPCE in the vehicle-treated group (mean 0.31 MNPCE/100 PCEs) in the current experiment was within the accepted spontaneous range for negative controls [Salamone and Marvournin, 1994]. Statistically significant increases in the incidence of MNPCE over the control value were observed following treatment with the positive controls MMC and COL ($P < 0.01$). The present results with the positive controls were in the same range as those of earlier studies [Schriever-Schwemmer and Adler, 1994; Attia et al., 2003; Attia, 2007]. These data confirmed the sensitivity of the experimental protocol followed in the detection of genotoxic effects.

In the control group, nearly half (42.7%) of the MN revealed double-labeling with the centromeric and telomeric signals, 28% of MN showed telomeric signals only, and 29.3% did not show any signal. The frequencies of MN with fluorescent signals as well as the distribution of

TABLE IV. Distribution of the Signal Frequencies in the Double FISH Labeled MN with the Minor Satellite and the Telomere DNA Probe after Induction with Mitomycin C (MMC), Colchicine (COL), and Lomefloxacin (LFLX)

Chemicals (mg/kg)	No. of double-labeled MN	One minor satellite and one telomere signal	One minor satellite and two telomere signals	One minor satellite and three telomere signals	One minor satellite and four telomere signals	Two minor satellite and one telomere signals	Two minor satellite and two telomere signals	Two minor satellite and three telomere signals	Two minor satellite and four telomere signals
Controls	32	9 (28.1%)	5 (15.6%)	3 (9.4%)	4 (12.5%)	3 (9.4%)	3 (9.4%)	4 (12.5%)	1 (3.1%)
MMC (2)	18	3 (16.7%)	1 (5.5%)	4 (22.3%)	2 (11.1%)	2 (11.1%)	1 (5.5%)	2 (11.1%)	3 (16.7%)
COL (2)	65	2 (3.1%)	12 (18.5%)	1 (1.5%)	8 (12.3%)	10 (15.4%)	9 (13.9%)	8 (12.3%)	15 (23.0%)
LFLX (160)	65	9 (13.8%)	14 (21.5%)	6 (9.3%)	7 (10.7%)	8 (12.3%)	6 (9.3%)	5 (7.7%)	10 (15.4%)
LFLX (320)	75	12 (16.0%)	13 (17.3%)	6 (8.0 %)	10 (13.3%)	6 (8.0 %)	7 (9.4 %)	6 (8.0 %)	15 (20.0%)

signals per MN were in the same range as observed in the control group of other studies [Schriever-Schwemmer and Adler, 1994; Schriever-Schwemmer et al., 1997; Attia et al., 2003; Attia, 2007]. However, the finding that 29.3% of the MN had no FISH signals seems to be higher than the 20.7% reported by Jie and Jia [2001] for the control. The difference between these values might be attributed to three variables: the solvent, the technical features of the FISH procedures, and the type of cells used in the different experiments. However, the solvent in the two studies was DMEM/F12 medium [Jie and Jia, 2001] or PBS (current study) and is unlikely to be responsible for the observed differences. Thus, other technical features of the hybridization procedures (such as damaged probes, inefficiency of probe labeling or label recognition) and the type of cells used may have produced the high frequencies of signal-negative MN in the current study. After treatment with the typical clastogen MMC the majority of MN (82.8%) did not show centromeric signals. This result agrees well with other studies [Schriever-Schwemmer and Adler, 1994; Jie and Jia, 2001; Attia et al., 2003; Attia, 2007]. Because of the strong clastogenic effect of MMC, MN were predominantly composed of acentric fragments. Our results indicate that 69 (79.3%) out of 87 centromere-negative MN had telomere signals, and 58% of MMC-induced MN with telomere signals were composed of three and four telomere signals; this observation is interpreted as MN containing acentric fragments (Table III). As expected for an aneugen, 76.5% of MN contained centromeric signals following treatment with COL. The present result is similar to other reports [Schriever-Schwemmer and Adler, 1994; Jie and Jia, 2001; Attia et al., 2003; Attia, 2007]. Our results, which are shown in more detail in Table IV, indicate that 18.5% of COL-induced MN with centromere signals were composed of one minor satellite signal and two telomere signals (probably contained a chromosome lagging after centromere separation). In addition, 23.0% of COL-induced MN with centromere signals were composed of two minor satellite signals and four telomere signals (most likely contained a chromosome lagging before centromere separation). Additionally, COL also increased the MN without a centromeric signal (23.5%); similar results have been reported in other studies [Schriever-Schwemmer and Adler, 1994; Jie and Jia, 2001; Attia et al., 2003; Attia, 2007]. This might be due to COL-induced MN containing the Y chromosome, which cannot be detected, but might also be due to a small potential clastogenic effect of COL. It has been reported that COL has clastogenic potential besides its aneugenic action [Arni and Hertner, 1997]. The percentage of MN with telomere and minor satellite signals induced by the typical aneugen COL was higher (76.5%) than that induced by the known clastogen MMC (17.2%); the distribution of centromere-negative and centromere-positive MN among the positive controls MMC and COL

was also different. Based on these results, it is clear that the relative aneugenic potential as well as the clastogenic activity of a particular agent can be identified by the combination of centromeric and telomeric probes used in these studies.

