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COMBINED EFFECTS OF CIRCULARLY POLARIZED MICROWAVES AND ETHIDIUM BROMIDE ON *E. coli* CELLS

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ABSTRACT

The significant difference in the effects of right- and left-handed polarized microwaves of millimeter range (MMW) on *Escherichia coli* cells and rat thymocytes has been shown previously. The helicity of DNA and supercoiling of DNA loops was suggested to cause the observed dependence of effects on polarization. It is known that DNA intercalators such as ethidium bromide (EtBr) are able to change the supercoiling of DNA loops and to affect the DNA helicity. In the present work, the combined effects of EtBr (1 μg/ml) and circularly polarized microwaves were studied in *E. coli* cells K12 AB1157 at the stationary stage of growth. The method of anomalous viscosity time dependencies (AVTD) was used to measure changes in the conformation of the genome. The cells were exposed to microwaves at 51.755 GHz and 0.1 mW/cm^2 for 10 min. Under these conditions of exposure, left-handed microwaves induced changes in the genome conformation, whereas right polarization was almost ineffective. The incubation of cells with EtBr inverted the effective polarization, and right-handed MMW became more effective than left polarization. The data obtained provide new evidence that DNA is a target of MMW effects on cells.

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INTRODUCTION

The biologic effects of nonthermal millimeter waves (MMW) have been investigated for more than 20 years (1–4). However, there is no accepted mechanism that can provide a predictive basis for potential detrimental or beneficial effects of MMW. Significant effects of nonthermal MMW on the genome conformational state (GCS) in *Escherichia coli* cells and thymocytes of rats have been described previously (5,6). Our experimental data have revealed the following regularities in the effects of low-intensity MMW on the GCS: frequency dependencies of a resonance type (5,6); dependence of resonance effects on polarization (selection rules on helicity) (7–9); and decrease in half-width of the resonances and rearrangement of frequency spectra of action with decrease in MMW power density (9–11). The MMW effect on *E. coli* cells has been shown to depend on the genetic peculiarities of the strain under study (12), the growth stage of the bacterial culture (13), the cell concentration, the static magnetic field during exposure (14), and the time between microwave exposure and recording of the effect (14). Such dependence on several genetic, physiologic, and physical parameters might be a reason why in some studies the authors failed to reproduce the original data of others (15,16).

It has been established that right- and left-handed circularly polarized (CP) MMW have different effects on the GCS at the same resonance frequency. In particular, right-handed MMW affected *E. coli* cells at the 41.32-GHz resonance frequency, whereas left polarization was ineffective, and vice versa: only left-handed polarization was effective at the resonance frequency of 51.755 GHz (7,8,13). It is known that most DNA in living cells has a right-handed helicity (B-form) but that a minor part, on the order of 1%, can be in the form of a left-handed helix (Z-form). It has been proposed that the spectrum of the MMW resonance frequencies is determined by genome structure and that the effective polarization is determined by the helicity of DNA sequences that interact with MMW (7,8). This assumption is supported by the findings that the spectra of the resonance frequencies were dependent on the genome length and that the effective polarization was determined by the supercoiling of the DNA domains (9,12). In particular, the effects of circularly polarized MMW at 51.755 GHz were modified by preirradiation of cells by X-rays. Without irradiation, only left-handed polarization was effective at the resonance frequency of 51.755 GHz. With increasing X-ray dose, the effect of left-handed polarization decreased and that of right-handed MMW increased. The effects of left- and right-handed MMW become the same at 50 cGy (9). At this dose, about one single-strand DNA break per haploid genome was induced, and the dose was too low to damage any cellular structure significantly except for DNA.

It is well known that a nucleoid in *E. coli* cells consists of domains (loops) of supercoiling (17). In *E. coli* cells, DNA supercoils are controlled enzymatically by DNA topoisomerase I and DNA gyrase (18). It is believed that the supercoiling of the DNA loops is very important for elementary genetic processes such as transcription, replication, recombination, and repair. Radiation-induced single-strand DNA breaks result in relaxation of these loops (19). On the other hand, supercoiling is associated with transition between the right B-form and the left Z-form in some DNA sequences (20). Therefore, the data suggested that the difference in biologic effects of polarized MMW is related to DNA helicity and supercoiling of DNA domains.

