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Terahertz Radiation Induces Spindle Disturbances in Human-Hamster Hybrid Cells

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The aim of this study was to investigate and quantify the production of spindle disturbances in A_L cells, a human-hamster hybrid cell line, by 0.106 THz radiation (continuous wave). Monolayer cultures in petri dishes were exposed for 0.5 h to 0.106 THz radiation with power densities ranging from 0.043 mW/cm² to 4.3 mW/cm² or were kept under sham conditions (negative control) for the same period. As a positive control, 100 μg/ml of the insecticide trichlorfon, which is an aneuploidy-inducing agent, was used for an exposure period of 6 h. During exposure, the sample containers were kept at defined environmental conditions in a modified incubator as required by the cells. Based on a total of 6,365 analyzed mitotic cells, the results of two replicate experiments suggest that 0.106 THz radiation is a spindle-acting agent as predominately indicated by the appearance of spindle disturbances at the anaphase and telophase (especially lagging and non-disjunction of single chromosomes) of cell divisions. The findings in the present study do not necessarily imply disease or injury but may be important for evaluating possible underlying mechanisms.

INTRODUCTION

Recently, Korenstein-Ilan *et al.* (*1*) reported on the induction of aneuploidy in human lymphocytes by continuous-wave 0.1 THz radiation (0.03 mW/cm²). Their findings are interesting, because little information has been reported on the interaction of radiation of the THz spectrum covering the frequency range between 0.1 and 10 THz (30 μm–3 mm in wavelength) with biological systems in inducing aneuploidy. However, owing to the rapidly increasing application of THz radiation in biology and biomedicine (*2*), it is crucial to identify any potential health risks related to THz radiation exposure. Since it has been demonstrated in the past years that non-ionizing radiation in the microwave region at a typical frequency of GSM mobile phones (0.9 GHz) can induce spindle disturbances during the anaphase and telophase of mitotic division in human-hamster hybrid (A_L) cells (*3, 4*) as well as in root meristematic cells of *Allium cepa* (*5*), we extended our investigations on the induction of spindle disturbances by 0.9 GHz to the corresponding analysis at 0.106 THz. Such functional chromosome alterations may lead to chromosome dislocations during mitosis, which may have implications as a potential source of the formation of aneuploidy (losses or gains of chromosomes in cells). Additionally, taking into account earlier results on biological effects after exposure to similar frequencies of mobile phone communication that were interpreted as production of numerical rather than structural chromosome aberrations (*6–8*), it generally seems to be justified that more consideration should be given to the question whether electromagnetic fields (EMFs) are capable of inducing aneuploidy.

The aim of the present study was to evaluate the potential of 0.106 THz radiation with power densities of 0.043, 0.43 and 4.3 mW/cm² in inducing spindle disturbances at the anaphase and telophase of mitotic division in A_L cells.

MATERIALS AND METHODS

Cell Culture Technique

We used A_L cells to study spindle disturbances because they have been used extensively to detect spindle disturbances after exposure to non-ionizing radiation (*3, 4*). Our A_L cells (*4*) show a modal number of 21 chromosomes (85% of analyzed cells), and 15% of the analyzed cells had a loss or gain of chromosomes. This cell line contains a standard set of Chinese hamster ovary-K1 chromosomes plus a single human chromosome 11 that confers the expression of the cell surface protein CD59. Thus the cells are suitable for studies to determine the frequency of radiation-induced mutations (*9*). Our cell culture technique has already been reported in detail (*4*). Briefly, the cells were grown as monolayer cultures in RPMI-1640 medium supplemented with 15% fetal calf serum and antibiotics at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The cells used during exposure were grown as a monolayer of 1.13 cm² in special cell culture dishes called μ-dishes (petri dishes) with an interior diameter of 29 mm (ibidi, Martinsried, Germany) containing 3 ml of culture medium. For the experiments, frozen aliquots from the same

