PROJECT REPORT

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EFFECTS OF 27 MHz RADIATION ON SOMATIC AND GERM CELLS

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16. Abstract (Limit: 200 words): A study was made of the dose dependence of effects of 27 and 2450 megahertz (MHz) continuous wave (CW) and pulse modulated (PM) radiation exposure of normal resting peripheral human lymphocytes, human glioma (LN71), HeLa, and Chinese-hamster-ovary (CHO) cells. Direct radiation effects on mouse germ cells were also investigated. Effects of high frequency electromagnetic radiation (EMR) on lymphocyte and glioma mitogenesis and the CHO cell cycle in-vitro provide evidence of direct dose or dose rate dependent alteration of a highly physiologically significant cellular endpoint. Although in-vitro data cannot be extrapolated directly to in-vivo responses, the experimental conditions of these in-vitro studies suggest that qualitatively similar effects may be induced by in-vivo exposure to electromagnetic fields of these frequencies. The author concludes that physiologically significant cellular alterations are induced by 27 and 2450MHz CW and PM radiation exposure under conditions that do not involve heating. The author suggests that the adequacy of radiofrequency occupational exposure guidelines that suggest that health effects are attributable to radiation induced tissue heating must be questioned.

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ABSTRACT

It is well documented that workers are routinely exposed to 27 MHz and other frequencies of electromagnetic radiation. Health protection guidelines are based upon the premise that adverse health effects are due to tissue heating. It is of primary importance for occupational safety and health to determine the validity of this premise. To this end studies are being conducted of the effects of continuous wave (CW) and pulse-modulated (PM) 27 MHz and 2450 MHz RF electromagnetic radiation on mammalian cells in vitro.

Studies conducted during this project provide firm evidence that these RF radiation frequencies affect cell mitotic activity under conditions that simulate occupational exposure. Whereas the mechanism is not well understood, it has been determined that it is a direct RF effect, not due to heating. These results indicate the need for detailed characterization of cellular effects of RF radiation to provide insight regarding: a) the adequacy of the basis for present occupational exposure guidelines, and b) mechanisms of direct RF-induced cellular alterations.

Cell suspensions were exposed to 27-MHz or 2450-MHz radiation under isothermal conditions in vitro. Viability and morphology were assayed immediately after exposure. Cells were cultured for 1-, 3-, or 5 days prior to assay (i.e., cell proliferation, mitogenesis, DNA, RNA, protein synthesis, sperm viability, and motility, in vitro fertilization). Cytofluorimetry was used to investigate the interaction of RF radiation with the cell cycle using synchronized populations of Chinese hamster ovary (CHO) and HeLa cells.

A single 2h isothermal (37 ± 0.2°C) exposure to either 27- or 2450 MHz RF radiation induced biphasic dose-dependent alterations in human lymphocyte mitogenesis 3d after exposure, or in DNA and RNA synthesis in glioma (LN71) cells 1, 3, or 5d after exposure. Exposure of either cell type to SARs in the range 5-50 W/kg stimulated biosynthetic processes, whereas exposure at >50W/kg suppressed cellular activity. Comparison of the effects of CW versus PM (duty cycle 0.377) 27 MHz RF radiation indicate similar biphasic effects on human lymphocyte activation and glioma proliferation. RF exposure of lymphocytes or glioma in vitro at elevated temperature (39°C) altered proliferation relative to exposure at 37°C. This finding is potentially significant since in vivo RF exposures in the work place are known to involve radiation-induced heating in some instances.

Dose-dependent RF-induced shifts in the cycle of synchronized CHO and HeLa cells indicated that the biphasic response resulted from cycle-specific effects on DNA/RNA synthesis. Maximum sensitivity for RF-induced cycle phase shifts appeared to occur during G_0/G_1 phase. A highly statistically significant reduction in the ability of mouse spermatozoa to fertilize mouse ova occurred following a 1 h exposure of sperm at 37 ± 0.2°C. RF exposure at SARs in the range of 5- to 200W/kg had no detectable effect on cell viability or morphology.