The supercoiling of DNA loops can be changed by means of DNA-specific intercalators such as ethidium bromide (EtBr). This approach has been used in anomalous viscosity time dependency (AVTD) studies of supercoiling in different cell types including *E. coli*

(10,21). Intercalation of EtBr between base pairs of DNA results in modification of DNA secondary structure and relaxation of negative DNA supercoils. Positive supercoiling arises with increase in concentration of EtBr. By changing the supercoiling, EtBr facilitates the transition of the left-handed DNA sequences (Z-form) to the right-handed B-form (22). If the effective circular polarization of MMW is determined by the helicity of specific DNA sequences or supercoils, the intercalation of EtBr in DNA can modify the effects of polarized MMW. To test this hypothesis, the combined effects of polarized MMW at the resonance frequency of 51.755 GHz and EtBr were studied in *E. coli* AB1157 cells.

MATERIALS AND METHODS

E. coli K12 AB1157 strain and conditions of cell growth have been described previously (5). The *E. coli* cultures were kept as spreads on Hottinger nutrient agar at 4°C for 1–14 days. The cells were grown in Luria broth (tryptone Sigma 10 g/l, yeast extract Sigma 5 g/l, NaCl 10 g/l) at 33°C without shaking. Over 18–20 h the cells reached an optical density of OD_{550} = 0.95–1.05, which corresponded to the early stationary phase of bacterial growth (1).

The cells were exposed at room temperature, 21°C, in Petri dishes (50 mm in diameter). Each dish contained 3.5 ml of a cell suspension. The experimental unit for cell exposure to circularly polarized MMW and specification of all exposure and measurement instruments has been described (8–11). Briefly, a backward wave oscillator was used for exposure. The voltage standing wave ratio (VSWR) did not exceed 1.4 at different points within the waveguide system. The frequency deviation was not more than 1 MHz. The output power was measured by a wattmeter with a thermistor detector. CP MMW was obtained by transformation of linearly polarized MMW with the help of quarter-wave mica plates. Average power density (PD) at the exposure site was calculated by dividing the output power by the projected area of the horn at the surface of the Petri dish. Two Petri dishes were simultaneously exposed to MMW at the same frequency of 51.755 ± 0.001 GHz but at different circular polarizations. Cells were exposed at the average PD of 0.1 mW/cm^2. The ellipticity coefficient was 1.02 along the propagation axis of circularly polarized MMW. The local distribution of PD on the surface of a styrofoam plate between the horn and exposed Petri dish was measured as previously described (5). The maximum deviation of local PD on this surface did not exceed 5 dB.

The Petri dishes were closed during exposure and pre-lysis incubation. The cells were exposed as previously described in a special chamber, which was shielded by a carbon particle-containing tissue matrix inside and by aluminum foil outside (10,11). The MMW incident power was decreased in suspension by a factor no less than 10^3. The reflected power was less than 5%. Reflections did not significantly enhance incident and absorbed power above the predicted levels. For each experiment, sham control exposures were carried out while the oscillator was working at the 51.755 GHz frequency and the MMW radiation was maximally attenuated by four attenuators down to PD < 10^{-14} W/cm^2 as described previously (10,11). Our exposure system was well clamped down to avoid mechanical vibrations. The temperature in the cell suspension was measured by a microthermocouple with an accuracy of 0.1°C. No heating was observed during exposure. Our unpublished data indicated that the effects of weak electromagnetic fields correlated with concentration of oxygen during exposure. In the present experiments, the concentration of oxygen was 6.5 ± 1.2 mg/l.

Before exposure, the cells were harvested by centrifuging at 2000*g* and diluted in M9 buffer (3 g/l KH_2PO_4, 0.2 g/l NH_4Cl, 6 g/l Na_2HPO_4, 1 g/l $MgSO_4$, 0.5 g/l NaCl, pH 7.0) to a concentration of 4×10^7 cells/ml. EtBr was added to a concentration of 1 μg/ml. It was shown that under these conditions of cell incubation, the kinetics of EtBr intercalation had a plateau beginning with 2 min after addition of intercalator (23). In our experiments, the incubation of cells with EtBr was not less than 30 min. Cells were exposed to MMW in Petri dishes for 10 min and then incubated for 40–150 min before lysis. During all incubations a metal chamber shielded the Petri dishes. As previously shown, a weak change in the static magnetic field could affect the GCS of *E. coli* cells (24). In this study, we controlled the static magnetic field as described previously (13). This field was 40 ± 5 μT in the places of growth, exposure, and incubation of cells.

