Linearly and Circularly Polarized, 50 Hz Magnetic Fields Did Not Alter Intracellular Calcium in Rat Immune Cells

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Abstract: This study was conducted to determine if exposure to 50 Hz magnetic field (MF) affects intracellular calcium ($[Ca^{2+}]_i$). We employed flow cytometry for real-time detection of possible changes in $[Ca^{2+}]_i$ in a large number of cells. A solenoid coil for vertical MF exposure and a multiple-loop square-coil for horizontal MF were utilized. In the laboratory, background 50 Hz MF was less than 0.08 μT when all necessary equipment was powered. Rat thymocytes were exposed to 0.1 mT horizontal or vertical field, or to 0.14 mT circularly polarized field for 30 min; the effects of consecutive exposures to vertical and horizontal MFs, or vice versa, for 20 min each also were examined. In addition, intact thymocytes or lectin-activated thymocytes, splenocytes and peripheral blood lymphocytes were exposed to the 5 mT vertical field for 30 min. In all these experiments, no alteration in $[Ca^{2+}]_i$ was evident. As a positive control, a calcium ionophore always was added to the medium following MF exposure to verify that the cells were capable of increasing $[Ca^{2+}]_i$ and the system would record the response. The data indicate that MF exposure had no effect on $[Ca^{2+}]_i$ under any of the conditions examined.

Key words: Magnetic Field Exposure, Power Frequency, Linearly or Circularly Polarized Field, Intracellular Calcium, Rat Immune Cells, Flow Cytometry, *In Vitro*

Introduction

Because epidemiological studies have suggested an association between magnetic field (MF) exposure and adverse health effects¹⁾, many *in vivo* and *in vitro* laboratory studies have been completed investigating the effects of MF^{2,3)}. At the cellular level, changes of intracellular calcium ($[Ca^{2+}]_i$) by MF exposure are often suggested. Control of $[Ca^{2+}]_i$ is a fundamental regulatory process affecting cell survival and function. Various important physiological activities, including actions of the endocrine system and the immune

system (e.g., activation of immune cells and antibody production), are regulated by $[Ca^{2+}]_i$.

Bawin *et al.*⁴⁾ and Blackman *et al.*⁵⁾ reported that electric field or MF exposure increased $^{45}Ca^{2+}$ efflux from embryonic brain tissue. Particular efforts have been devoted to the exposure regimen called cyclotron resonance, which Liboff *et al.*⁶⁾ demonstrated by measuring the calcium response *in vitro*. More recently, influx of $^{45}Ca^{2+}$ and changes in $[Ca^{2+}]_i$ have been reported from several laboratories. For example, using spectrofluorimetry, Liburdy⁷⁾ showed that a 60 Hz electric field exposure increased $[Ca^{2+}]_i$ of thymocytes, loaded with fura-2, stimulated with the mitogen concanavalin A (Con A). A microfluorometric experiment also indicated

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that the normal oscillatory changes in $[Ca^{2+}]_i$ were altered in a leukemic cell line exposed to MF⁸⁾.

There are suggested mechanisms to explain how MF interacts with *in vitro* biological systems. Alternating MF exposure induces alternating currents, called eddy currents, in the culture medium in which targeted cells are suspended or adhered. Because the plasma membrane interfaces with the medium, MF-induced currents could act on membranous channels and enzymes^{9, 10)}. The current might disturb these regulatory components, which eventually modifies the intrinsic nature of the cells. Another hypothesis is that the induced currents change the affinity of ligands to bind to membrane receptors¹¹⁾ that trigger intracellular signals. The signals are transmitted to membranous calcium channels, causing an increase in $[Ca^{2+}]_i$. Considering the fundamental roles that $[Ca^{2+}]_i$ is regulating a wide variety of cellular activities, MF exposure might create electrically modulated biological signals that could perturb normal cellular functions.

However, inability to reproducing the original findings of $[Ca^{2+}]_i$ changes were reported in literature^{12, 13)}. Other studies have demonstrated that MF exposure did, or did not, alter $[Ca^{2+}]_i$ or inhibit the $^{45}Ca^{2+}$ influx depending on the cellular status^{14, 15)}. Sontag¹⁶⁾ showed that an exposure of differentiated HL-60 cells to electric fields, 0 to 100 Hz, in a fluorescence spectrometer cuvette did not change $[Ca^{2+}]_i$. The Electric and Magnetic Fields Research and Public Information Dissemination Program (EMF RAPID) conducted by the Department of Energy and the National Institute of Environmental Health Sciences was initiated to examine the possible interactions between MF exposures and biological systems, including effects on $[Ca^{2+}]_i$. Based on this background, the authors chose $[Ca^{2+}]_i$ as the one of the cellular functions reported to be responsive to MF. The objective of this study was to clarify whether or not MF exposure changes $[Ca^{2+}]_i$.