RESEARCH OUTLINE AND RATIONALE

Research conducted during the past 5 years with support of Grant No. 2R01OHO2148 has been directed toward quantitating direct effects of continuous wave (CW) and pulse modulated (PM) 27 MHz radiofrequency (RF) radiation on mammalian cells in vitro. Parallel studies have been conducted of effects of CW and PM 2450 MHz microwave radiation to provide an assessment of the frequency-dependence of cellular effects of these radiations. Exposure systems were constructed for simultaneous exposure of cells to 27 MHz and 2450 MHz radiation under conditions of precise temperature control. By controlling temperature during exposure it was possible to differentiate direct radiation effects on cells from indirect effects due to radiation-induced heating.

The general rationale for this investigation was to provide data of pertinence to the assessment of the adequacy of occupational exposure guidelines for these types of radiation. Specifically, by differentiating direct versus indirect thermal effects, these data afford a means of testing the central premise upon which occupational exposure guidelines are based. This premise is that adverse health effects of occupational exposure to radiation at frequencies, such as 27- or 2450 MHz, are attributable solely to induced tissue heating. Consequently, exposure guidelines are designed to prevent tissue heating, without consideration of nonthermal radiation effects.

We investigated the dose-dependence of effects of isothermal (37 ± 0.2°C) 27- and 2450 CW and PM radiation exposure of: a) normal resting peripheral human lymphocytes; b) human glioma (LN 71); and c) Chinese hamster ovary (CHO) cells. A single 2 hour radiation exposure caused direct dose-dependent effects on cell proliferation that were detectable for at least 6 days after exposure. Cellular proliferation rates were increased at doses (Specific Absorption Rates (SARs)) of less than 50 W/kg, but decreased at higher doses. In the dose range investigated, there were no effects on cell viability or morphology. Differences have been detected in the effects of PM versus CW exposures, especially at the low and high end of the dose range. Whereas responses to 27- versus 2450 MHz radiation were generally similar there is evidence of differences in the lower dose range. On the basis of these data, and data obtained from studies using synchronized CHO cells, it was hypothesized that effects on cell proliferation were due to dose-dependent radiation-induced alterations in specific phases of the mammalian cell-cycle.

Direct radiation effects on germ cells were also investigated. A one hour exposure of mouse spermatozoa caused a statistically significant reduction in fertilization of mouse ova in vitro. The effect, which resulted from exposure to either 27- or 2450 MHz radiation, was dose-dependent.

To test the hypothesis that 27- or 2450 MHz radiation-induced effects on mammalian cells were due to radiation-induced alterations in membrane permeability, studies were conducted of radiation effects on: a) rubidium (Rb) transport in CHO cells, and b) cytosine arabinofuranoside (ARA-C) transport in large unilamellar liposomes. These studies provided no evidence to support this hypothesis.

SPECIFIC TASK SUMMARIES

A. Effect of Isothermal Radiofrequency Radiation (RF) Exposure on Normal Resting Human Peripheral Lymphocytes

Whole human blood was exposed or sham-exposed in vitro for 2 h to 27 or 2,450 MHz radio-frequency electromagnetic (RF) radiation under isothermal conditions (i.e. 37 ± 0.2°C). Immediately after exposure, mononuclear cells were separated from blood by Ficoll density-gradient centrifugation and cultured for 3 days at 37°C with or without mitogenic stimulation by phytohemagglutinin (PHA). Lymphocyte proliferation was assayed at the end of the culture period by 6 h of pulse labeling with ^{3}H-thymidine (^{3}H-TdR). Exposure to radiation at either frequency at specific absorption rates (SARs) below 50 W/kg resulted in a dose-dependent, statistically significant increase of ^{3}H-TdR uptake in PHA-activated or unstimulated lymphocytes. Exposure at 50 W/kg or higher suppressed ^{3}H-TdR uptake relative to that of sham-exposed cells. There were no detectable effects of RF radiation on lymphocyte morphology or viability. Notwithstanding the characteristic temperature dependence of lymphocyte activation in vitro, the isothermal exposure conditions of this study warrant the conclusion that the biphasic, dose-dependent effects of the radiation on lymphocyte proliferation were not dependent on heating.

A detailed summary of this research task is provided in the manuscripts: Lymphocyte Proliferation Modulated In Vitro by Isothermal Radiofrequency Radiation Exposure, and Functional Alteration of Mammalian Cells by Direct High Frequency Electromagnetic Field Interactions.