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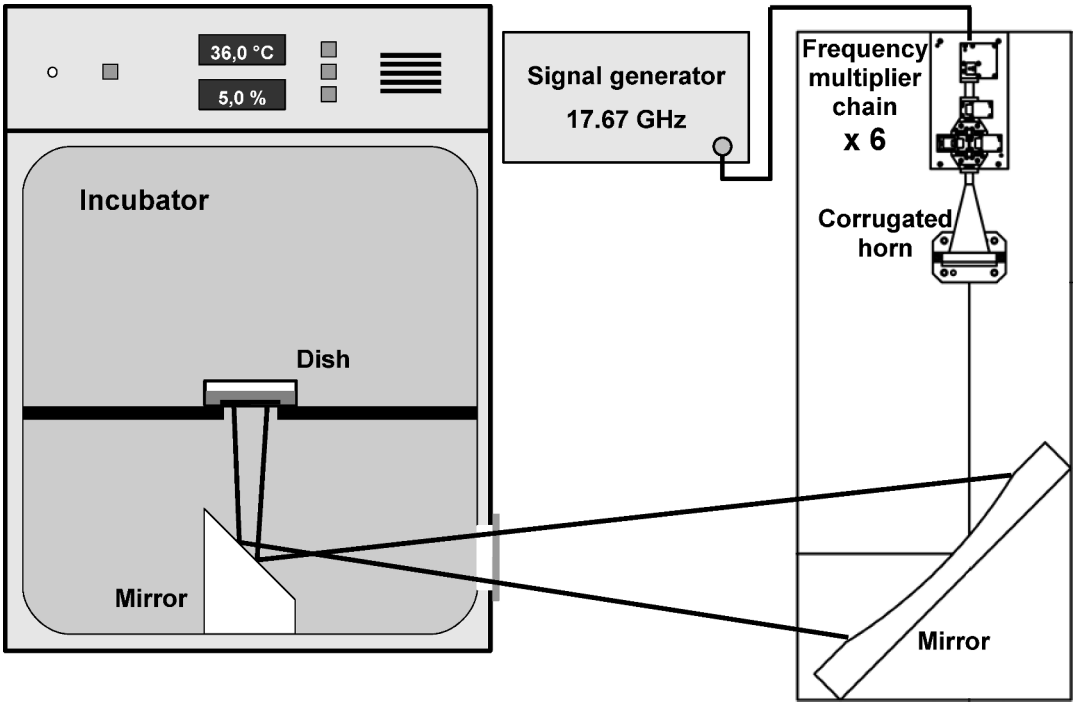

FIG. 1. Setup for field exposure experiments at 0.106 THz. A signal from a generator is sextupled and directed from below onto the μ-dishes containing the cells. The frequency multiplier chain is shown from above (tilted by 90°) for clarity.

stock culture of cells were thawed and seeded as exponentially growing unsynchronized cells in normal cell culture dishes. After incubation for at least 72 h at 37°C, the cells were transferred to an area of 1.13 cm^2 of the μ-dishes and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Twenty hours later, μ-dish cultures were exposed for 0.5 h to 0.106 THz radiation with power densities ranging from 0.043 mW/cm^2 to 4.3 mW/cm^2 or were sham-exposed. As a positive control, cells were incubated with 100 μg/ml of the insecticide trichlorfon. This typically aneuploidy-inducing chemical (*10*) requires an exposure time of 6 h to induce spindle disturbances (*4*). For each exposure protocol, two separate replicate experiments were performed.

Terahertz Radiation Source and Experimental Setup

The cell culture dishes were irradiated from below with a Gaussian beam of electromagnetic radiation at 0.106 THz originating from a frequency multiplier cascade as shown in Fig. 1. A continuous wave signal at approximately 17.67 GHz from a frequency synthesizer (Agilent E8257D) was sextupled in a Schottky multiplier. The wave was fed into a round corrugated horn antenna via a variable attenuator that allowed adjustment of the radiated power between 0 and approximately 155 mW. The beam was collimated to a beam width (full-width half-maximum) of 2 cm at the location of the μ-dishes using a metallic mirror. The μ-dishes containing the cells were irradiated in a modified incubator with a transparent window on one side and an additional flat metallic mirror that was used to direct the radiation onto the dishes from below. The incubator window as well as the μ-dish showed radiation transmission of more than 90%. During exposure, the sample containers were kept in defined environmental conditions of 36°C and 5% CO_2 in a modified incubator as required by the cells. All relevant experimental parameters such as temperature, humidity and CO_2 content within the incubator and RF power were monitored continuously during the exposures (Almemo Datalogger, Ahlborn, Holzkirchen, Germany). The power density at the location of the cell culture dishes was adjusted traceable to the SI units using a photo-acoustic detector based on a closed air-cell and a pressure transducer (Thomas Keating Power Meter, Thomas Keating Ltd., UK). Calibration was provided by Ohmic heating of a thin metal film within the detector head. The given power densities represent averages over the exposure spot area with a diameter of 12 mm in which cells had been evaluated. The local power distribution was measured and verified experimentally using a very small aperture. Temperature measurements indicated that irradiation with 4.3 mW/cm^2 resulted in a temperature increase of approximately 1.4°C. Thermal influences of irradiation with 0.86 mW/cm^2 and lower intensities did not exceed the fluctuation of the temperature regulation of the incubator (<0.3°C).