In our study, freshly prepared immune cells in an incubator were exposed to linearly or circularly polarized MF, produced by a solenoid coil and a square coil, and $[Ca^{2+}]_i$ was measured with flow cytometry. We developed the temperature-controlled cell incubator specialized for flow cytometry to investigate $[Ca^{2+}]_i$ ¹⁷⁾. Flow cytometry detects changes of $[Ca^{2+}]_i$, the net of inward and outward calcium fluxes across cell membrane, in real-time because the fluorescent dye binds only the cytoplasmic free calcium, not the calcium in culture medium, adhering on cell surface, or stored in the endoplasmic reticulum. The fluorescent dye indo-1 AM penetrates the plasma membrane and changes its fluorescent intensity rate when it binds calcium. The acetoxymethyl

ester dye itself is not able to bind extracellular calcium in the medium, but hydrolysis by cytosolic esterases change the indo-1 AM to indo-1, the form that chelates calcium^{18, 19)}.

The solenoid coil used was fabricated in the first attempt to use the same type of coil in different laboratories for replication studies²⁰⁾; the cells exposed were the same types of cells that were employed in several studies suggesting MF effects on $[Ca^{2+}]_i$ ^{21, 22)}. Exposure times of 30 or 40 min and intensities of 0.1, 0.14, or 5 mT were used because they were comparable to the MF intensities and exposure durations used in other MF-calcium studies. Thirty minutes is longer than most of studies^{23, 24)} employing fluorescent dyes for measurement but the same as, or shorter than, studies using $^{45}Ca^{2+}$ ^{25–27)}. Linearly polarized MF produces time-varying (50 Hz) eddy currents in the medium, whereas circularly polarized MF induces constant eddy current²⁸⁾. The circularly polarized MF is an exposure characteristic that is suggested to cause melatonin reductions in rat plasma and pineal gland²⁹⁾. Thus we included circularly polarized MF in our test conditions.

International Non-ionizing Radiation Committee of the International Radiation Protection Association³⁰⁾ set the interim guidelines applied to human exposure based on the calculation that the induced current produced by continuous MF exposure should not exceed 10 mA/m² in the head and trunk when the whole body was exposed for a substantial part of the day. Thus 5 mT has been set as the limiting magnetic flux density for occupational, short-term exposure, and 0.1 mT was set as the limiting flux density for general public who can be exposed for up to 24 hr per day. The maximum induced current in our study of 167 mA/m² (5 mT, vertical MF) is about 16 times higher than the basis of the occupational guideline; in case of 0.1 mT vertical MF, the current experienced by the immune cells in this study was about one-third of the guideline.

Materials and Methods

Cell preparation

Thymus and spleen were aseptically excised from specific pathogen free (SPF), Sprague-Dawley rats (Japan SLC, Inc., Japan) 6 to 9 wks of age. Removed thymus glands were gently torn in ice-cold, calcium-magnesium free, phosphate-buffered-saline (PBS; Dalbecco 21600-069, USA) and filtered through 53 μ m stainless steel mesh. Meshed cells were suspended in PBS and washed then were centrifuged at 200 g and dispersed in PBS. This procedure was repeated twice. Total cell number was counted with hemocytometer.

Viability was determined by trypan blue exclusion test. The viability always was more than 96%. Viable cell concentration was adjusted to 5×10^6 cell/ml.

Splenocytes were collected by gentle tapping the spleen on 53 μm stainless steel mesh with PBS. Erythrocytes were lysed with 0.16 M of NH_4Cl -Tris buffer, pH 7.65. After lysis, cells were washed three times with PBS and then treated in the same way as the thymocytes.

To obtain peripheral blood lymphocytes (PBLs), rats were anesthetized lightly by inhalation of diethyl ether and whole blood was collected by intracardiac puncture. Heparinized blood was placed on separating solution (Sigma, HISTOPAQUE - 1077, USA) and centrifuged at 200 g for 20 min. Buffy coat was collected and the enriched cells were washed three times with PBS then counted.

Intracellular calcium measurement

Indo-1 AM (I-1223, Molecular Probes, Inc., USA) was utilized as a probe of detecting $[\text{Ca}^{2+}]_i$ change, and the assay was performed using a flow cytometer (EPICS ELITE, Coulter Co., USA), equipped with UV laser. Real-time $[\text{Ca}^{2+}]_i$ was expressed as the ratio of the fluorescent intensities at 381 and 535 nm. The cell incubator, which is described later, could hold a special annular glass-culture-dish that could contain 3 ml of cell medium, allowing flow cytometry measurement for more than an hour.

Fifty μg of indo-1 AM was first dissolved in 100 μl of dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries Ltd., Japan) and then the mixture was dissolved in 1400 μl of PBS. Two ml of cells at 5×10^6 cell/ml was loaded with 360 μl of the indo-1 AM solution at 37°C for 30 min. To remove the excess indo-1 AM, the cells were centrifuged, washed again and re-suspended, this time at 3×10^6 cell/ml in 3 ml of 37°C RPMI-1640 (Sigma, USA) containing 5%, heat-inactivated fetal bovine serum and 30 μl of 100 mM CaCl_2 .

In the series of MF exposure experiments, measurements were made for a total of 60 min, which included baseline, MF exposure, post-exposure, and positive control periods. In some cases, cells were stimulated with Con A prior to the MF exposure period.