The cells were lysed as previously with some modification (5). Briefly, the cell suspension was distributed to polyallomer test tubes, 1 ml in each. Solutions of lysozyme (Sigma, 1.5 mg/ml) 0.3 ml, sarcosyl (Serva, 2%) 1 ml, and papain-glycerol (Merck, 3 mg/ml, 10%) 0.7 ml were added to each test tube. All solutions were prepared in a lysing buffer (0.25 M Na_2EDTA, 0.01 M Tris-base, pH 7.15). The lysates were kept in the dark for 40–45 h at 33°C, and the GCS changes were measured in lysates using the AVTD method as described previously (5,10). Briefly, this method is based on the radial migration of large DNA-protein complexes in the high-gradient hydrodynamic field of a rotary viscometer. Radial migration of molecular complexes toward the rotating rotor causes anomalous changes of viscosity that can be recorded by measuring the rotor rotation period as a function of time (Fig. 1). This anomalous viscosity time dependence strongly depends on the conformational state of the genome, which in turn is dependent on DNA parameters such as molecular weight and the number of proteins bound to the DNA. Each AVTD curve is a set of experimental points (period of rotation *vs.* time of measurement), which

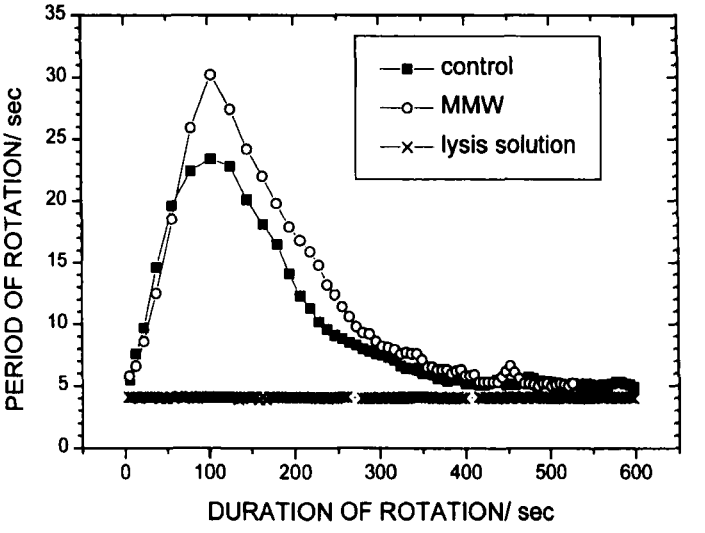

FIGURE 1. Typical AVTDs in the lysates of the *E. coli* AB1157 cells, which were lysed as described in Materials and Methods after exposure to left-handed MMW (51.755 GHz, 0.1 mW/cm^2) or sham exposure. The dependence of rotation period on duration of rotation for the cell-free lysing solution is also shown.

were recorded by an IBM PC. The AVTDs were measured at a shear rate of 5.8 s^{-1} and a shear stress of 0.0065 N/m^2. The maximum period of rotation (T_{max}) corresponds to maximum viscosity and has previously been shown to be the most sensitive AVTD parameter. The significance of differences between mean values in irradiated samples $\langle T_{max\ irr} \rangle$ and control samples $\langle T_{max\ con} \rangle$ was evaluated with Student's t-test for each experiment. Maximum relative viscosity (η) was used to determine the MMW effect on the GCS: $\eta = \langle T_{max\ irr} \rangle / \langle T_{max\ con} \rangle$. Results were considered as significantly different at $p < 0.05$. Each version of the experiment included not less than three measurements that were compared with corresponding variants of control and sham exposed cells using Student's t-test and the paired t-test. Comparison of control with sham control revealed no significant differences.

RESULTS

In four experiments, the *E. coli* AB1157 cells at a concentration of 4×10^7 cells/ml were exposed to left- or right-handed CP MMW at 51.755 GHz. The PD was 0.1 mW/cm^2 in all experiments. A significant response of *E. coli* cells to low-intensity MMW was observed. The AVTD peaks increased up to 30% after exposure to MMW (Fig. 1). Similar to previous data (7–9), left-handed CP MMW affected the GCS, whereas right polarization was almost ineffective (Fig. 2). The left-handed polarized MMW was about 2–3 times more effective than right-handed circular polarization at all time points. This difference was statistically significant at 90 and 120 min after exposure ($p < 0.05$).

