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# EFFECTS OF ELECTROMAGNETIC FIELDS ON SIGNAL TRANSDUCTION PATHWAYS LEADING TO HL-60 CELL DIFFERENTIATION

BY

QI TAO

A dissertation submitted to the Graduate faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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12/20/99
Date

Chair of Examining Committee
Dr. Ann Henderson, Hunter College

1/13/00
Date

Executive Officer
Dr. Richard L. Chappell

Dr. Shirley Raps, Hunter College

Dr. David Foster, Hunter College

Dr. Martin Blank, Columbia University

Dr. Reba Goodman, Columbia University

Supervising Committee

The City University of New York

**Abstract**

EFFECTS OF ELECTROMAGNETIC FIELDS ON SIGNAL TRANSDUCTION PATHWAYS LEADING TO HL-60 CELL DIFFERENTIATION

By

Qi Tao

Advisor: Professor Ann S. Henderson

The purpose of this study was to examine the hypothesis that cancer-related changes could occur in cells exposed to extremely low frequency electromagnetic fields (elf-EM fields). The research was designed to provide a means of simply confirming or negating a connection between electromagnetic field exposure and cell responses. Several studies were carried out. The first study showed that low energy electromagnetic field exposure promotes cellular differentiation in HL-60 cells in a manner similar to treatment with a known tumor promoter, TPA. The results using microscopic analyses demonstrated that similar morphological changes appeared following either EM field exposure or TPA treatment. Result of studies that used flow cytometry analyses showed a significant increase in the number of phagocytic cells that was observed after HL-60 cells were exposed to 1 G and 2G EM field at 24 hours. There is a linear dose-response in HL-60 cells to low concentrations of TPA as low as 50-250 pg/ml. The relative effect of a 1 G EM field on HL-60 cells at 24 hours is approximately equivalent to that of 250-500 pg/ml TPA. The data also showed that there was an additive effect when EM field exposure and TPA were used together at lower concentrations of TPA (50-500 pg/ml). The additive effect declined as the TPA

concentration was increased, *i.e.*, the effects were swamped out by TPA at concentration of 1 ng/ml and above. This study suggests that EM fields and TPA could share common transduction pathways.

The second part of the study was designed to determine if exposure to EM fields affects protein kinase C (PKC) membrane translocation. TPA can cause significant PKC-α translocation and total PKC-α is down-regulated. Cells exposed to 60 mG EM fields showed changes in PKC translocation, but the differences were not statistically significant. Cells exposed to 1 G EM field exposure showed a trend that was consistent with PKC translocation. However, at most time points, the increase was not significant. Total PKC-α is not down-regulated in long term-EM field exposure. This suggest that either PKC is unaffected by EM exposure or that the relative effect of an EM field is very low.

This study implies that both EM field exposure and TPA treatment share the ability to initiate a set of common consequences in cell differentiation and molecular signal transduction. PKC, however, responds to TPA treatment and EM field exposure differently. The insignificant changes of PKC translocation in cells exposed to EM field implies that alternative signaling pathways, *i.e.*, cell surface membrane tyrosine kinase receptors may be involved.

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# Introduction

## I. Background

It has been known for some time that ultraviolet (UV) irradiation and exposure to ionizing radiation can cause various types of cancer ranging from skin carcinomas to leukemia (Beissert *et al.*, 1996). The results of exposure to EM fields of extremely short wave length, high frequency and high energy can directly damage DNA structure leading to carcinogenesis (Goodman *et al.*, 1995). At the lower end of the electromagnetic spectrum, the consequences of exposure are more debatable. The possible negative health effects of extremely low frequency electromagnetic fields (elf-EM fields, ≤ 3000 Hz), produced from such sources as high voltage power lines, daily appliances, video display terminals and cellular phones have driven scientists to broaden the range of the studies (Figure 1).

The negative health effects of elf-EM field studied by epidemiological studies suggest a significant linkage between increased acute childhood leukemia and EM field exposures resulting from unusual power line configurations (Creasey *et al.*, 1990). The presence of extenuating environmental factors makes interpretation of the effects of EM fields exposure difficult, however, and the evidence has been inconsistent and controversial. Further laboratory investigations have not provided enough convincible cause-effect evidence to confirm the linkage between elf-EM field exposure and the increased cancer incidence (McCann *et al.*, 1993).