Results were timecourse figures giving the ratio of fluorescence at 381 and 535 nm, which provided $[\text{Ca}^{2+}]_i$. Each fine dot on a timecourse figure represented cells that had the same $[\text{Ca}^{2+}]_i$. Because of the nature of flow cytometry, direct, in-line measurement of absolute $[\text{Ca}^{2+}]_i$ value was not feasible. Instead, at the end of each exposure, cells were treated with an ionophore (A23187; Sigma, USA) that served as a positive control inducing an increment of the $[\text{Ca}^{2+}]_i$ in the cells.

In a separate experiment calibration of indo-1 itself was performed using a commercial kit (Molecular Probes Inc., USA) and a fluorescent spectrophotometer (F-3000, Hitachi, Ltd., Japan). Calibration of $[\text{Ca}^{2+}]_i$ in intact cells also was conducted separately with a known method using ionophores^{31, 32}.

Cell incubator and culture dish

The authors designed and fabricated a temperature-controlled cell incubator (Fig. 1) that allows flow cytometry and can be placed within the type of solenoid coil used at Lawrence Berkeley Laboratory (LBL). The cylindrical incubator, which was made of MF-permeable, vinyl chloride, was 26 cm in height and 13 cm in diameter. Warm water for cell incubation was continuously provided with a remotely operating water bath and pump unit. When the room temperature was $23 \pm 2^\circ\text{C}$, the cell medium was at $37 \pm 0.1^\circ\text{C}$.

Fig. 1. Schematic drawing (side view) of a temperature-controlled, cell incubator for flow cytometry.

Cells were suspended in a culture dish covered with an airtight, screw lid that has nozzles for flow cytometry measurement. Cells were incubated with warm water that was remotely circulated by a heater and pump unit.

Use of a remote incubator was necessary to avoid any interfering MFs associated with the heater and pump. The lid had nozzles for cell sampling, pressured air, and reagent inlet. Cells could reach the flow cytometer within a second by passing from the incubator through a narrow Teflon tube.

An annular culture dish that was made of silicon-coated-glass and was equivalent to a commercial annular dish in size (Falcon 3037, Becton Dickinson and Company, USA), was placed in the incubator. The outer ring of the dish was used in these experiments to have the maximum induced current available by vertical MF exposure. The vessel was 0.8 cm wide by 1 cm in depth, and diameter of outer and inner portion of the vessel was 4.4 cm and 3.6 cm, respectively. To avoid use of a magnetic stirrer, the incubator and coils were placed on a rotating shaker (NR-3, TAITEC Co., Japan) set at 20 rpm shaking to prevent cell sedimentation and to stir the culture medium upon reagent administration.

Exposure coils

The exposure system consisted of a two-channel signal generator (FC-120, Yokogawa Electric Corporation, Japan), an ampere meter (65AG, Yokogawa Electric Corporation, Japan), two power amplifiers (#4015, #4505; NF Corporation, Japan), and two coils; one the LBL solenoid coil for vertical MF, and another, a multiple-bobbin-square coil for horizontal MF (Fig. 2). These coils were energized independently with different phases of alternating currents to create circularly polarized field. The circularly polarized MF consisted of superimposed horizontal and vertical fields at the same intensities but with their phases shifted by 90 degrees.

Details of the solenoid coil were described elsewhere²⁰. Briefly, coil length was 14 cm and diameter was 17 cm. It had 392 turns of enamel-coated, square lead-line that made 2.34 Ohms. The multiple-square coil was composed of five bobbins; each bobbin was aligned in horizontal direction with the same distance. The width, height, and length of the coil was 41 × 41 × 35 cm; turn number ratio of the bobbins was 11:2:4:2:11. The resistance was 2.4 Ohms. General characteristics of multiple coils were described by other researchers^{33, 34}.

Magnetic fields

MFs were 50 Hz sine-waves, and exposure was continuous. The coils were characterized using two flux density meters (MFM-12A, Shoden Corporation, Japan; HM-201, MTI Co., Ltd., Japan) at the location and with the configurations used in the exposure experiments. For MF at the culture dish site of up to 6 mT, the solenoid coil showed a linear

Fig. 2. Photograph of coils, cell incubator, and connected flow cytometer.

An outer square coil produced horizontal magnetic field (MF), and an inner solenoid produced vertical MF. Cell incubator was inside the solenoid coil, and this exposure system was placed on a rotating shaker.

relationship between current and MF; throughout this paper, the MF always is expressed in root mean square (rms) unit. At 50 Hz, a current of 2.7 A was required to produce 6 mT. Inside the coil, flux density varied 3% in the horizontal direction. At the culture dish site, the variation was 1% or less. Flux density, however, varied widely in the vertical direction. Because the most uniform MF existed in the middle of the coil, i.e., at a height of 5 to 9 cm, the culture dish site was placed at the 7 cm height.

The multiple-bobbin coil generated a uniform MF, with less than 2% in variation anywhere inside the coil. The uniformity was preserved at the actual culture dish site that was surrounded by the solenoid coil. Characterization also revealed a linear correlation between current and MF up to 0.2 mT. There was no appreciable heat, vibration, or noise associated with either coil currents.