B. Modulation of Glioma Proliferation by Isothermal RF Radiation Exposure

Isothermal (37 ± 0.2°C) exposure of glioma cells (LN71) for 2 h to 27 or 2450 MHz continuous-wave radiofrequency (RF) radiation in vitro modulated the rates of DNA and RNA synthesis 1, 3, and 5 days after exposure. The alterations indicate effects on cell proliferation and were not caused by RF-induced cell heating. The dose response for either frequency of the radiation was biphasic. Exposure to specific absorption rates (SARs) of 50 W/kg or less stimulated incorporation rates of tritiated thymidine (^{3}H-TdR) and tritiated uridine (^{3}H-UdR), whereas higher SARs suppressed DNA and RNA synthesis. Statistically significant time-dependent alterations were detected for up to 5 days postexposure, suggesting a kinetic cellular response to RF radiation and the possibility of cumultive effects on cell proliferation.

The results of this study are summarized in detail in the manuscripts: Glioma Proliferation Modulated In Vitro by Isothermal Radiofrequency Radiation Exposure, and Functional Alteration of Mammalian Cells by Direct High Frequency Electromagnetic Field Interactions.

C. Cell Synchronization

A modified method to synchronize CHO and HeLa cells was developed based upon a combined shaking-off and chemical blockage. This method has effectively blocked quiescient cells which is the main obstacle of high degree synchronization. Flowcytometry data show the improvement on the degree of synchronization and yield compared to previously used methods.

Chinese hamster ovary (CHO) cells were monolayer-cultured with DME/F12 medium plus 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. HeLa S-3 cells were monolayer-cultured with DMEM medium plus fetal calf serum, penicillin and streptomycin as for CHO culture. Both cell lines were incubated in 5% CO_2 at 37°C. Under these culture conditions the generation time of CHO cells was 13 hours (5.5 hours for G_1-phase, 5 hours for S-phase and 2.5 hours for G_2/M-phase). The generation time of HeLa cells was 23 hours (11 hours for G_1, 9 hours for S and 3 hours for G_2/M). HeLa cells were also cultured in spinner flasks at a spin speed of 60 rpm at concentrations between $3x10^5$/ml and 10^6/ml, under the same conditions used for monolayer culture. In this culture the generation time was approximately 24 hours.

CHO and HeLa cell generation time was determined from growth curves obtained by cell counting. Cell cycle phase durations were determined from flow cytometry data and growth curves of partially synchronized cell cultures by double TdR block.

Method 1. CHO and HeLa cells were synchronized by double thymidine block. Thymidine was added to random monolayer cell cultures to a final concentration of 5 mM and incubated for a period somewhat longer than the sum of G_2, plus M, plus G_1 portions of the cycle (9 hours for CHO, 14 hours for HeLa). The cultures were released from thymidine block by reculture in normal medium for a period that slightly exceeded S-phase (5.5 hours for CHO and 9.5 hours for HeLa). This procedure was repeated for a second cycle block. Synchronization of suspension - cultured HeLa cells was attempted by multiple thymidine block. The cells underwent repeated cycles of a 14-hour exposure to 2 mM TdR followed by a 10-hour exposure to normal medium for periods of up to 4 weeks. After the final release cells were assayed cytofluorometrically [2] to check synchronization.

Method 2. CHO cells were seeded into culture dishes and allowed to grow for 18 hours. Thymidine (5 mM) was added and cells were incubated for 9 more hours and then released from blockage by charge of medium. Six hours after the release, mitotic cells were selectively detached from the monolayer by gentle agitation and collected from the decanted medium. This shake-off procedure was repeated 2-4 times with 15-minute intervals to increase cell yield. More shake-off gave higher yield but increased the percentage of nonmitotic cells. Mitotic cells were held at 4°C during collection. Cells were incubated in the presence of 2 mM HU for 9 hours after collection and then sampled for degree of synchronization after release.