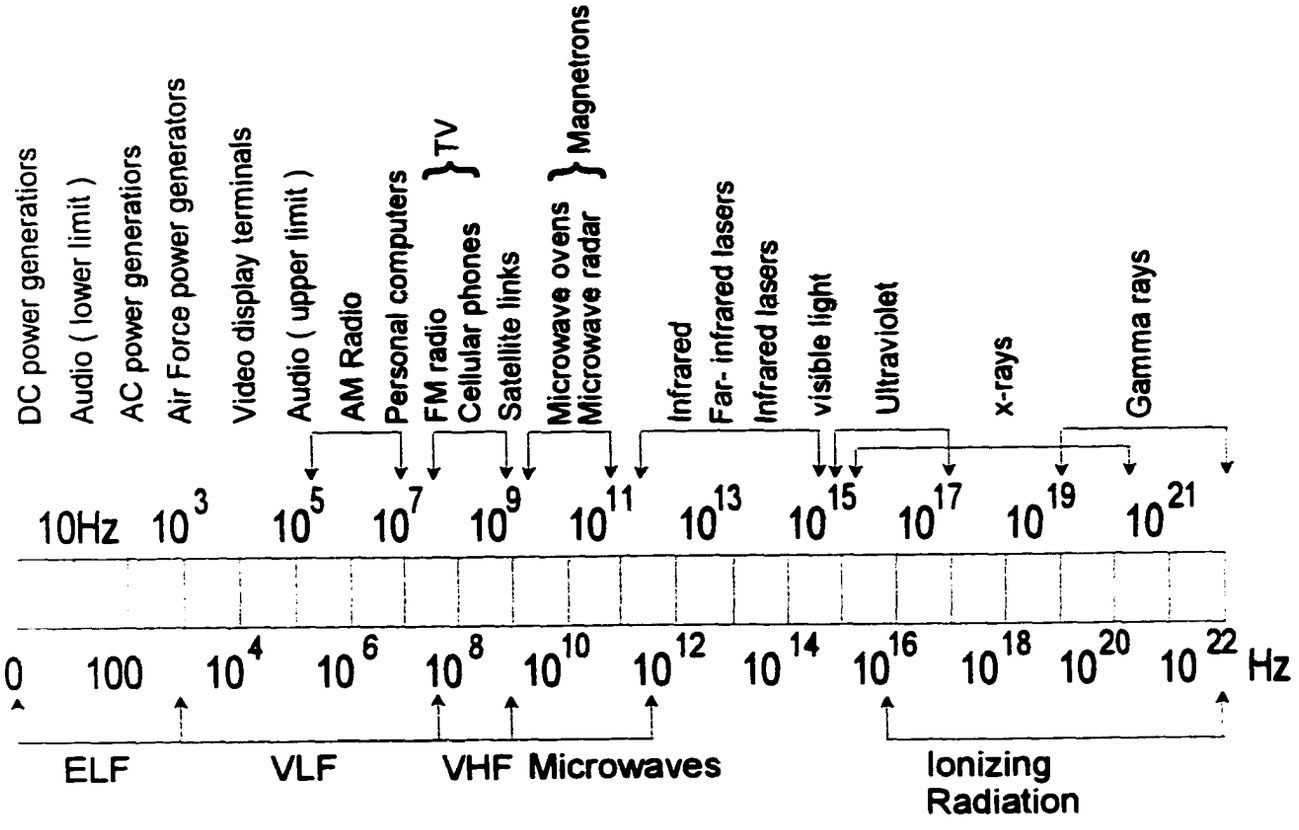
The results from using cell cultures and animal models, imply that elf-EM field exposure can cause diverse biological effects. Yet the underlying mechanisms are still not clear.

To further study the effects to elf-EM field exposure on living systems, it is necessary to re-evaluate previous findings including epidemiological, molecular, biological and physical

Figure 1. Spectrum of Electromagnetic Fields

Examples of typical field strengths from various power sources. X-rays, visible light, microwaves, radio waves, and Electric and magnetic fields (EM fields) are all forms of electromagnetic energy. EM fields are produced by power lines, electrical wiring, and electrical equipment. Electric fields are produced by voltage, magnetic fields result from the flow of current through wires or electrical devices. Electric fields are shielded or weakened by materials that conduct electricity. Magnetic fields pass through most materials and are difficult to shield. Both electric and magnetic fields decrease as the distance from the source increases. Abbreviations: ELF, extremely low frequency; VLF, very low frequency; VHF, very high frequency.

# ELECTROMAGNETIC SPECTRUM



studies. The evaluation done by the International Agency for Research on Cancer (IARC) in 1998 concluded that elf-EM field is a possible carcinogen based on limited evidence, mainly epidemiological studies. One conclusion from the National Academy of Science (NAS) review[1] and extensive evaluation by National Institute of Environmental Health Sciences (NIEHS) was that notably induced changes in cultured cells exposed to EM fields at 50-60 Hz could be related to signal transduction events only at field strengths that far exceed typical residential field strengths (in particular, fields greater than 50 mG). One goal of the proposed research is to verify this conclusion to determine if elf-EM fields exposure could cause the cell responses and to verify the underlying mechanisms. Scientists have long suspected that a possible linkage between elf-EM field exposure and carcinogenesis could be due to EM field-cell surface interaction and the consequential activation of signal amplification pathways (Adey, 1983).