Fig. 3. Increase of intracellular calcium ($[Ca^{2+}]_i$) of mouse thymocyte stimulated with anti-CD3epsilon monoclonal antibody, IgG crosslinking antibody, and calcium ionophore.

Real-time $[Ca^{2+}]_i$ is expressed as the ratio of the fluorescent intensities at 381 and 535 nm. Each fine dot on a timecourse figure represents cells that have the same $[Ca^{2+}]_i$. Arrow A indicates anti-CD3epsilon monoclonal antibody stimulation, arrow B indicates IgG crosslinking antibody stimulation, and arrow C indicates calcium ionophore treatment.

In the laboratory, background MF at 50 Hz was less than $0.08 \mu T$ when all necessary equipment was powered; otherwise it was less than $0.01 \mu T$. The geomagnetic field at the site was $42.3 \mu T$. The cell media had a conductivity of 10.6 mS/m at $37^\circ C$, as measured by a conductivity meter (Model SC82, Yokogawa Electric Corporation, Japan). When the 5 mT vertical MF was applied to cells, the induced current in the cell culture medium was calculated, by Faraday's law, to be 167 mA/m^2 , based on the measured conductivity. In case of 0.1 mT vertical field, the value was 3.33 mA/m^2 .

Results

System validation

First, our biological system was validated with calcium stimulation and exclusion experiments. Antibody and its cross-linking are known to increase $[Ca^{2+}]_i$ ³⁵. Both the primary antibody (anti-mouse CD3epsilon monoclonal antibody,

Fig. 4. Change in intracellular calcium ($[Ca^{2+}]_i$) of rat thymocyte exposed to 0.1 mT, horizontal magnetic field (MF) for 30 min followed by calcium ionophore treatment.

Real-time $[Ca^{2+}]_i$ was expressed as the ratio of the fluorescent intensities at 381 and 535 nm. Each fine dot on a timecourse figure represented cells that had the same $[Ca^{2+}]_i$. Arrow A indicates calcium ionophore treatment.

hamster IgG; 500A2, $5 \mu g/ml$; Pharmingen, USA) and the cross-linking antibody (anti-hamster IgG monoclonal antibody; G94-56, $10 \mu g/ml$; Pharmingen, USA) increased $[Ca^{2+}]_i$ in thymocytes from SPF, C57BL/6 male mice. The cells then were stimulated with 100 nM of ionophore A23187 (Sigma, USA); further increases in $[Ca^{2+}]_i$ were observed (Fig. 3).

In the resting state the normal cell contains extremely low concentration of $[Ca^{2+}]_i$ that can be released by calcium ionophore treatment in a calcium-free medium (PBS and EGTA (Dojindo, Japan)). This slight decrease in $[Ca^{2+}]_i$ of rat thymocytes was detectable with 100 nM A23187 treatment. These experiments demonstrated that the system was sensitive enough to detect the increase caused by anti-CD3 antibody and the small decrease of $[Ca^{2+}]_i$ leaked from the cells.

Horizontal or vertical MF exposures at 0.1 mT

The $[Ca^{2+}]_i$ of intact rat thymocytes was established during a 10-min baseline period (Fig. 4); then a 30 min MF exposure was followed by short post-exposure period. The gap at 46 min represents a short break in measurement because of the

Fig. 5. Change in intracellular calcium ($[Ca^{2+}]_i$) of rat thymocyte exposed to 0.1 mT, vertical magnetic field (MF) for 20 min and consecutive 0.1 mT, horizontal MF for 20 min followed by calcium ionophore treatment.

Real-time $[Ca^{2+}]_i$ was expressed as the ratio of the fluorescent intensities at 381 and 535 nm. Each fine dot on a timecourse figure represented cells that had the same $[Ca^{2+}]_i$. Arrow A indicates calcium ionophore treatment.

addition of ionophore, 1 μ M A23187. The ionophore treatment caused a robust elevation of $[Ca^{2+}]_i$. This demonstrates three things: (1) the cells kept their integrity and were able to increase $[Ca^{2+}]_i$ until the termination of the experiment, (2) the flow cytometry system was able to detect an increase in $[Ca^{2+}]_i$ when it occurred with the addition of ionophore, and (3) horizontal MF exposure for 30 min did not produce an increase in $[Ca^{2+}]_i$. Vertical MF exposure resulted in the same pattern as horizontal MF. The $[Ca^{2+}]_i$ was consistently low during the baseline, MF exposure, and post-exposure periods; then a pronounced rise occurred by positive control of 1 μ M ionomycin.

Consecutive exposures of vertical and horizontal MFs at 0.1 mT

Vertical and horizontal MFs were independently applied sequentially to thymocytes. Other than the MF orientation, the experimental approach was kept the same as that used in the previous experiments. Immediately after the 20-min

Fig. 6. Change in intracellular calcium ($[Ca^{2+}]_i$) of rat thymocyte exposed to 0.14 mT, circularly polarized magnetic field (MF) for 30 min followed by calcium ionophore treatment.