No significant difference was observed between control cells and cells incubated with EtBr. Incubation of cells with EtBr inverted the effective polarization (Fig. 3). Right-handed CP MMW was more effective than left polarization. In experiments on the combined effect of MMW with EtBr, Student's t-test revealed no difference between right

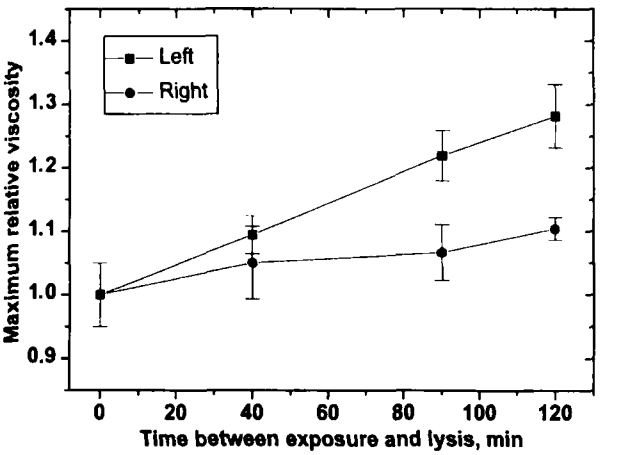

FIGURE 2. Effects of CP MMW. Kinetics of changes in the maximum relative viscosity after exposure of the stationary *E. coli* cells (4×10^7 cells/ml) to circularly polarized MMW (10 min, 51.755 GHz, 0.1 mW/cm^2) at left-handed or right-handed circular polarization. Incubation of cells in M9 buffer without EtBr. Mean of four experiments and standard error (SE).

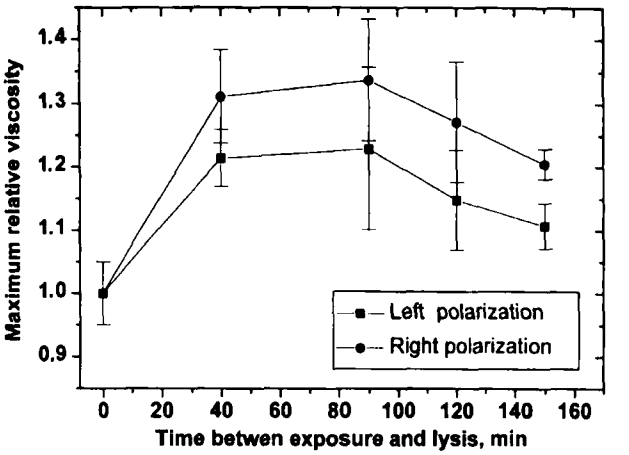

FIGURE 3. Combined effects of CP MMW and EtBr. Kinetics of changes in the maximum relative viscosity after exposure of the stationary *E. coli* cells (4×10^7 cells/ml) to circularly polarized MMW (10 min, 51.755 GHz, 0.1 mW/cm^2 at left-handed or right-handed circular polarization. Before and after exposure to MMW, the cells were incubated in M9 buffer with EtBr at a concentration of 1 μg/ml.

and left polarization when the data from separate time points were compared. However, the paired *t*-test showed that the effect of right-handed MMW was significantly greater ($p < 0.005$). Linear correlation was observed between the effects of CP MMW ($p < 0.02$). The effects of the right polarization with and without EtBr were significantly different at 40 min ($p < 0.03$) and 90 min ($p < 0.04$) compared with Student's *t*-test. The paired *t*-test also revealed a significant difference ($p < 0.02$). Thus, the preincubation of cells with EtBr resulted in a significant increase in the effect of right-handed polarization and inverted the effective polarization.