In a previous in vivo study carried out by Itoh et al. [2002a], single doses of 25 or 50 mg/kg lomefloxacin were given by oral intubation followed by light irradiation and the mice were killed on days 2, 3, 4, 5, or 8 after treatment. Lomefloxacin at either 25 or 50 mg/kg caused significant increases in the MN frequency, which peaked on day 4. Nevertheless, no significant increase in MN frequency was observed after treatment without light irradiation at these doses. In the chromosomal aberration assay, Singh et al. [2003] found that lomefloxacin increased the frequencies of different types of chromosomal aberrations (including chromatid and chromosome breaks) and induced mitotic depression in the bone marrow cells of mice in vivo; however, a significant increase in chromosomal aberrations and reduction in mitotic index was observed only at the highest dose of the drug (160 mg/kg). In agreement with the above-cited reports, the present experiment showed that exposure to 40 or 80 mg/kg lomefloxacin yielded a mean value of 0.35 or 0.45% MNPCE, respectively, which is slightly but not significantly higher than that of the mean value of MN in the control groups (mean 0.31%). At high concentrations, however, it is evident from the results of the present study that lomefloxacin is a genotoxic agent as it produced significant increases in the number of PCE with MN. Furthermore, lomefloxacin caused significant depression of erythroblast proliferation, most likely as a consequence of mitotic arrest. At 24 hr after the treatment, the MNPCE showed a significant increase in mice treated with 320 mg/kg lomefloxacin, as compared to the corresponding vehicle control. Moreover, at 48 hr after treatment, the MNPCE showed significant increases in mice treated with 160 or 320 mg/kg lomefloxacin. The responses were also directly correlated with bone marrow cytotoxicity.

A combination of minor satellite and telomere double FISH labeling was used to characterize the content of MN induced by lomefloxacin. A total of 135 MN from the 160 mg/kg lomefloxacin-treatment group were analyzed by FISH and 65 (48.2%) MN of these were double labeled with minor satellite and telomere probes, indicating that they were formed by lagging chromosomes and reflecting an aneugenic activity of lomefloxacin. Similarly, 70 (51.8%) of the induced MN were centromere-negative, indicating that they were formed by acentric fragments and reflecting the clastogenic activity of lomefloxacin. For 320 mg/kg of lomefloxacin, a total of 150 MN were analyzed by FISH assay and 75 MN (50.0%) of induced MN were centromere-positive and 75 MN (50.0%) were centromere-negative (Table II and Fig. 1). Since the centromere probe labeled mitotic chromosomes

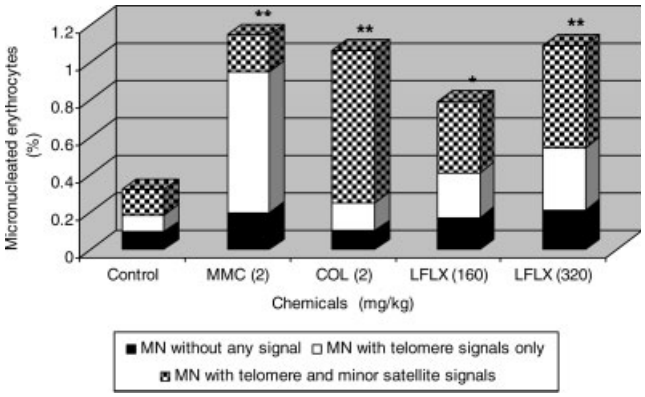

Fig. 1. Percentage of telomere- and/or minor satellite-positive MN in bone marrow cells of mice treated with mitomycin C (MMC), colchicine (COL), and lomefloxacin (LFLX). Telomere-positive MN only (white solid boxes), telomere and minor satellite-positive MN (slashed boxes). $*P < 0.05$ and $**P < 0.01$, compared with the solvent control (conventional staining; Mann-Whitney U-test and χ^2 test). Data combined from Tables I and II.

