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Inhibition of DNA Synthesis and Enhancement of the Uptake and Action of Methotrexate by Low-Power-Density Microwave Radiation in L1210 Leukemia Cells[1]

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ABSTRACT

We have studied the effects of low-power-density microwave (MW) radiation (continuous and pulsed wave with average power density of 10 milliwatts/sq cm and range of 5 to 50 milliwatts/sq cm; frequency, 1.0 GHz) on the uptake and action of methotrexate (MTX), the inhibition of DNA synthesis in L1210 murine leukemia cells *in vitro*, and the MTX treatment of mice bearing this leukemia. Using short-term tissue culture techniques, MTX concentrations of 0.2 μM, and MW exposure times of 20 min, we have found that continuous-wave low-power-density MW irradiation enhances the uptake of [^{3}H]MTX as compared to nonirradiated controls. The enhancement is observed in only a small range of power densities (5 to 25 milliwatts/sq cm) and is in an inverted-U-shaped relationship. MW irradiation alone has an inhibitory effect on the [^{3}H]deoxyuridine incorporation into DNA. Compared to cell suspensions treated with MTX alone, groups treated with MW irradiation followed by MTX exhibit an augmentation of inhibition of DNA synthesis as measured by [^{3}H]deoxyuridine incorporation. Combined treatment of L1210-bearing mice with MW irradiation and MTX *in vivo* prolonged the duration of survival over that of animals treated with MTX alone, indicating a greater killing of leukemia cells. These results suggest that the therapeutic index of MTX may be improved by the use of MW irradiation at low power densities.

INTRODUCTION

The major disadvantage in cancer chemotherapy is the toxicity of antitumor agents against the normal tissues. Various attempts have been made to circumvent this disadvantage and to increase the therapeutic index of these agents through (*a*) chemical modification of the agents to increase the selectivity against the tumor cells, (*b*) subsequent administration of metabolic intermediates to override the effects of the agent on host tissues (the so-called "rescue" regimens), (*c*) combination of multiple agents to achieve an additive or synergistic effect without increasing the overall toxicity, and (*d*) synchronization of tumor cells to render them more sensitive to drugs.

An alternative approach is to enhance the drug uptake by the tumor cells. Since many major chemotherapeutic agents enter the cells via membrane transport, electromagnetic (MW[3]) radiation was chosen to influence such mechanisms. The exact mechanism of effects of MW radiation on cellular metabolism is unknown. It is presumed that such radiation may involve a change in the dielectric properties of biological molecules, in energy metabolism, and in the functional orientation of the cell membrane (9).

MTX was chosen for our study because it is a drug with a wide range of applicability in the treatment of human neoplasms. The agent has been and continues to be under active investigation. It is known to be taken up by dividing cells (both normal and malignant) via an active transport mechanism (4). Since MTX is thought to exert its major effect by inhibiting *de novo* DNA synthesis (1, 6, 10), we have also investigated the effect of low-power-density MW radiation alone on DNA synthesis. Based on our work using L1210 murine leukemia cells, *in vitro* and *in vivo*, the data suggest that MW radiation, at certain low power densities, may augment the cellular uptake of MTX and may also have an inhibitory effect on DNA synthesis.

MATERIALS AND METHODS

L1210 Murine Leukemia Cell Line. The cell line was originally obtained from Southern Research Institute (courtesy of Dr. W. W. Brockman). L1210 cells were serially passaged in DBA/2 mice (The Jackson Laboratory, Bar Harbor, Maine) on a weekly basis by i.p. inoculation of 10^5 cells/mouse. Prior to each *in vitro* experiment, the animals were sacrificed by cervical dislocation, the peritoneal cavity was opened under sterile conditions, and the ascitic fluid containing the L1210 cells was aspirated using a syringe coated with heparin. The cells were harvested 5 to 6 days after inoculation of the animals. When the ascitic fluid was grossly bloody, the cells were centrifuged over a Ficoll (9%)/Hypaque (50%) gradient (3/1, v/v; specific gravity, 1.08) to remove the RBC.