Assay for Spindle Disturbances and Scoring Criteria

Immediately after exposure, the dishes were coded and cells were fixed on the μ-dishes using standard procedures (*10*); i.e., for fixation, one-third of the medium was replaced by a mixture of three parts of 99% ethanol and one part of glacial acetic acid. The fixation procedure was repeated three times over 15 min. With this method the cytoplasm of the cells remained well preserved and chromosome loss due to the preparation procedure was avoided. After air-drying, the cells were stained with 2% acetic orcein (Gurr, Wycombe, UK). For scoring damaged mitotic figures, mitoses with spindle disturbances at metaphase, anaphase and telophase were distinguished according to the conditions described previously (*4*). In brief, partial or complete disturbance of the spindle apparatus was indicated by the appearance of initial c-metaphases where chromosomes were not distributed over the entire cell area or were concentrated in the center of the cell. In addition, typical c-metaphases (contracted chromosomes completely scattered in the cytoplasm) and improper alignment of single chromosomes onto the metaphase plate (non-congression) were scored. Spindle disturbances of anaphase and telophase figures were analyzed, particularly those exhibiting configurations with lagging or non-disjunction and multipolar distribution of individual chromosomes. In Fig. 2, spindle disturbances at anaphase and telophase are

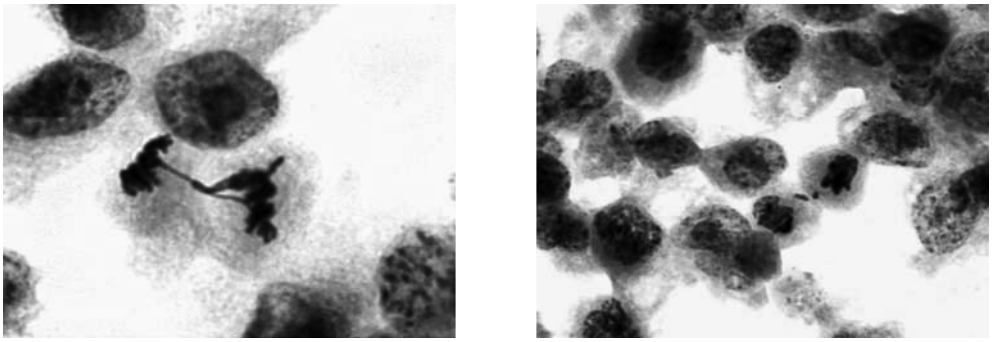

FIG. 2. A_L cells with spindle disturbances in the anaphase (left) and telophase (right) of the cell cycle.

shown that exhibit configurations with lagging or non-disjunction of chromosomes. The appearance of anaphases and telophases with bridges and/or breaks was considered as an indicator of clastogenic activity, i.e., the induction of structural chromosome aberrations. The mitotic index was determined by scoring $3 \times 1{,}000$ cells in different areas of the cell monolayer on the μ-dishes.

Statistics

A null hypothesis that the proportions of cells with spindle disturbances in each of two replicates with the same experimental procedure are not different was tested using the method of Kazmier and Pohl (*11*). For each exposure condition (0.106 THz radiation with different power densities of 0.043, 0.43 and 4.3 mW/cm^2 as well as positive and negative controls), the proportions were calculated to determine both the standard error of the difference between proportions and the estimate of the population proportions. Since the conditions for the applications of the normal probability distribution hold for these data (i.e., both sample sizes are much larger than 100 cells and the products of sample sizes and proportions are greater than five), the observed difference between the two sample proportions could be converted into *z*-test statistics. A difference with a two-sided P value <0.05 was considered statistically significant. The same statistical procedure was applied to compare the results for 0.106 THz radiation exposure obtained under different exposure conditions compared to the corresponding sham-exposed controls.