at both chromatids, it can be concluded that equal proportions of MN contained single chromatids (chromosome loss) and biarmed chromosomes (nondisjunction). These results indicate that lomefloxacin is clastogenic and aneugenic during mitotic phases. At both doses, an average of 30.5% of the analyzed MN was telomere-positive and must be interpreted as MN harboring terminal acentric fragments. Additionally, an average of 20.4% of the analyzed MN had no FISH signals and must be interpreted as MN harboring interstitial acentric fragments (Table II). Minor satellite plus telomere double-labeled MN that gave one minor satellite signal and two telomere signals (21.5 and 17.3% with 160 and 320 mg/kg of lomefloxacin, respectively; Table IV) probably contained a chromosome lagging after centromere separation. MN with two minor satellite signals and four telomere signals (15.4 and 20.0% with 160 and 320 mg/kg of lomefloxacin, respectively; Table IV) most likely contained a chromosome lagging before centromere separation or two chromatids after centromere separation.

The finding of the present study indicates that lomefloxacin acts as a genotoxic agent and produces chromosomal damages that end up as MN. The mechanisms by which lomefloxacin cause this adverse effect appear related, in part, to primary clastogenic and aneugenic events. Several hypotheses can be suggested to account for the genotoxic effects of lomefloxacin, including the formation of adducts and/or damage at the level of DNA or chromosomes [Fisher et al., 1989; Averbeck, 1994; Marrot et al., 2003]. Lomefoxacin could also activate cells to enhance their intracellular production of reactive oxygen species, of which the stable and diffusible forms could damage nuclear DNA [Rosen et al., 1997; Martinez et al., 1998; Marrot et al., 2003], and contribute to

development of antibiotic resistance [Pillai et al., 2001]. If the cellular repair mechanisms are overloaded, primary DNA damage may lead to mutations or chromosomal damage and eventually cause tumors [Loveday, 1996; Nataraj et al., 1996; Klecak et al., 1997]. The recommended therapeutic dose of lomefloxacin for humans is 400 mg once daily for 7–14 consecutive days. Taking into consideration 70 kg as an average weight of human body, the limit of the drug per day is 5.7 mg/kg. Thus the 40, 80, 160, and 320 mg/kg of lomefloxacin used in the current study are 7, 14, 28, and 56 times higher, respectively, than the human therapeutic dose. Despite years of using lomefloxacin at high doses, there is no clear indication of increased cancer risk in human after treatment with this agent.

It should be noted that FISH with a combination of centromeric and telomeric DNA probes allows further characterization of the chromosomal composition of MN and analysis of the numerical distribution of chromosomal breakages and whole chromosomes inside individual MN, with some restrictions. One problem is that the Y chromosome cannot be detected with the minor satellite probe [Miller et al., 1992; Schriever-Schwemmer and Adler, 1994]. However, the probability of the Y chromosome being included in a MN should be less than 1/40 of all MN induced according to random loss theory, and this problem is thus negligible [Broccoli et al., 1990; Moens and Pearlman, 1990]. Another problem is that up to 25% of the telomeres of metaphase chromosomes could not be detected with the telomeric probe [Miller et al., 1992; Miller and Nüsse, 1993; Schriever-Schwemmer and Adler, 1994; Jie and Jia, 2001; Lindberg et al., 2008], suggesting that FISH can have a lower efficiency in interphase cells. A possible reason is that the length of the telomeric sequence in different chromosomes of the mouse varies from 20 to 200 kb [Kipling and Cooke, 1990], leading to corresponding differences in signal brightness. Thus, the absolute number of fragments enclosed in a MN could not be quantified precisely and it was impossible to distinguish between MN containing exactly one or two whole chromosomes and those containing both whole chromosomes and acentric fragments. However, since 75–82% of the telomeres could be detected with the telomeric probe [Cornforth et al., 1989; Wu et al., 1998; Bolzan and Bianchi, 2006], the number of fragments enclosed in a MN could be estimated. Of course, for more accurate quantitative determination of the number of acentric fragments and chromosomes in MN, the efficiency of labeling and detecting the telomeric probe should be further improved, by such means as using PCR to label telomeric probes and using confocal fluorescence microscopy to detect some faint telomeric signals.

In summary, by using FISH assay with centromeric and telomeric DNA probes for erythrocyte MN it could be shown that lomefloxacin is not only clastogenic but also aneugenic in somatic cells in vivo. The assay also showed that chromosomes can be enclosed in a MN before and after centromere separation. Based on the current results, multicolor FISH with centromeric and telomeric DNA probes shows promise as a technique for analyzing the chromosomal composition of MN. However, centromeric FISH alone appears to be adequate for differentiating clastogens from aneugens.

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