Real-time $[Ca^{2+}]_i$ was expressed as the ratio of the fluorescent intensities at 381 and 535 nm. Each fine dot on a timecourse figure represented cells that had the same $[Ca^{2+}]_i$. Arrow A indicates calcium ionophore treatment.

exposure to a 0.1 mT, vertical MF ended, a 20-min horizontal MF exposure of 0.1 mT was provided (Fig. 5). A 5-min post-exposure observation was made; then 1 μ M A23187 was added to the medium; this evoked a large increase in $[Ca^{2+}]_i$. In other experiments, horizontal MF exposure preceded vertical MF exposure. Once again, no $[Ca^{2+}]_i$ increase occurred until the positive control period.

Intact or stimulated thymocytes exposed to 0.14 mT, circularly polarized MF

Two orthogonal MFs of 0.1 mT were superimposed to make a 0.14 mT (resultant value), circularly polarized MF in which the phases of the horizontal and vertical MFs differ from each other by 90 degrees. Exposure was for 30 min, and the positive control was 500 nM A23187; cell types were the same as those used previously. Once again, MF exposure had no effect on the $[Ca^{2+}]_i$ of the exposed cells (Fig. 6).

Liburdy³⁶⁾ and Lindström *et al.*⁸⁾ examined the relationship between cellular status and effectiveness of exposure

Fig. 7. Change in intracellular calcium ($[Ca^{2+}]_i$) of stimulated rat thymocyte exposed to 0.14 mT, circularly polarized magnetic field (MF) for 30 min followed by calcium ionophore treatment.

Real-time $[Ca^{2+}]_i$ was expressed as the ratio of the fluorescent intensities at 381 and 535 nm. Each fine dot on a timecourse figure represented cells that had the same $[Ca^{2+}]_i$. Arrow A indicates concanavalin A stimulation, and arrow B indicates calcium ionophore treatment.

suggesting that stimulated cells exhibited a MF-induced calcium response but resting cells were insensitive to the MF exposure. To examine this possibility, thymocytes were stimulated, prior to baseline period, with a lectin, 2 μ g/ml of Con A (Sigma, USA). However, regardless of the pre-stimulation, exposure to circularly polarized MF did not change the $[Ca^{2+}]_i$ (Fig. 7).

Stimulated or non-stimulated thymocytes exposed to vertical MF at 5 mT

Non-stimulated and stimulated thymocytes were exposed to 5 mT vertical MF for 30 min. In the stimulated condition, 2 μ g/ml of Con A was added to the medium shortly before the initiation of baseline measurement; $[Ca^{2+}]_i$ was measured in baseline, MF exposure, post-exposure, and positive control periods as the same to previous experiments. MF exposure did not increase $[Ca^{2+}]_i$ in the Con A stimulated cells (Fig. 8).

In a separate experiment conducted using the same approach, the positive control was replaced by negative

Fig. 8. Change in intracellular calcium ($[Ca^{2+}]_i$) of stimulated rat thymocyte exposed to 5 mT, vertical magnetic field (MF) for 30 min followed by calcium ionophore treatment.

Real-time $[Ca^{2+}]_i$ was expressed as the ratio of the fluorescent intensities at 381 and 535 nm. Each fine dot on a timecourse figure represented cells that had the same $[Ca^{2+}]_i$. Arrow A indicates concanavalin A stimulation, and arrow B indicates calcium ionophore treatment.

control to verify that positive $[Ca^{2+}]_i$ response of the cells was not caused by the DMSO used as the solvent vehicle for the ionophore. Cells that showed no $[Ca^{2+}]_i$ alteration by the MF were treated with DMSO. The cells did not respond to the negative control.

Stimulated splenocytes or peripheral blood lymphocytes exposed to 5 mT, vertical MF

Two sets of Con A stimulated splenocytes and one set of PBLs were exposed to vertical 5 mT MF for 30 min followed by treatment with the positive control. In this series of experiments, all cells were stimulated by Con A during the baseline period. Although 2 μ g/ml of Con A added at 6 min increased the $[Ca^{2+}]_i$ of splenocytes, MF exposure did not alter the initial increment. PBL experiments demonstrated the same response pattern.

Summary of all experiments

Obtained results from nine different experimental

Table 1. Respose of intracellular calcium in cells exposed to magnetic fields

Exposure intensity (mT)	Characteristic of magnetic field	Exposure time (min)	Number of replications	Cell type	Response to magnetic field	Response to positive control
0.1	Horizontal	30	3	Thymocyte	Not Detected	Detected
0.1	Vertical	30	6	Thymocyte	Not Detected	Detected
0.1	Horizontal + Vertical	20 + 20	6	Thymocyte	Not Detected	Detected
0.14	Circularly	30	6	Thymocyte	Not Detected	Detected
0.14	Circularly	30	3	Stimulated thymocyte	Not Detected	Detected
5	Vertical	30	6	Thymocyte	Not Detected	Detected
5	Vertical	30	6	Stimulated thymocyte	Not Detected	Detected
5	Vertical	30	6	Stimulated splenocyte	Not Detected	Detected
5	Vertical	30	3	Stimulated peripheral blood lymphocyte	Not Detected	Detected

conditions of experiments were summarized in Table 1. In each condition, three to six replications were made to insure that observed response was consistent. In every experiment, MF exposure produced no measurable change in $[Ca^{2+}]_i$ but positive control treatment with ionophore produced a large increase in $[Ca^{2+}]_i$.