Method 3. Modified method: This method consisted of double thymidine block followed by mitotic shake-off and hydroxyurea block. Double thymidine block, as described above, was used to induce a large mitotic index and hence increase the yield from the shake-off procedure. Gentle shaking of treated CHO cells yielded up to 15% of total cells. HeLa cells were more adherent. Gentle shaking yielded a small number of mitotic cells whereas violent shaking detached cells in phases other than M. To avoid this, "low trypsin concentration (0.05%; 1/5 of the concentration for normal trypsinization) was used to treat cell cultures for a maximum of 3 minutes at room temperature. Regular medium with 10% FCS was added and shaking was performed. Mitotic cells collected from shaking were then cultured, with 2 mM HU; 9 hours for CHO cells, 12 hours for HeLa cells. HU prevented cells from entering S phase but did not otherwise alter their physiological state. After 1 hour the medium was changed to remove unattached cells and culture was continued in the presence of 2 mM HU. 2 mM HU was selected for synchronization after different concentrations were tested. We found that 0.1 mM HU did not effectively block cells at the G_1/S boundary.

Flowcytometry was used to determine relative DNA contents of cells to check the degree of cell synchronization. Monolayer-cultured CHO or HeLa cells were trypsinized and washed with calcium/magnesium-free phosphate-buffered saline, PH 7.0 and centrifuged for 10 min at 500G. Cell pellets were vortexed, dyed with PIF stain and filtered through a 37 μm screen filter. The final cell concentration was adjusted to 10^6/ml. Cell samples were analyzed by an EPICS753 flow cytometer with an excitation wavelength of 488 nm. The flourescence was measured at wavelengths above 630 nm. Data were collected and analyzed by an MDADS microcomputer and DNA distributions were plotted.

Results

The modified method utilized a double TdR block and shake-off plus HU blockage. This improved the degree of synchronization since: a) double TdR block induced a high mitotic index, which increased the yield, b) discarding the medium with unattached cells after cells have settled, and using low concentration trypsin to treat the HeLa cell culture before shaking increased the yield and purity of M-phase cells. For both CHO and HeLa cells the yield was high (>15% of total cells) and the synchronization was significantly improved. In both cases cells were sampled and stained for DNA analysis by flowcytometry at times shown after the final release from HU block. Cells entered S-phase shortly after release and progressed through the normal cycle. By sampling at various times after release synchronized cell samples can be obtained in any phase.

The methods used and results of this research are summarized in the manuscript: Modified Method of Cell Synchronization Improves Yield and Degree of Synchronization,

D. Effect of Isothermal RF Exposure on the Cell-Cycle of CHO Cells

To test the hypothesis that modulation of cell proliferation by isothermal RF-radiation exposure was due to a direct effect on the cell-cycle we studied the effects of 27- and 2450 MHz CW radiation on synchronized cells. Cells were exposed under conditions we had determined affected proliferation of human lymphocytes and glioma. Initial studies involved Chinese hamster ovary (CHO) and HeLa cells since synchronization procedures had been developed for these cell lines.

Significant effects were detected on the cycle of G_0/G_1 cells exposed to 25 W/kg 2450-MHz CW radiation. The relative magnitude of the effect of 2450-MHz microwave radiation versus 27-MHz at the same SAR (25 W/kg) was time-dependent. Microwave exposure increased the relative number of cells in G_0/G_1 and reduced M-phase cell numbers on days 2, 3, and 5. Four days after exposure there was a significant increase in S-phase cells relative to G_0/G_1. These data indicate qualitative and quantitative differences in the effect of 25 W/kg 27-MHz versus 2450-MHz radiation on G_0/G_1-synchronized CHO cells. A similar differential effect of 25 W/kg 27-MHz versus 2450-MHz CW radiation on the CHO cell cycle was detected in cells exposed during S-phase. Microwave-exposed S-phase cells had a greater number of G_0/G_1-phase cells, in contrast to cells exposed to 27-MHz which had a greater number of S- and M-phase cells.

Synchronized CHO cells in G_0/G_1-, S-, and G_2/M-phase were exposed isothermally to 27- or 2450-MHz CW RF radiation for 2-h at a SAR of 50 W/kg. Effects on the CHO cell-cycle were qualitatively similar to effects of exposure to 25 W/kg described above. In general, however, the magnitude of the effect, as indicated by the peak heights of the

differential DNA-distributions, was less following 50 W/kg exposure to either frequency, compared to 25 W/kg exposure.