RESULTS

For each 0.106 THz radiation exposure level and the coincident negative and positive controls, two replicate samples were analyzed to demonstrate reproducibility and to identify any inter-test variability. As shown in Table 1, the numbers of mitoses with normal and damaged mitotic figures determined in a total of 6,365 mitotic cells ranged between 339 and 886, independent of the exposure condition. The mitotic index within the whole cell population declined slightly with increasing power density of the 0.106 THz radiation, but this change was only marginal (data not shown). In Table 2, the pooled data from the two replicate samples of each exposure condition are shown together with the corresponding fractions of anaphases and telophases. Pooling these data is justified, because no significant differences between the samples with regard to the most frequent damage, i.e. anaphases and telophases with spindle disturbances, were observed. As shown in Table 3, none of the differences were significantly different at the 5% level; i.e., all P values exceed 0.05.

TABLE 1
Number of Mitoses with Normal and Damaged Mitotic Figures in A_L Cells Induced by 0.5 h Exposure to 0.106 THz Radiation

Exposure condition	0 mW/cm^2	0 mW/cm^2	0.043 mW/cm^2	0.043 mW/cm^2	0.43 mW/cm^2	0.43 mW/cm^2	4.3 mW/cm^2	4.3 mW/cm^2	Trichlorfon	Trichlorfon
Cells analyzed	438	623	493	695	709	820	339	886	804	558
Normal mitotic cells	431	619	488	685	699	797	332	870	788	539
Prophases	124	169	143	198	179	245	91	203	178	159
Metaphases	106	266	238	226	304	282	168	429	391	209
Anaphases-telophases	201	184	107	261	216	270	73	238	219	171
Mitoses with spindle disturbances	7	4	5	10	10	23	7	16	16	19
Metaphases with										
initial c-mitosis	0	0	0	1	0	0	1	1	0	0
c-mitosis	0	1	0	0	0	1	1	0	1	0
non-congression	0	0	0	0	0	1	0	0	1	1
Anaphases-telophases with										
multipolar distribution	0	0	1	1	0	0	0	0	0	1
lagging	6	1	3	3	5	11	2	10	9	6
non-disjunction	1	2	1	5	5	10	3	5	5	11

TABLE 2
Number of Mitoses with Normal and Damaged Mitotic Figures in A_L Cells

Exposure condition	Mitotic cells analyzed	Total anaphases-telophases	Normal anaphases-telophases	Anaphases-telophases with spindle disturbances	Fraction of anaphases-telophases with spindle disturbances
0 mW/cm^2	1061	395	385	10	0.025
0.043 mW/cm^2	1188	382	368	14	0.037
0.43 mW/cm^2	1529	517	486	31	0.060
4.3 mW/cm^2	1225	331	311	20	0.060
Trichlorfon	1362	422	390	32	0.076

Note. Results are shown as pooled data from both replicates.

As shown in Table 4, any proportions of the fractions of anaphases and telophases with spindle disturbances for the different exposure conditions (0.106 THz radiation or trichlorfon) were significantly higher than the corresponding control except the value of 0.037 ± 0.010 for a power density of 0.043 mW/cm^2, which was not significantly different from the corresponding control value of 0.025 ± 0.008 ($P = 0.18$). The corresponding dependence of the fractions of anaphases and telophases with spindle disturbances on 0.106 THz radiation at different power densities as well as on the positive control trichlorfon is shown in Fig. 3. Therefore, the present results indicate that exposure of A_L cells to 0.106 THz radiation at power density levels of 0.43 and 4.3 mW/cm^2 induces statistically significant spindle disturbances in anaphase and telophase. In contrast to these functional chromosome alterations in exposed cells, no indication of structural chromosome aberrations like breaks or asymmetrical exchanges (anaphase or telophase bridges) were observed under the applied exposure conditions.

DISCUSSION

Since the completion of the research activity of the THz-BRIDGE project (*12*) funded by the European Commission, little further information has been available on the potential damages that could be induced after exposure of living cells to THz radiation. The variety of techniques and biological assays employed within THz-BRIDGE to clarify any potential hazard induced in cells by EMFs between 100 and 120 GHz did not find any biological damage: neither induction of micronuclei, alteration of cell cycle kinetics, DNA strand breaks, nor alkali-labile sites. Therefore, in general, the results obtained in the THz-BRIDGE project indicate that THz radiation exposure cannot produce genotoxic effects by directly causing DNA damage. However, under some specific exposure conditions, an induction of aneuploidy has been observed to occur in human lymphocytes.