**Epidemiological Studies-- EM fields and public health**

Epidemiological studies resulted in the first caution to the public about health and elf-EM fields exposure. An epidemiological study conducted by Wertheimer and Leeper (1979) reported that an increased incidence of acute childhood leukemia was related to residential exposure resulting from higher magnetic fields emitted from unusual power line configurations (odds ratio >2)[2]. The investigators estimated the magnetic fields using wire coding, or on the basis of the

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1. "Possible Health Effects of Exposure to Residential Electric and Magnetic Fields" (NRC-NAS, October 31, 1996); "Assessment of Health Effects from Exposure to Power-Line Frequency Electric and Magnetic Fields" NIEHS Working Group Report, NIEHS, report to Congress, 1999

2. Odds ratio: ratio of the incidence rates of the disease in the exposure group to those in the control group.

characteristics of the power lines visible from outside of the home and the distance between the power lines and the home. Wire code configurations were developed as a surrogate method of estimating long-term exposure to EM fields from nearby distribution lines. The coding was classified as high-current and low-current configurations. In this case-control study, cancer mortality was examined from death certificates for residents of Denver, Colorado who died at less than 19 years of age during 1950-73. A total of 344 cases of childhood cancer was identified, and 344 population controls were selected from Denver area birth certificates matched on the basis of birth month and county. Exposure was assessed from the wire coding of the homes occupied at the time of birth and occupied at the time of death. The equivalent information for controls was not available. They noted that children who died of cancer were more likely to have lived in homes classified as high-current configuration than in homes classified as low-current configuration.

Savitz *et al.* (1988) conducted a case-control study of residential exposure in the same area as that studied by Wertheimer and Leeper (1979). They studied Denver residents under 15 years of age and assessed the exposure in homes occupied at the time of cancer diagnosis and two years before diagnosis. Cases were obtained from population-based cancer registries and hospital records for the years 1976-83. A total of 356 appropriate childhood cancer cases were found, 278 population controls were also identified. Exposure in the home occupied at the time of diagnosis was assessed by the binary wire coding scheme of Wertheimer and Leeper (1979), and by spot measurements of EM fields taken 1-9 years after diagnosis. The relative risk for all cancers among children living in a home in the high-current classification was 1.5 (95% CI, 1.0-2.3).

Wiring configurations was analyzed to elucidate a dose-response relationship. The

estimated relative risks tend to increase in a linear fashion up to an almost tripling of risk in the highest exposure category of 2.8 (95% CI, 0.9-8.4), representing a statistically significant linear trend. For leukemia, a relative risk of 1.5 (95% CI, 0.9-2.6) was reported when comparisons were made between high and low-current classifications. Spot measurements taken inside the residence showed an association between wire codes and magnetic fields, but no association between wire codes and electric fields were found.

London *et al.* (1991) conducted a case-control study of 232 cases and 232 population controls in 1991. They focused strictly on the incidence of childhood leukemia, and assessed exposure in selected homes. The population base in this study consisted of all children under 10 years of age living in Los Angeles County during the years 1980-87. Exposure was assessed by three methods, a five-level wire code configuration, spot measurements in children's bedroom and 24 hour measurements of magnetic fields under the bed in the child's room. The relative risk for leukemia in relationship to wire code configurations (high- versus low- current) was 1.7 (95% CI, 1.1-2.5). With a five-level wire coding, the estimated relative risks tended to increase in a linear fashion (a statistically significant linear trend) up to more than a doubling of risk in the highest exposure category 2.2 (95% CI, 1.1-4.3). The result with 24 hours magnetic field measurements indicated an increased risk only for the highest cut-point used (≥2.7 mG) with a relative risk of 1.5 (95% CI, 0.7-3.3).

After Wertheimer and Leeper (1979) suggested that residential exposure to magnetic field might be carcinogenic, further epidemiological investigations of the correlation between workplace exposure to EM fields and various diseases began. The early studies were based on job titles, but lacked measurement-based validation. After a number of these studies showed

association between cancer and work in an electrical environment, all further studies included measurements of exposure to elf-EM fields. Sahl *et al.* (1993) studied 36,000 electric utility workers who worked for at least one year between 1960 and 1988 in California. Workers were classified as "electric" and "non-electric" workers. Electrical workers were defined as "craft occupations who work near energized equipment." The age adjusted relative risk (RR)[3] for electrical occupations was calculated for all cancers combined (a total of 261) and was not significant 1.1 (95% CI, 0.92-1.3). Further studies conducted nested case-controls in the cohort (comprising 44 cases of leukemia)[4]. The odds ratio for leukemia was close to one. It was also one in 32 cases of brain cancer in a nested case-control study.