Chemicals and Reagents. [^{3}H]MTX (specific activity, 7.0 to 13.4 Ci/mmol) was obtained from Amersham/Searle, Chicago, Ill. The purity was determined by paper chromatography with 0.5% sodium carbonate as solvent. The [^{3}H]MTX used in our experiments was 85% pure. Unlabeled MTX, Reference Standard Grade, was courtesy of Lederle Laboratories, Pearl River, N. Y. [^{3}H]dUrd (specific activity, 20 Ci/mmol) was from New England Nuclear, Boston, Mass. Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., New York, N. Y.) was used throughout for suspending cells. Toluene-Permablend (Packard Instrument Co., Downers Grove, Ill.) was used for scintillation fluid. BNS was used for MTX uptake experiments as previously suggested (4).

MW Equipment. MW source was from Airborne Instrument Laboratories (AIL 125). All experiments were conducted at a

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[3] The abbreviations used are: MW, microwave; MTX, methotrexate; dUrd, deoxyuridine; BNS, 0.85% NaCl solution buffered to pH 7.3 with $NaHCO_3$; PBS, phosphate-buffered saline (0.155 M NaCl-0.0097 M Na_2HPO_4-0.00169 M NaH_2PO_4, pH 7.4); CW, continuous wave; PW, pulsed wave; TCA, trichloroacetic acid.

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frequency of 1.0 GHz, with variation of power density from 5 to 50 milliwatts/sq cm of CW and at an average power density of 10 milliwatts/sq cm for PW with peak power from 111 to 121 milliwatts/sq cm. A double-ridged waveguide horn antenna was used. A radiation hazard meter (General Microwave Corporation) was used to measure power densities without the samples present. A Model 402 thermistor probe (Yellow Springs Instruments), calibrated to accuracy within 0.1° against a National Bureau of Standards mercury thermometer, was used. With probe oriented perpendicular to the MW electric field, there was no appreciable interference in temperature readings by the MW field. Temperatures were recorded for experiments performed at 25 and 50 milliwatts/sq cm (CW) and were not allowed to exceed 38°.

[^{3}H]MTX Uptake Experiments. The following method for determining the uptake of MTX was adapted from that of Goldman *et al.* (4). L1210 cells were harvested as described above and suspended in Roswell Park Memorial Institute Medium 1640. The cells from one mouse were used for each experiment. Viability and cell count were determined in 0.2% trypan blue (Grand Island Biological Co.). The cell suspension was then divided into 2 treatment groups (volume, 4.5 to 6.5 ml) and placed in Falcon tissue culture flasks (1.2 x 3.7 x 8 cm). The control group was given [^{3}H]MTX alone; the other was given MW radiation and [^{3}H]MTX. MW radiation was given from above with the tissue culture flask placed on its side. The depth of the fluid was 1.5 to 2.0 mm. The samples were located 15 to 20 cm from the mouth of the horn antenna and were exposed to the radiation at a given power density for 20 min at room temperature. The control group was kept at room temperature for the period of radiation.

Immediately following the MW exposure, [^{3}H]MTX was added to the flasks to a final concentration of 0.2 μM. The cell suspensions were divided into 1-ml aliquots in tissue culture tubes, with duplicates for each incubation time, and were then incubated at 37° in a 5% CO_2 incubator. The incubations were terminated at 1 or 2 hr (except for a few experiments in which cells were labeled continuously for 4 hr) by the addition of 4 ml of ice-cold BNS, centrifuged at 0°, and then washed twice in BNS to remove unincorporated [^{3}H]MTX. The cells were then resuspended in 0.1 ml of buffer, pipetted onto Whatman 2.3-cm filter paper discs, dried, and counted in a Beckman liquid scintillation counter. Results were expressed as cpm/10^6 cells.