DISCUSSION

The resonance effect of low-intensity MMW on the GCS of *E. coli* K12 AB1157 cells has been observed in several frequency windows with resonance frequencies of 41.32 ± 0.01, 51.674 ± 0.003, 51.755 ± 0.001, 51.805 ± 0.002, and 51.835 ± 0.005 GHz (5,10,11). The body of our data, including measurements of local PD on the surface of the exposed cell suspension, showed that the observed MMW effects could not be explained by the total heating or local hot-spots (10,11,25) and ref. therein). This paper confirmed the difference in effects of right- and left-handed CP MMW and provided new experimental evidence for nonthermal MMW effects. The dependence of resonance frequencies on the genome length obtained strongly supports the concept that DNA is a target of the MMW resonance effects in *E. coli* cells (12). These results suggested that the whole genome, including the noncoding part, may play an important role in gene regulation by creating self-consistent spectra of acoustic, mechanical, and electromagnetic oscillations (12,26). According to our data, left-handed circularly polarized MMW was more effective than right polarization at three of five resonances studied. 51.675 ± 0.001, 51.755 ±

0.001, and 51.835 ± 0.005 GHz (10,11). In contrast, left-handed polarized MMW was ineffective at two other resonance frequencies (41.32 ± 0.01 and 51.805 ± 0.02 GHz), whereas right-handed polarization affected the cells. These data suggested that at any resonance of MMW frequency, one of the two possible circular polarizations is more effective than the other. These data provide evidence that the target for resonance interaction of MMW with living cells should possess a chiral asymmetry and should alternate between right and left chiral forms. DNA satisfies these properties. In this paper, we incubated the cells with the specific DNA intercalator EtBr before exposure to MMW. EtBr relaxes the negatively supercoiled DNA loops and stabilizes the right-handed B-form of DNA (22). We exposed cells at the resonance frequency of 51.755 GHz. The left-handed circular polarization was effective at this resonance frequency without EtBr. The incubation with EtBr inverted the effective circular polarization, resulting in significant increase in the effect of right polarization. This is in good accordance with data showing stabilization of B-form DNA by EtBr. At the same time, left-handed MMW also resulted in an effect. Probably, EtBr did not stabilize all DNA sequences that were responsible for the interaction with MMW in B-form.

EtBr changes the supercoiling of DNA loops starting at a concentration of about 1 μg/ml as measured with the AVTD in lysates of different cell types including *E. coli* (10,21,27). In living cells, the concentration of EtBr is higher than in the incubation media due to accumulation of EtBr by binding to nucleic acids (23). According to the data of Lambert *et al.* and Ohta *et al.* (28,29), the AB1157 *E. coli* cells are extremely resistant to EtBr. These cells are still able to grow almost normally when 0.1 mg/ml of EtBr is present in the medium (28). At concentrations up to 0.1 mg/ml, ethidium was unable to induce DNA damage as measured with the SOS system in *E. coli* (28,29). This means that treatment with even higher concentration of EtBr than in the present paper is not genotoxic and does not result in DNA damage. As was shown previously, the AVTD technique is highly sensitive to DNA damage (9,30,31). The absence of DNA damage after incubation with EtBr in our experiments was also supported by the AVTD data because we saw no differences between controls and cells incubated with EtBr. Therefore, the observed modification of the MMW effect by EtBr almost certainly was not caused by DNA damage.

Likely candidates for interaction with MMW in *E. coli* cells are repeated extragenic palindromes (REPs), which contain binding sites for DNA gyrases (topoisomerase II) and topoisomerase I (32). These enzymes are located at the attachment sites for supercoiled domains at the cell membranes and control the supercoiling of a nucleoid (18). A possible role of DNA gyrases in effects of nonthermal MMW is supported by our data about inhibition of such effects by means of nalidixic acid (Belyaev *et al.*, ms. in preparation). This antitumor drug is believed to be a specific inhibitor for DNA gyrases. It is important that REPs alternate in a left-to-right orientation. These alternations may be responsible for the observed difference in the effects of left- and right-handed circularly polarized MMW.

The AVTD technique is a rather new method, and the relationship of the AVTD data to changes in the genome conformational state should be directly verified with a well-established technique. Recently, such verification was performed using single-cell gel electrophoresis (33). A possible biologic significance of the AVTD changes that were induced by MMW in *E. coli* cells has been discussed elsewhere (13,14). In particular, the relationship between AVTD changes and changes in cell growth (14) and the spectrum of DNA-bound proteins (13) was observed.

In conclusion, the data provide new evidence that DNA is a target for the effects of MMW on living cells.

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