Discussion

In this study, freshly prepared thymocytes, splenocytes, and PBLs were exposed to 50 Hz, linearly or circularly polarized MF at 0.1, 0.14, or 5 mT for 30 to 40 min and $[Ca^{2+}]_i$ were examined. Under the conditions tested, MF exposures had no effect on $[Ca^{2+}]_i$.

In general, extracellular free calcium tends to adhere to glycoproteins over the cell surface. The possibility exists that MF-induced current in the medium somehow acts to increase opportunities for cations to encounter negatively charged residues of cell surface proteins. If this happens, increased $^{45}Ca^{2+}$ counting, from absorption rather than uptake, can occur when the extracellular calcium is radiolabeled. Another possibility is that influx of free calcium from the medium indeed was increased by MF exposure as measured with $^{45}Ca^{2+}$ and efflux to the medium or flux to the internal storage was concurrently and equivalently accelerated. The increases in Ca^{2+} fluxes might be attributed to the presence of magnetically induced current in cell culture media. Consequently, Ca^{2+} concentration could have remained at a constant level despite changes in the Ca^{2+} fluxes.

Although the patch-clamp method is the definitive way to monitor single cell response, the $[Ca^{2+}]_i$ response as a large cell population, such as in an organ, is beyond the scope of the method. Microscopic observation requires pre-

treatment and pre-selection of cells, as occur with cover slip attachment and appropriate rate of spontaneous calcium spiking activity. Limitations to the cell number that can be studied might lead to focusing on relatively a small group of cells that might not represent typical status of the original intact cells. However, numerous cells that are not methodologically selected can be measured with flow cytometry.

Presumably our experiments are not limited by lack of the sensitivity required to detect the reported effects. A study with indo-1 demonstrated that MF exposure induced a $[Ca^{2+}]_i$ response which was elevated 2 to 5 times higher in comparison with the baseline of unexposed cells³⁷⁾. Lindström *et al.* also indicated in their literature^{8, 24)} that the magnitude of MF-response in $[Ca^{2+}]_i$ was as intense as anti-CD3epsilon antibody stimulation. Some of their observations showed more than a 5-fold increase in $[Ca^{2+}]_i$ from baseline, a change which was about twice that produced by the antibody stimulation. However, there also were unresponsive cells that maintained a constant level of $[Ca^{2+}]_i$ despite MF exposure. These observations suggest two things: (1) a positive MF-response can occur, with the effect magnitude being, at least, as intense as anti-CD3epsilon antibody stimulation or larger, to occasionally 5-fold increase from the baseline, and (2) positive cells were apparently distinguishable from unresponding cells, suggesting either there were, at least, two subsets of cells or that the response for each cell was "on-or-off". The responses to anti-CD3epsilon mAb stimulation and ionophore treatment were clearly detected in our flow cytometry system. Detection of these increases in the fluorescence ratio implies that the system would have been able to detect a $[Ca^{2+}]_i$ elevation, if the MF-response had produced an increase similar to the

anti-CD3 antibody stimulation to a 5-fold increase from the baseline fluorescence intensity. Flow cytometry was designed to distinguish different cell characteristics from a group of heterogeneous cells so that existence of subsets in terms of $[Ca^{2+}]_i$ should be apparent with the assay, e.g., responders and non-responders after the stimulation of antibodies (Fig. 3 after arrows A and B). On the other hand, such subtype could make spectrofluorescent measurements rather insensitive because all subjective cells were suspended in a single cuvette and the reading gave a total fluorescence intensity.

There is accumulating evidence from different laboratories that $[Ca^{2+}]_i$ is not altered by MF exposures. With carefully designed exposure apparatus, Höjevik *et al.*³⁸⁾ demonstrated negative results in the $[Ca^{2+}]_i$ mobilization under resonance-tuned MF exposure. An ongoing calcium study³⁹⁾ from the EMF RAPID Program observed negative results differing from the original positive findings; they used an equivalent experimental set-up and the same type of cell line as had been used by Lindström *et al.*²⁴⁾. Lyle *et al.*⁴⁰⁾ suggested, based on experiments using flow cytometry and the same LBL solenoid coil that was employed in this study, that the lack of $[Ca^{2+}]_i$ change in the cells exposed to the induced electric field produced by a 60 Hz MF of 23.8 mT. In another study⁴¹⁾, they demonstrated that exposure of anti-CD3-stimulated Jurkat E6-1 cells to 0.15 mT, 60 Hz MF and 78.2 μ T DC MF did not affect $[Ca^{2+}]_i$ measured by flow cytometry with fluorescent dye, fluo-3. Also, Fritz *et al.*⁴²⁾ used the same LBL solenoid coil, our cell incubator, and their flow cytometry with an indo-1 system. They reported that their results suggest that oxidatively stressed cells do not show any changes in $[Ca^{2+}]_i$ in response to 0.1 mT MF exposure for 60 min. Taken together, these might be an implication that MF exposure could change fluxes of calcium or the rate of oscillatory spikes under specific experimental conditions, although the exposure did not alter net cytoplasmic free calcium concentration, i.e., $[Ca^{2+}]_i$, that could be biologically significant.