RF exposure had less effect on S- or G_2/M-phase cultures relative to G_0/G_1-phase cultures at SARs of 25 W/kg or 50 W/kg. An apparent exception was noted, however, in S-phase cultures exposed to 27- or 2450-MHz radiation at 50 W/kg. The unexpectedly large relative magnitude of these responses was tentatively attributed to the fact that cytofluorimetric assays were performed 12 h after exposure (and 1 d intervals thereafter) instead of 24 h after exposure as in all previous studies. The heightened response may be due to a time-dependent (reversible) effect of RF-radiation on the CHO cell-cycle. The kinetics of RF-induced CHO cell-cycle alterations are currently being investigated.

In summary, isothermal exposure of synchronized CHO cells to 27- or 2450-MHz RF radiation affected the cell-cycle. The nature and magnitude of the RF effect depends upon: a) SAR, b) RF-frequency, c) cell-cycle stage (i.e. G_0/G_1, S, or G_2/M), and d) time after exposure. Effects were detected up to 6-d after exposure. In general, RF-effects on the CHO cell-cycle are consistent with previously detected RF effects on lymphocyte and glioma proliferation. Direct comparisons, however, will require determination of temporal patterns of DNA and RNA synthesis in RF-exposed CHO cells and additional cytofluorimetric dose-response data.

E. In Vitro Fertilization of Mouse Ova by Spermatozoa Exposed Isothermally to RF Radiation

Mouse spermatozoa were exposed in vitro for 1 h to 27- or 2450-MHz CW RF radiation at SARs of 0 to 90 W/kg, under isothermal (37 $\pm$ 0.2°C) conditions. Exposure at either frequency to RF radiation at SARs of 50 W/kg or greater resulted in statistically significant reduction in the ability of irradiated sperm to fertilize mouse ova in vitro ($p<.05$). Over the range of SARs there was no apparent difference in the effects of 27- vs. 2450-MHz RF radiation. There were no readily detectable exposure effects on spermatozoan morphology, ultrastructure, or capacitation. The reduction of in vitro fertilization is attributed to a direct effect of RF radiation on spermatozoa rather than to heating.

A detailed account of this research is provided in the manuscript: In Vitro Fertilization of Mouse Ova by Spermatozoa Exposed Isothermally to Radiofrequency Radiation.

F. RF Effects on Liposome Permeability

Large unilamellar dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) liposomes loaded with an aqueous chemotherapeutic drug, cytosine arabinofuranoside (ARA-C) were exposed for 30 min to 60 W/kg continuous-wave (CW) 100-MHz or 2.45-GHz radiation in vitro at temperatures between 37°C and 43°C. Liposomes were exposed in HEPES buffer or in HEPES buffer supplemented with 44% by volume fetal calf serum (FCS). Characteristic phase transition responses were detected in the range of 39°C to 40°C with the presence of FCS, increasing maximum % release of ^{3}H-ARA-C by 20% relative to HEPES suspension. Neither frequency of electromagnetic radiation had any detectable effect on liposome permeability or the location of the phase transition in the presence or absence of FCS.

The effects of RF radiation on liposome permeability are summarized in detail in the manuscript: Effects of 2.45 GHz Microwave and 100 MHz Radiofrequency Radiation on Liposome Permeability at the Phase Transition Temperature.

GENERAL SUMMARY

Effects of high frequency EMR on lymphocyte and glioma mitogenesis and the CHO cell-cycle in vitro provide evidence of direct dose or dose-rate dependent alteration of a highly physiologically significant cellular endpoint. Although in vitro data cannot be extrapolated directly to in vivo responses the experimental conditions of these in vitro studies suggest that qualitatively similar effects may be induced by in vivo exposure to EMR fields of these frequencies. In applying in vitro data obtained under isothermal exposure conditions to in vivo exposure effects it must be kept in mind that in the latter case attainment of isothermal exposure conditions is difficult if not impossible. Finally, it may be concluded that in at least a general sense, the results of in vitro cellular studies are consistent with reported effects of high frequency EMR on laboratory animals and human beings. The biphasic dose-dependence detected in in vitro studies provides an explanation for the troublesome and previously unexplained variability of in vivo data.

In conclusion, these results indicate that physiologically significant cellular alterations are induced by 27- and 2450 MHz CW and PM radiation exposure under conditions that do not involve heating. Consequently, the basic premise used in formulating RF occupational exposure guidelines, namely that health effects are attributable to radiation-induced tissue heating, must be questioned, as must, therefore, the adequacy of the guidelines themselves.

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