A more detailed picture of this special investigation has been given by Korenstein-Ilan *et al.* (*1*). Isolated lymphocytes stimulated by phytohemagglutinin (PHA) for 1–6 h before exposure were exposed to continuous-wave radiation at 0.1 THz for 1, 2 and 24 h and harvested by common cytogenetic procedures about 70 h after the onset of exposure. The changes in the levels of aneuploidy as a genetic marker for genomic instability were evaluated by an interphase fluorescence *in situ* hybridization (FISH)-based technique using directly labeled commercial probes for the centromeric repetitive DNA arrays. Measuring losses or gains of chromosomes 11 and 17, aneuploid cells could be observed when the 0.1 THz exposure period exceeded 1 h. However, the appearance of these aneuploid cells should not be compared directly with the present findings after exposure of A_L cells to 0.106 THz radiation. Different underlying mechanisms may be responsible for the production of aneuploid cells reported by Korenstein-Ilan *et al.* (*1*) and the presence of chromosome segregation errors during mitosis observed in the present investigation. Although it is well known that in general spindle disturbances can cause numerical chromosome aberrations giving rise to aneuploidy in daughter cells, differences in the nature of the primary mechanisms should be taken into consideration based on

TABLE 3
***Z*-Test Statistics for the Difference in the Proportions of Anaphases-Telophases with Spindle Disturbances between Both Replicate Experiments (Fractions F1 and F2) Obtained under Different Exposure Conditions**

Exposure condition	Fraction F1 (± SE) of anaphases-telophases with spindle disturbances	Fraction F2 (± SE) of anaphases-telophases with spindle disturbances	*z* value	Two-sided *P* value
0 mW/cm^2	0.034 ± 0.013	0.016 ± 0.009	1.11	0.13
0.043 mW/cm^2	0.045 ± 0.020	0.033 ± 0.011	0.54	0.30
0.43 mW/cm^2	0.072 ± 0.015	0.044 ± 0.014	0.91	0.09
4.3 mW/cm^2	0.064 ± 0.026	0.059 ± 0.015	0.16	0.44
Trichlorfon	0.095 ± 0.021	0.060 ± 0.016	1.36	0.09

Note. Differences are significant at $P < 0.05$.

TABLE 4
***Z*-Test Statistics for the Difference of Proportions of Anaphases-Telophases with Spindle Disturbances between the Different Exposures (Fraction F1) and the Sham Exposures as Negative Control (Fraction F2)**

Exposure condition	Fraction F1 (± SE) of anaphases-telophases with spindle disturbances	Fraction F2 (± SE) of anaphases-telophases with spindle disturbances	*z* value	Two-sided *P* value
0.043 mW/cm²	0.037 ± 0.010	0.025 ± 0.008	0.91	0.18
0.43 mW/cm²	0.060 ± 0.010	0.025 ± 0.008	0.99	0.01
4.3 mW/cm²	0.060 ± 0.013	0.025 ± 0.008	0.99	0.01
Trichlorfon	0.076 ± 0.012	0.025 ± 0.008	1.00	0.00

Note. Differences are significant at $P < 0.05$.

an earlier observation by Kadhim *et al.* (*13*). Measuring the DNA content in PHA-stimulated lymphocytes by fluorescence-activated cell sorting, they observed that, even 6 h after addition of PHA, almost all cells were still in G_0/G_1 phase of the cell cycle, i.e., predominately in the DNA pre-synthetic phase of cell division. Also, Preston *et al.* (*14*) described the general cell cycle duration in human blood lymphocytes after PHA stimulation: G_1 phase from 6–8 h, S phase from 24–30 h, G_2 phase from 45–48 h, and first mitosis from 48–52 h. Therefore, it should be assumed that the lymphocytes would still be in G_1/S phase of the cell cycle after PHA stimulation for 1–6 h before exposure and 1, 2 and 24 h after THz radiation exposure (*1*) and that THz radiation thus could not act directly on mitotic figures to induce aneuploidy. In contrast, only mitotic cells were examined in the present investigation, because the chromosome segregation errors during anaphase and telophase were analyzed after a 0.5-h exposure of A_L cells to 0.106 THz. This short exposure period was chosen based on the exposure time for the induction of spindle disturbances in A_L cells in our studies on 0.9 GHz radiation (*3, 4*). Since the 0.5-h exposure period is about half the length of a mitosis, a direct effect on the mitotic apparatus during metaphase or predominately anaphase-telophase must be assumed. As discussed earlier (*4*), such time-dependent effects can be assumed owing to the observation of lower fractions of anaphases and telophases with spindle disturbances after 0.17 h exposure, whereas the fractions of anaphases and telophases with spindle disturbances were not higher for exposure periods between 0.5 and 2 h.