Conclusion

Our observation of results differing from positive calcium changes reported by other researchers could be attributable to differences in exposure equipment, MF dose, exposure time, cell type, or analytical protocol. This might suggest that MF exposure is effective only under the specifically described biological and electromagnetical conditions. Alternatively, the MF exposure might affect calcium fluxes

or oscillatory spikes, though it might not alter $[Ca^{2+}]_i$. The obtained results lead the authors of this paper to conclude that under the conditions evaluated, MF exposures did not change $[Ca^{2+}]_i$ in our experimental model.

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References

- 1) Savitz DA (1993) Overview of epidemiologic research on electric and magnetic fields and cancer. *Am Ind Hyg Assoc J* **54**, 197–204.
- 2) Anderson LE (1993) Biological effects of extremely low-frequency electromagnetic fields: *In vivo* studies. *Am Ind Hyg Assoc J* **54**, 186–96.
- 3) Cleary SF (1993) A review of *in vitro* studies: Low-frequency electromagnetic fields. *Am Ind Hyg Assoc J* **54**, 178–85.
- 4) Bawin SM, Adey WR, Sabbot IM (1978) Ionic factors in release of $^{45}Ca^{2+}$ from chicken cerebral tissue by electromagnetic fields. *Proc Natl Acad Sci USA* **75**, 6314–8.
- 5) Blackman CF, Benane SG, Kinney LS, Joines WT, House DE (1982) Effects of ELF fields on calcium-ion efflux from brain tissue *in vitro*. *Radiat Res* **92**, 510–20.
- 6) Liboff AR, Rozek RJ, Sherman ML, McLeod BR, Smith SD (1987) Ca^{2+} -45 cyclotron resonance in human lymphocytes. *J Bioelectr* **6**, 13–22.
- 7) Liburdy RP (1992) Calcium signaling in lymphocytes and ELF fields. Evidence for an electric field metric and a site of interaction involving the calcium ion channel. *FEBS Lett* **301**, 53–9.
- 8) Lindström E, Lindström P, Berglund A, Lundgren E, Mild KH (1995) Intracellular calcium oscillations in a T-cell line after exposure to extremely-low-frequency magnetic fields with variable frequencies and flux densities. *Bioelectromagnetics* **16**, 41–7.
- 9) Adey WR (1988) Cell membranes: The electromagnetic environment and cancer promotion. *Neurochem Res* **13**, 671–7.
- 10) Blank M, Soo L (1992) Threshold for inhibition of Na, K-ATPase by ELF alternating currents. *Bioelectromagnetics* **13**, 329–33.
- 11) Chiabrera A, Grattarola M, Viviani R (1984) Interaction