Incorporation of [^{3}H]dUrd into DNA. Following harvesting of transplanted L1210 cells from mice and their suspension in culture medium, the cells were placed in tissue culture flasks and divided into 4 treatment groups: Group 1, control; Group 2, MW alone; Group 3, MTX alone; and Group 4, MW and MTX. After MW irradiation of Groups 2 and 4 for 20 min, unlabeled MTX (0.2 μM) was added to Groups 3 and 4. All 4 groups were incubated at 37° in a 5% CO_2 incubator for 30 min. These 4 separate cell suspensions were then divided into 1-ml aliquots in duplicate for each incubation period. Labeling time with [^{3}H]dUrd (0.7 μCi/ml of culture) was 60 min. Incubations were terminated at 1 and 2 hr by the addition of 4 ml of ice-cold PBS, and the cells were pelleted and washed with 4 ml of PBS. DNA was precipitated from the cell pellet with 1 ml of cold 5% TCA, followed by hydrolysis with 0.5 N NaOH to remove possible contaminating RNA and reprecipitation of the DNA with addition of 1.1 ml of 10% TCA. The final TCA precipitates were collected on Whatman No. 3MM glass fiber filters (diameter, 2.4 cm) and washed with 10 ml 5% TCA and 95% ethanol. The filters were dried and counted in 2 ml of toluene-Permablend.

***In Vivo* Effect of MW and MTX Treatment.** The experimental model was adapted from the model designed by Skipper *et al.* (8). L1210 cells (10^5) were inoculated i.p. into 40 mice, which were then divided into 4 separate groups of 10. Six days after the inoculation, when abdominal enlargement was evident (mean survival for the inoculated animals, 9 days), Group 1 was given i.p. 0.85% NaCl solution injections every other day as controls; Group 2 was given MTX (3 mg/kg i.p.; a dose which would partially prolong the survival) every other day; Group 3 received MW radiation (5 milliwatts/sq cm for 20 min) every other day; and Group 4 was given MW radiation immediately followed by MTX injection in dosages and power densities identical to those of Groups 2 and 3. All animals received treatment until death ensued.

RESULTS

MW Effect on [^{3}H]MTX Uptake

The uptake and transport of MTX in L1210 cells has been studied extensively by Goldman *et al.* (4). In our laboratory, the cellular uptake of 0.2 μM MTX was more linear within the first hr of incubation, beyond which the rate began to slow down. Chart 1 shows that the cellular tracer uptake was 38 and 32% higher than the uptake in the control cells at 5 and 10 milliwatts/sq cm and 78% higher at 25-milliwatt/sq cm power densities of CW radiation. Fifty milliwatts of CW radiation per sq cm and 10 milliwatts of PW radiation per sq cm resulted in a reduction of tracer uptake below the control level. The temperature recorded from the cell suspension was 23.5 ± 1°

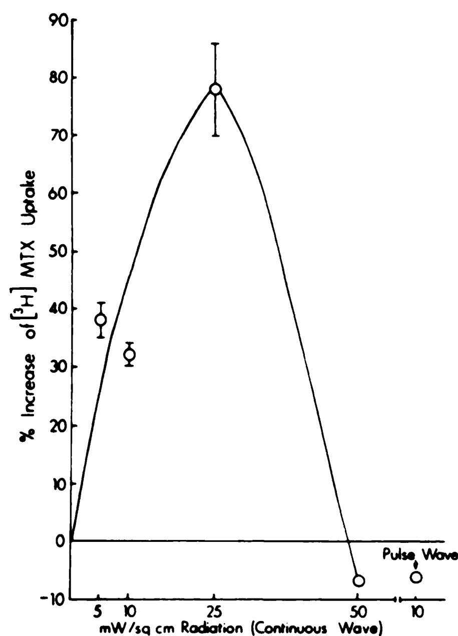

Chart 1. [^{3}H]MTX uptake. L1210 leukemia cells (cell counts adjusted to 10^6/ml) were divided into irradiated and unirradiated groups. Sixty min after addition of [^{3}H]MTX (unlabeled, 0.2 μM), the cells were removed from incubation at 37° and washed twice with cold BNS before they were counted for incorporated radioactivity. *Ordinate*, percentage of increase of exposed cells as compared to unexposed controls; *bars*, S.E. of 4 independent experiments; *mW*, milliwatts.