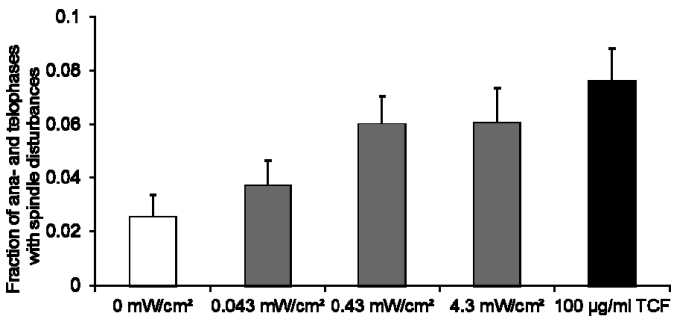

FIG. 3. Anaphases and telophases with spindle disturbances after exposure to 0.106 THz radiation at different power densities or incubation with trichlorfon (TCF) determined in two replicate experiments. Data are presented as means ± SE.

The present results are essentially consistent with the previous findings obtained for 0.9 GHz (*3, 4*); i.e., EMFs in a broad frequency range can induce spindle disturbances within a very narrow time window within the cell cycle and thus may induce numerical chromosome aberrations in proliferating cells. Based on this finding, it can be assumed that all the arguments associated with our previous experiments are essentially valid for the results of the present investigation. Therefore, it is an adequate approximation to relate the observed effects to functional disturbances of the spindle apparatus but not to structural alterations of the DNA. However, the small number of structural chromosome aberrations observed in the present experiments for a 0.5-h exposure period (1 break and no bridges in 6,365 analyzed cells) can provide only limited evidence regarding whether 0.106 THz radiation may have chromosome-breaking (clastogenic) potential, because anaphase-telophase analysis is not as precise and efficient as the analysis of colcemid-arrested metaphases; i.e., only a small proportion of structural changes induced at the G_0 or G_1 stages of the cell cycle are detectable as bridges and breaks in subsequent anaphases-telophases. However, cytogenetic observations in human lymphocytes exposed to 0.12 THz radiation suggested the absence of direct structural chromosome aberrations using either the micronucleus test (*15*) or the micronucleus test and the alkaline comet assay (*16*). These results are not surprising because the energy of this radiation quality is much smaller than the energy of about 1 eV needed to break the weakest chemical bonds in DNA (*17*). Thus it can be suggested that the THz radiation in our experiments appears to have an effect on the spindle apparatus but not directly on the DNA.

The present finding of spindle disturbances suggests that 0.106 THz radiation is a spindle-acting agent in human-hamster hybrid cells preferably at the anaphases and telophases (especially lagging and non-disjunction of single chromosomes) after an exposure time of only 0.5 h. In general, spindle disturbances and chromosome dislocations may have implications as a potential source of formation of aneuploid cells. Missegregation of chromosomes during mitosis can cause numerical chromosome aberrations, giving rise to aneuploidy in daughter cells,

which could potentially lead to severe adverse effects in humans; e.g., it can be associated with carcinogenesis (*18*). Therefore, it remains to be determined whether THz radiation can produce aneuploid cells. A suitable method for analyzing this question in more detail would be the application of the micronucleus test including kinetochore staining or the FISH painting technique.

CONCLUSION

The question of whether the increasing widespread presence of radiation at GHz or even THz frequencies at a broad range of power densities in today's urban environment represents a potential health hazard is a current subject of debate. Although microwave radiation has been implicated in producing spindle disturbances during mitosis of proliferating cells, the potential consequences or the relevance of exposure for humans are still under debate. Up to now, no reasonable explanations are available that might help to elucidate the underlying mechanisms involved in the formation of spindle disturbances, which can be induced by non-ionizing radiation qualities. Our recent information that it might be reasonable to assume that an external electric force like the electrical component of an EMF can affect the equilibrium resulting from the continuous binding (polymerization) and releasing (depolymerization) of the tubule proteins could be a first step in an interpretation of the underlying mechanisms. Further investigations are needed to evaluate in more detail the capacity of THz radiation to act during the narrow time-dependent window of the mitotic cycle, i.e., the passage from metaphase to anaphase-telophase. Since such functional chromosome alterations may lead to chromosome dislocations during mitosis, the possibility that they could be a potential cause of formation of aneuploidy should not be excluded. However, it should be taken into account that aneuploidy is often associated with tumors. Whether aneuploidy is a cause or a consequence of cancer remains to be determined (*19*). The findings in the present study do not necessarily imply disease or injury but may be important for evaluating possible underlying mechanisms.

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