A Canadian and French cohort research, led by Thériault *et al.* (1994) conducted a study of 223,292 workers at two large utilities in Canada and a national utility in France. A nested case-control approach within these three was to study all cancers combined among the male population of the three companies. The estimated exposure to magnetic fields was determined based on the combined job history of each case and control with time-weighted average (TWA) exposure to magnetic fields from a job-exposure matrix (JEM) for each job. No association was found between the risk for all cancers combined and cumulative exposure to elf-EM fields. However, in analyses of specific leukemia subtypes, only the risk for acute non-lymphocytic leukemia was found to be related to exposure to magnetic fields 2.4 (95% CI, 1.1-5.4) among workers with TWA exposures above the median compared to those below the median. For other leukemia subtypes,

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3. Relative risk: ratio of the incidence rates of the disease in the exposure group and those in the control group

4. Nested case controls: groups of individuals are assembled on the basis of outcome and their previous experiences with various exposures are compared.

no significant associations were reported.

In summary, epidemiological studies suggest that there existed a possible linkage between residential EM fields exposure and specific types of cancer. Surrogate wire codes were considered to be of sufficient quality to be used in evaluation of the association between the incidence of childhood leukemia and exposure to magnetic fields. Investigations testing occupational exposure to EM fields also showed major changes in the incidence of chronic lymphocytic leukemia, but no significant change in other cancers.

**Physical Studies**

Physical principles do not favor a clear explanation of biological and health effects of elf-EM field exposure. The cell-EM field interaction is extremely complex, dynamic in nature, and involves multiple levels of organization in organisms. Biological systems are electrically very complex and have widely ranging conductivities and dielectric properties.

Physical interaction mechanisms have been proposed to explain the possible biological effects of EM fields. But controversy arises in that the field strengths and resulting forces associated with exposure are relatively very small and the weak signals produced by field exposure must compete with the noisy background of endogenous electric fields and normal thermal fluctuation.

Studies that address theoretical signal-to-noise were conducted by Weaver *et al.* (1990). They examined the thermodynamic constraints that would be imposed on EM field-cell membrane interactions by the thermal noise associated with random membrane fluctuations in a single cell. They calculated that an electric field intensity of about 1 mV/cm would be able to influence cells,

even in the absence of biological amplification mechanisms. Provided that field effects are frequency specific and taking into account signal average, their computations predicted that a field as weak as 0.001-0.01 mV/cm could still significantly affect membrane-associated proteins such as receptors, enzymes, or channels without violating the thermal noise limit. In most reported biological experiments, the estimated electric fields are around this computed value. More recent theoretical analyses have demonstrated that spatial and temporal averaging processes (*e.g.*, arrays of ion channels, synchronized cell populations, multiple receptors, and charged species on cell membranes) substantially reduce the predicted detection thresholds.

Blank *et al.* (1992a) tested the effects of electric fields on the Na/K ATPase ion pump in cell membranes. They reported that electric fields of 30-300 Hz applied to membrane preparations at a current density of 55 uA/cm$^2$ (1.1 uV/cm) can cause either increases or decreases in enzyme activity depending on the concentrations of sodium and potassium ions in the medium. They estimated that the threshold for effects was magnetic field strength of approximately 5 uV/cm across the membrane, and this threshold value, although low by comparison with ambient electric fields in air near power lines, is much higher than those believed to be induced by environmental exposure to electric fields.

It has been proposed that nonlinear, nonequilibrium phenomena play an important role in biological systems (Adey *et al.*, 1975, 1981, 1983). This interpretation implies that biological systems are sensitive to initial conditions. Subtle changes in one or more parameters can significantly alter the state of the system. The phenomena assumes the possible occurrence of stochastic resonance (McNamara *et al.*,1993). The output signal-to-noise ratio improves at first with increasing input noise before it decreases as the noise is further increased. The application

of this theory is based on a threshold triggered systems in which a weak, below-threshold signal is raised above the threshold by the presence of noise (Moss *et al.*, 1995).

The complexity of living tissue makes the explanation of the effect of low energy elf-EM fields exposure on living systems debatable. Adey's nonlinear, nonequilibrium theory and other calculable implications such as stochastic resonance, the enhancement of the output signal/noise ratio, the sensitivity of biological system and the ion channel related membrane enzyme activities provide other plausible explanations of the effects of EM fields exposure.

**Clinical Studies**

Epidemiological investigations suggesting higher risks of certain cancers with EM fields exposure raised concerns about EM field related environmental carcinogenesis. The results from clinical studies, however, are less controversial. Before any reports of the possible negative health effects of elf-EM field, it had been demonstrated that electric and magnetic fields are clinically useful in accelerating bone fracture non-unions, bone remodeling in osteoporosis and in retarding a vascular degeneration present in a vascular necrosis of the head of the femur (Bassett, 1990).