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(S.E.) without radiation, 30.7 ± 3.5° with 25 milliwatts/sq cm, and 37.4 ± 0.9° with 50 milliwatts/sq cm. In 4-hr continuous labeling experiments, the observed increase associated with radiation was transient, present only in the first hr following a 20-min radiation. When the rates of tracer efflux were compared after removal of [^{3}H]MTX from the cell suspension, both exposed and unexposed cells demonstrated a 65% loss of radioactivity from the cells into their respective supernatants in the ensuing 60 min. This finding suggests that MW exposure is associated with an increase in uptake rather than a slowing of the efflux process.

Effect of MW and MTX on DNA Synthesis in L1210 Cells

Effect of MW on *de Novo* DNA Synthesis. To examine the effect of MW on the inhibition of DNA synthesis by MTX, the incorporation of [^{3}H]dUrd into DNA of L1210 cells was used (4). Inhibition by MTX will result in the reduced transfer of a methyl group necessary for the conversion of phosphorylated dUrd to phosphorylated thymidine before its incorporation into DNA (1, 6, 10). Chart 2 shows that MW exposure (20 min of CW) alone results in a consistent reduction in the incorporation of [^{3}H]dUrd. The effect lasted for at least 120 min. This inhibition appeared to have a curvilinear (inverted-U) relationship as the power density increased. The peak effect was seen between power densities of 15 and 25 milliwatts/sq cm. PW exposure at 10 milliwatts/sq cm gave an inhibition similar to that seen with CW radiation.

Combined Effect of MW and 0.2 μM MTX. A power density of 15 milliwatts/sq cm was chosen for these experiments. MTX (unlabeled, 0.2 μM) was selected in order to give an intermediate level of inhibition of DNA synthesis so that additional suppression would be demonstrable. It had also been the level used in the tracer uptake experiment. Chart 3 shows that combined MW exposure and MTX suppressed DNA synthesis to a somewhat greater extent than did either alone.

Combined *in Vivo* Effects of MW and MTX

The purpose of this experiment was to examine whether the *in vivo* effect of combined treatment with MTX and MW on mice bearing L1210 leukemic cells was beneficial in comparison to treatment with MTX or MW alone. The survival of these animals reflects the number of viable L1210 cells capable of producing

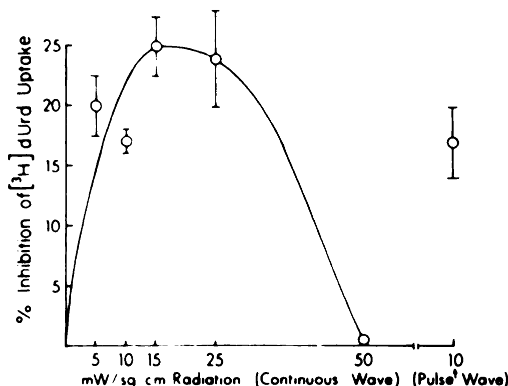

Chart 2. [^{3}H]dUrd incorporation. Incubation conditions are as described in Chart 1. *Ordinate*, percentage of reduction of [^{3}H]dUrd incorporation into DNA of 10^6 exposed L1210 cells as compared to unexposed cells; *bars*, S.E. of 4 independent experiments; *mW*, milliwatts.

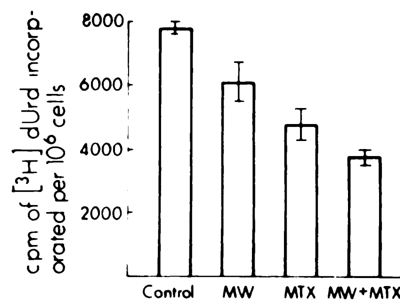

Chart 3. Combined effect of MW and 0.2 μM MTX. The experimental conditions are identical to those shown in Chart 1, with MW irradiation given at 15 milliwatts/sq cm and 0.2 μM MTX. *Ordinate*, [^{3}H]dUrd cpm/10^6 L1210 cells under various exposures. Values are averages of 4 experiments. *Bars*, S.E.

death in the animal (8). The median survival of control animals from this experiment was 8.3 (range, 7.5 to 10.5) days, indicating an equivalent of 10^5 cells inoculated according to the method of Skipper *et al.* MTX (3 mg/kg), given every other day, prolonged the median survival to 11.3 (range, 9.5 to 15.5) days, indicating an equivalent of 5×10^3 cells inoculated (or 1.5-log kill of L1210 cells). MW alone did not significantly alter the survival of 7.5 (range, 7.5 to 8.5) days. Combined treatment with MW followed by MTX also prolonged the survival of these animals to 13.3 (range, 10 to 19.5) days, indicating an equivalent of 5×10^2 cells inoculated (or 2.5-log kill of L1210 cells).