- between electromagnetic fields and cells: Microelectrophoretic effect on ligands and receptors. *Bioelectromagnetics* **5**, 173–91.
- 12) Parkinson WC, Hanks CT (1989) Search for cyclotron resonance in cells *in vitro*. *Bioelectromagnetics* **10**, 129–45.
 - 13) Prasad AV, Miller MW, Carstensen EL, Cox C, Azadniv M, Brayman AA (1991) Failure to reproduce increased calcium uptake in human lymphocytes at purported cyclotron resonance exposure conditions. *Radiat Environ Biophys* **30**, 305–20.
 - 14) Walieczech J, Budinger TF (1992) Pulsed magnetic field effects on calcium signaling in lymphocytes: dependence on cell status and field intensity. *FEBS Lett* **314**, 351–5.
 - 15) Coulton LA, Barker, AT (1993) Magnetic fields and intracellular calcium: effects on lymphocytes exposed to conditions for 'cyclotron resonance'. *Phys Med Biol* **38**, 347–60.
 - 16) Sontag W (1998) Action of extremely low frequency electric fields on the cytosolic calcium concentration of differentiated HL-60 cells: nonactivated cells. *Bioelectromagnetics* **19**, 32–40.
 - 17) Nishimura I, Shigemitsu T, Yamazaki K (1994) Characteristics of newly developed cell incubators and exposure coils for magnetic field study. In: *Project Abstracts of The Annual Review of Research on Biological Effects of Electric and Magnetic Fields from the Generation, Delivery & Use of Electricity*, P-9, 61.
 - 18) Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**, 3440–50.
 - 19) Cobbold PH, Rink TJ (1987) Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem J* **248**, 313–28.
 - 20) Liburdy RP, Morris JE, Blackman CF, Lyle DB, Sheppard A (1991) ELF *in vitro* group cooperative study: Objectives, goals, and first experimental plan. In: *Abstract Book of Bioelectromagnetics Society thirteenth Annual Meeting*, E-1-1.
 - 21) Walieczech J, Liburdy RP (1990) Nonthermal 60 Hz sinusoidal magnetic-field exposure enhances $^{45}\text{Ca}^{2+}$ uptake in rat thymocytes: dependence on mitogen activation. *FEBS Lett* **271**, 157–60.
 - 22) Yost MG, Liburdy RP (1992) Time-varying and static magnetic fields act in combination to alter calcium signal transduction in the lymphocyte. *FEBS Lett* **296**, 117–22.
 - 23) Carson JJJ, Prato FS, Drost DJ, Diesebourg LD, Dixon SJ (1990) Time-varying magnetic fields increase cytosolic free Ca^{2+} in HL-60 cells. *Am J Physiol* **259**, C687–92.
 - 24) Lindström E, Lindström P, Berglund A, Mild KH, Lundgren E (1993) Intracellular calcium oscillations induced in a T-cell line by a weak 50 Hz magnetic field. *J Cell Physiol* **156**, 395–8.
 - 25) Lyle DB, Wang X, Ayotte RD, Sheppard AR, Adey WR (1991) Calcium uptake by leukemic and normal T-Lymphocytes exposed to low frequency magnetic fields. *Bioelectromagnetics* **12**, 145–56.
 - 26) Liburdy RP, Callahan DE, Harland J, Dunham E, Sloma TR, Yaswen P (1993) Experimental evidence for 60 Hz magnetic fields operating through the signal transduction cascade. *FEBS Lett* **334**, 301–8.
 - 27) Fitzsimmons RJ, Ryaby JT, Magee FP, Baylink DJ (1994) Combined magnetic fields increased net calcium flux in bone cells. *Calcif Tissue Int* **55**, 376–80.
 - 28) Misakian M (1991) *In vitro* exposure parameters with linearly and circularly polarized ELF magnetic fields. *Bioelectromagnetics* **12**, 377–81.
 - 29) Kato M, Honma K, Shigemitsu T, Shiga Y (1993) Effects of exposure to a circularly polarized 50-Hz magnetic field on plasma and pineal melatonin levels in rats. *Bioelectromagnetics* **14**, 97–106.
 - 30) International Non-ionizing Radiation Committee of the International Radiation Protection Association (1990) Interim guidelines on limits of exposure to 50/60 Hz electric and magnetic fields. *Health Phys* **58**, 113–22.
 - 31) Shapiro HM (1998) Optical probes of intracellular calcium, In: *Practical flow cytometry*. eds. by Alan R, 192–4, Liss Inc., New York.
 - 32) June CH, Rabinovitch PS (1990) Flow cytometric measurement of intracellular ionized calcium in single cells with indo-1 and fluo-3. In: *Methods in cell biology*, vol. 33, *Flow Cytometry*, eds. by Darzynkiewicz Z, Crissman HA, 37–58, Academic Press Inc., San Diego, California.
 - 33) Kirschvink JL (1992) Uniform magnetic fields and double-wrapped coil systems: Improved techniques for the design of bioelectromagnetic experiments. *Bioelectromagnetics* **13**, 401–11.
 - 34) Shigemitsu T, Takeshita K, Shiga Y, Kato M (1993) 50-Hz magnetic field exposure system for small animals. *Bioelectromagnetics* **14**, 107–16.
 - 35) Ledbetter JA, June CH, Grosmaire LS, Rabinovitch PS (1987) Crosslinking of surface antigens causes

- mobilization of intracellular ionized calcium in T lymphocytes. *Proc Natl Acad Sci USA* **84**, 1384–8.
- 36) Liburdy RP (1992) Biological interactions of cellular systems with time-varying magnetic fields. In: *Biological effects and safety aspects of nuclear magnetic resonance imaging and spectroscopy*, Volume 649 of the *Annals of the New York Academy of Sciences*, March **31**, 74–95.
- 37) Barbier E, Dufy B, Veyret B (1996) Stimulation of Ca^{2+} influx in rat pituitary cells under exposure to a 50 Hz magnetic field. *Bioelectromagnetics* **17**, 303–11.
- 38) Höjevik P, Sandblom J, Galt S, Hamnerius Y (1995) Ca^{2+} ion transport through patch-clamped cells exposed to magnetic fields. *Bioelectromagnetics* **16**, 33–40.
- 39) Wey HE, Conover DL, Mathias P, Toraason MA, Lotz WG (1997) Studies of magnetic field effects on intracellular calcium in Jurkat cells, In: *Abstract Book of The Second World Congress for Electricity and Magnetism in Biology and Medicine*, P-167-B, 263.
- 40) Lyle DB, Doshi J, Fuchs TA, Casamento JP, Sei Y, Swicord ML (1992) Intracellular calcium signaling by human T-leukemic cells exposed to an induced 1 mV/cm 60 Hz, sinusoidal electric field. In: *Abstract Book of The First World Congress for Electricity and Magnetism in Biology and Medicine*, B-7.
- 41) Lyle DB, Fuchs TA, Casamento JP, Davis CC, Swicord ML (1997) Intracellular calcium signaling by Jurkat T-lymphocytes exposed to a 60 Hz magnetic field. *Bioelectromagnetics* **18**, 439–45.
- 42) Fritz LK, Morris JE, Stevens RG, Thrall BD, Anderson LE (1996) Investigation of EMF-induced calcium mobility in oxidatively stressed HL-60 cells. In: *The Abstract Book of Bioelectromagnetics Society Eighteenth Annual Meeting*, P-37A.