Bassett *et al.* (1982) reported that pulsing magnetic fields (approved by the FDA in 1979), can successfully treat ununited fractures and failed arthrodeses. Treatment with pulsing electromagnetic fields (PEMF) was effective in 75% of 332 patients with an average 4.7-year disability duration and a mean of 3.4 previous operative failures. In 84% of carpal navicular and 82% femoral neck-reochanteric nonunions, they united after treatment. After attempted arthrodeses could not salvage a failed total-knee prosthesis, PEMF promoted healing in 85% of patients. Bassett *et al.* (1982) found that pulsing electromagnetic fields induce weak electric

currents in bone through the use of external coils on casts or skin.

Ottani *et al.* (1988) reported studies on the effects of pulsing extremely-low-frequency magnetic fields on skin wounds in the rat. In his studies, rats with skin-wounds surgically created on their backs were exposed immediately after surgery and every 12 hours thereafter to pulsed, extremely-low-frequency magnetic fields (50 Hz, 80 G peak). The rate of healing of skin wounds was evaluated microscopically and by light and electron microscopy at 6, 12, 21 and 42 days after operations. A significant increase in the rate of wound contraction was found in rats treated with magnetic fields. Forty-two days after surgery, all exposed animals showed fully closed wounds, while control rats at the same time intervals still lacked a final 6% of the wound surface to be covered. Exposed rats showed earlier cellular organization, collagen formation and maturation, and a remarkable feature of the very early appearance of a newly formed vascular network. The collagen fibers underwent a faster maturation in the extracellular matrix which represented a new aspect of the effects that magnetic fields may affect on the regeneration of soft tissues.

The beneficial effects of PEMF and elf-PEMF on nonunited bone fracture and skin wounds provided evidence that the effects of EM fields can be observed physically, and that there are no adverse side effects resulting from exposure to electromagnetic fields relevant to bone repair.

**Melatonin Studies**

It has been reported that melatonin can slow the growth of some cancer cells, including breast cancer cells (Blask, 1984). A proposed relationship was made between reduced serum melatonin and EM field exposure by Stevens *et al.* (1997) to account for the linkage of residential exposure to EM fields and increased cancer risks.

Laboratory experiments supported the proposal that EM field exposure alters melatonin levels in hamsters and rats. Reiter *et al.* (1988) reported that electric field exposure reduced the night pineal melatonin levels in Sprague-Dawley rats. Groups of six to eight pregnant rats were exposed throughout gestation, and their pups were exposed from birth to day 23. The exposures was to 0, 10, 65, or 130 kV/m linearly polarized 60 Hz electric fields for 19 hours/day by a parallel plate system, with the rats in electrical contact with the reference ground electrode. Significant decreases in pineal melatonin were found at 2 hours after lights were off ($p \leq 0.001$), when the concentration of melatonin would be expected to be highest. Wilson *et al.* (1981) and Grota *et al.* (1994) also found decreased night serum or pineal melatonin in electric fields exposed Sprague-Dawley rats.

Other studies were done which ranged from short-term to long-term exposure to magnetic fields. Kato *et al.* (1993) reported that six-week exposure to circular polarized 50 Hz magnetic fields (1-10 G) from a modified Helmholtz coil can reduce the nocturnal pineal or serum concentrations of melatonin in male Wistar-King rats. In a further study, no significant change in plasma melatonin or pineal melatonin concentration was found (Kato *et al.*, 1994). However, a series of laboratory and environmental measurement of the blood concentration of melatonin and its major urinary metabolite in human, showed no overall effect of EM field on melatonin levels during laboratory studies and environmental studies (Wilson *et al.*, 1990; Graham *et al.*, 1996; Selmaoui *et al.*, 1996; Burch *et al.*, 1998).

Liburdy *et al.* (1993) demonstrated that growth inhibition of melatonin on human estrogen-responsive breast cancer cells (MCF-7) was blocked by exposure to a 60 Hz, 12 mG sinusoidal magnetic field when melatonin was present at physiological concentrations (1 nM). A field of 2