DISCUSSION

The experiments shown in this report attempted to demonstrate whether the cellular uptake and action of MTX could be enhanced by low levels of MW radiation in *in vitro* and *in vivo* systems. From power densities of 5 to 50 milliwatts/sq cm, a stimulation of [^{3}H]MTX uptake by L1210 cells in an inverted-U relationship was observed. Enhancement of uptake is unlikely to be the result of thermal stimulation, inasmuch as the effect was absent or even inhibited at 50 milliwatts/sq cm when the temperature reached 37°, the level more suitable for cell growth and metabolism. This curvilinear relationship has also been seen previously in MW-induced lymphocyte transformation by us (5) and in the nervous system by others (7). This relationship indicates that there exists only a small range of power level in which stimulatory effect may be seen. This observation offers an explanation for those studies which fail to show MW effects when the power densities chosen are outside of the effective "window." The phenomenon was observed again in our recent study of MW effect on the blood-brain barrier in dogs in which maximum disruption was found at 25 milliwatts/sq cm (2). The "window" phenomenon not only applies to the power density but may also vary with regard to frequency of MW radiation (7). Also, different cell types respond to MW stimulation of MTX uptake somewhat differently. Blood granulocytes take up 4 times as much [^{3}H]MTX as do lectin-stimulated or resting lymphocytes following MW exposure.[4]

Suppression of DNA synthesis as measured by incorporation of [^{3}H]dUrd follows a similar curvilinear relationship as a function of power densities, with a peak effect at 25 milliwatts/sq

[4] Unpublished data.

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cm. A comparable degree of inhibition of RNA synthesis ([^{3}H]-Urd uptake) and a somewhat greater reduction of protein synthesis (^{3}H-amino acid uptake) were also observed.[3] The increased membrane permeability noted in MW-irradiated cells may account for this moderate inhibition, because a greater flux of ^{3}H-nucleosides into exposed cells may offset the actual inhibition to some extent (9). When MW radiation is combined with MTX, the inhibition of DNA synthesis is further increased. The lack of a greater degree of synergism may be due to the suppression of *de novo* DNA synthesis by MW masking or interfering with the effect of MTX.

In vivo treatment of L1210-bearing mice and measurements of their survival were chosen to study degrees of cell kill by MTX and a combination of MW and MTX. This method of study is modeled after the much-used and reproducible system previously designed by Skipper *et al.*, except for the concurrent exposure of animals to MW radiation. A lower power density of MW (5 milliwatts/sq cm) in these experiments is used to circumvent a potentially complicating systemic thermal effect on the animal which can occur at higher power levels (1° rise in rectal temperature at 25 milliwatts/sq cm) (5). The combined treatment with MW and MTX altered the duration of survival to an equivalent tumor cell kill of 2.5 logs in comparison to 1.5 logs with MTX alone. This *in vivo* difference is in agreement with our *in vitro* observations of inhibition of DNA synthesis and drug uptake.

The basic mechanism of MW cellular effect is unclear. It is possible that the electromagnetic field in which the cells are exposed may change the orientation or resonance of polar molecules or polar organelles within them. This type of low-energy radiation can be a clinically useful adjunctive treatment, since it has few or no known additive side effects, such as neurotoxicity, seen when vincristine is used to enhance the suppressive effect of MTX (3) and marrow suppression, or cumulative tissue damage, as is the case with ionizing radiation. The local thermal effect at the power densities tested is physiologically negligible. Its application offers a viable and safe modification, potentially useful in the future development of cancer chemtherapy.

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