mG (essentially background levels) had no significant effect. Growth inhibition was not affected in the absence of melatonin. The cells grow rapidly in the presence of normal concentrations of estrogens, but the growth rate is decreased in the presence of melatonin. Thus, there is evidence that EM fields may affect the growth of cancer cells at the cellular level involving receptor and signal transduction. Replication experiments reported similar effects of 12 mG fields on inhibition of MCF-7 cell growth (Blackman *et al.*, 1998). Luben *et al.* (1996) in their replication study of 12 mG EM field effects on melatonin responses of MCF-7 breast cancer cells showed that an 18% increase in growth and a net 28% difference between the cell growth could not be accounted for by differences in cell viability, cell density, or incubator location. The replication study done by Blackman *et al.* (1999) reported that the cell numbers were significantly reduced by 16.6% in the melatonin-treated cultures after 7 days of incubation compared to control culture. In the presence of 12 mG, 60 Hz magnetic fields, the melatonin treated, culture had the similar cell numbers as the control cultures. These results are consistent with the Liburdy observation of 17.1% reduction in cell number in the melatonin-treated cells, compared to controls, and an elimination of this growth reduction upon magnetic field exposure. In another study, Tamoxifen reduced the cell growth by 18.6% and 25% on days 6 and 7, respectively, while in 12 mG fields, tamoxifen reduced the cell growth only by8.7% and 13.1%, respectively (Blackman *et al.*, 1999). Tamoxifen is used clinically to manage estrogen-positive breast cancer. Tamoxifen competitively binds to estrogen receptors and reduces estrogen-induced proliferation stimuli in breast cancer cells (MacGregor and Jordan, 1998). This experiment indicates that EM fields may affect cellular surface receptors.

There are no overall reduced pineal and serum melatonin levels that can be observed in

human studies. The effects of exposure to electric and/or magnetic fields on blood concentrations of melatonin and its major urinary metabolite 6-hydroxymelatonin sulfate (6-OHMS) in humans have been examined. All-night exposure of human volunteers to magnetic fields under controlled exposure and lighting conditions in the laboratory had no apparent effect on nocturnal blood concentrations of melatonin when compare with equivalent sham-exposure conditions. This phenomenon may be explained as the easy measurable alterations of melatonin in rats and the individualized variation in human melatonin levels. The blockage of growth inhibition in MCF-7 cells by EM field exposure suggested the underlying environmental-level field interactions could happen between cellular receptors and magnetic fields.

**Promotion and Co-promotion**

Carcinogenesis is a multi-step and multifactorial process. Due to the lack of evidence that elf-EM field exposure can cause genetic changes, researchers began to consider the possibility of alternative roles for EM fields in tumor promotion and/or co-promotion.

Skin tumor development in mice is a well-accepted, convenient model for the study of multistage carcinogenesis. McLean *et al.* (1991) described results in which SENCAR mice were previously exposed to a sub-carcinogenic dose of 7,12-dimethyl benzanthracene (DMBA), then exposed and sham exposed to 60 Hz, 20 G magnetic fields. None of the mice developed papillomas following 20 G magnetic field exposure alone. In another group of mice, a sub-carcinogenic dose of DMBA applied to the dorsal skin of mice was followed at weekly intervals by an application of TPA. These mice were then exposed or sham exposed to an EM field. A slightly earlier development of tumors was observed in animals exposed to both the magnetic field

and TPA, but the difference in the time of onset was not statistically significant. In the TPA and EM field treated group of mice, the observations also included an enlarged spleen, a greater number of mononuclear cells in spleens and depression in NK-cell activity. This indicated that development of tumors may be due to the suppression of the immune surveillance system. However, the results from other two studies were not consistent (Rannug *et al.*, 1993; Rannug *et al.* 1994; Sasset *et al.*, 1998).

More direct effects related to tumor promotion have been described. One conjecture is that chronic exposure to a 60 Hz EM field and a chemical tumor promoter disrupt communication between normal and transformed cells and influence membrane-related events. This is based on experimental evidence which showed that EM field (60 Hz, 1G) alone did not increase focus formation in C3H/10$T_{1/2}$ cells (transformed/normal), but it could co-promote with TPA to increase focus formation in co-culture. Cain *et al.* (1993) conducted this experiment. In his experiment, disruption of communication between transformed cells and normal cells was assumed to be involved in tumor promotion. Parental C3H/10$T_{1/2}$ cells suppress growth of the transformed UV-TDTx10e fibroblast cells by preventing 10e cells from growing in multilayered foci, as occurs when 10e cells grow alone. The tumor promoter TPA promotes focus formation of mutant 10e cells by relieving the suppression imposed by C3H/10$T_{1/2}$ cells. They tested the co-promotion effect of EM field on TPA-induced focus formation in co-culture of C3H/10$T_{1/2}$ cells with transformed UV-TDTx10e fibroblast cells. One hour of a 60-Hz magnetic field exposure at 1 G, four times daily for 28 days, neither affected the growth curves of C3H/10$T_{1/2}$ and of UV-TDTx10e nor promoted focus formation of UV-TDTx10e in co-culture with C3H/10$T_{1/2}$ in the absence of TPA. However, video image analyses of experiments showed that in field-exposed

cultures, there was an increased TPA-induced focus formation (by 1.5-2.0 fold), as well as an increase in the size of the foci. Focus formation arose more rapidly in field exposure cultures with increasing TPA concentration at 10-100 ng/ml. The investigators suggested that magnetic field exposures may involve membrane-related events. EM field exposure may enhance the ability of TPA to disrupt gap junction communication and eventually lead to co-promotion of focus formation.

Based on skin tumor development and foci formation experiments, the results do not favor the proposal that electromagnetic field exposure alone can promote tumor promotion. They do leave space to consider co-promotion of magnetic fields with a known tumor promoter. In another words, the focus formation experiment suggested that EM field exposure would have effects on cell-cell communications at the cell surface.

**Electromagnetic Fields and Gene Expression**

The possible modulation of gene expression by EM field exposure is one of the mechanisms that has been used to explain the increased relative risks of cancer following electromagnetic field exposure. The effects of elf-EM fields may be restricted to a group of normally expressed transcripts involved in growth and/or differentiation (Goodman and Henderson, 1991). An early experiment using magnetic field exposed dipteran salivary glands suggested that an increase in mRNA levels was likely to be due to an increased rate of transcription rather than the modification of existing RNA (Goodman *et al.*, 1983). They also reported that elf-EM field induced increased *c-myc* mRNA levels in human HL-60 leukemia cells (Goodman *et al.*, 1989). Other specific transcripts including β-actin, β-tubulin, histone H2B, *c-src*

were also elevated after EM field exposure (Goodman *et al.*, 1992). Supportive experiments suggested that elf-EM field can increase *c-myc*, c-*fos*, *c-jun* and PKC-β gene expression in Daudi cells and other cell lines (Phillips *et al.*, 1992).

An experiment in the yeast *S. cerevisiae* to identify the specific genes responsible to magnetic fields stimulation was done by Benninger *et al.* (1996). Using differential display, they exposed the yeast *S. cerevisiae* to 60 Hz sinusoidal field at 200 mG for 15 generations (24 hours). New transcripts present following magnetic field exposure were called EMF-α DNA and the EMF-β DNA. A third cDNA clone (EMF-δ DNA) is one whose expression appears to be inhibited by EM field exposure. The predicted amino acid sequence for EMF-β indicates that it may encode a serine protease inhibitor was found. EMF-δ cDNA is derived from the *SUR*4 gene of yeast which is involved in the transcriptional control of a plasma membrane $H^+$-$K^+$ATPase.

Gold *et al.* (1994) reported an increased expression of large T antigen mRNA and protein in SV-40 transformed fibroblasts exposed to a 60 Hz, 60 mG field at 20 minutes. They suggested that this was an adapted stress reaction comparable to the heat shock response. The transcription and translation of heat-shock proteins were also found by Goodman *et al.* (1994). In experimental exposure to a 60 Hz, 60 mG magnetic field increased heat shock protein HS-70 mRNA in cultured human cells.

Gene expression is often considered as the early response of cells to environmental stimulation. The low level of gene expression induced by magnetic fields is not as significant as that stimulated by other ligands, and the different strains of the same cell line in different labs demonstrated different responses to magnetic fields (Jin *et al.*, 1997).

## Magnetic Fields and Second Messenger

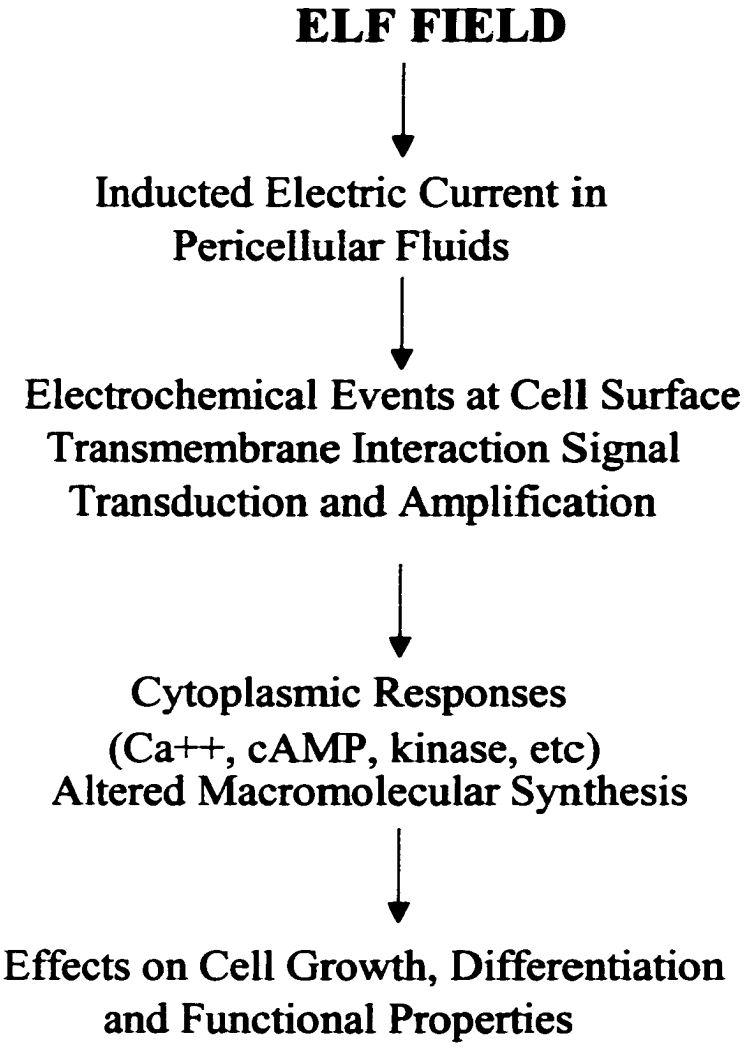
Investigators have studied the signaling pathways that could mediate a weak external EM field signal. It is proposed that EM field triggers a cell membrane change which can amplify the weak signal to the nucleus (Adey, 1988) (Figure 2). Some laboratory data suggested that calcium ions and/or the cell membrane receptors could mediate this pathway. Calcium ions serve as a second messenger to regulate cell activities including protein kinases, phosphatases, phosphodiesterases, cytoskeletal components and ion channels.

Alteration of calcium flux induced by EM field exposure is a consistent and a confirmed evidence of the effects of EM fields. The effects of nonthermal EM field on calcium ions were first demonstrated using nonlymphoid tissues. Work done by Bawin *et al.* (1976) showed that calcium eflux from chick brain can be measurably altered by exposure to an elf-modulated radio frequency carrier wave *in vitro*. During the past decade, field affects calcium membrane influx or efflux have been reported. One important idea was that elf-EM field frequencies below 100 Hz seemed to be most effective in modifying calcium ion regulation, and in some cases field responses appeared to be frequency-specific (Blackman *et al.*, 1985; Rozek *et al.*, 1987).

Walleczek and Liburdy (1990) observed that 60 Hz magnetic fields increase calcium ion influx by 50-200% in rat thymocytes exposed to a 220 G magnetic field for 60 minutes at $37^0$ C in the presence of concanavalin A (Con A). Lindstrom *et al.* (1993) showed that application of a 50 Hz 1 G magnetic field increased intracellular calcium signaling in the Jurkat T-cell line, and the effect was similar to that obtained with an anti-CD3 monoclonal antibody used as a positive control. Independent studies investigated possible nonthermal effects of time-varying magnetic fields of intensities in the range from 1 G to 10 G. The role of calcium signals in mediating

Figure 2. Proposed signaling mechanism of EM field effects on biological systems

Adapted from: Tenforde, T.S. (1993) Cellular and Molecular Pathways of Extremely Low Frequency Electromagnetic Interactions with Living Systems. *In* Blank, M; ed. Electricity and Magnetism in Biology and Medicine. San Francisco: San Francisco Press. p.5



immune cell field effect at very weak field intensities (≤ 1 G) cannot yet be assessed. In contrast, for higher field intensities (>1 G), there is enough information to allow examination of the potential role of calcium in induction of cellular field effects (Wallaczek *et al.*, 1992). Exposure of phytohaemaglutagen (PHA) activated human peripheral blood lymphocytes (HPBL) to 60 Hz, 2 G sinusoidal magnetic field proved ineffective in modifying a cell cycle progression rate. On the other hand, a 50 Hz sinusoidal magnetic field with a 25-fold higher intensity (50 G) slightly but significantly (8%, $p < 0.0001$), increased the proliferation index of PHA-treated HPBL. Furthermore, there is evidence that exposure periods of 24 hours, or in some cases even less, can induce field effects on DNA synthesis (Conti et al., 1986).

The alteration of calcium flux caused by magnetic fields reflects some aspects of EM field bioeffects and could be interpreted as a possible mechanism for explaining some of the effects of EM field exposure. Experimental results suggested that the duration, intensity and frequency of magnetic fields exposure involves calcium flux and that the cellular response to magnetic fields, proliferation and transcription, is also dependent on the calcium homeostasis (Walleczek, 1995).

**Magnetic Fields and Receptors**

Experiments support the hypothesis that EM fields cause changes that are initiated at the cell surface and which may affect receptor binding activity. The amplification of weak signals of EM fields at the cell surface could affect proteins, second messengers, or other charged structures (Adey, 1988).

Luben *et al.* (1993) studied bone cells exposed to sinusoidally varying 60 Hz, 1 G magnetic field. The field significantly inhibited cAMP accumulation in osteoblasts in the presence of 1 nM

parathyroid hormone and the inhibitory activity was decreased at higher doses of parathyroid hormone, with no significant inhibition at 100 nM of parathyroid hormone. The result showed that the receptor protein for parathyroid hormone is at least one transducer of magnetic field effects. The magnetic field does not alter ligand binding to the receptor, but appears to influence transmission of the signal from the cell membrane to the cytoplasm.

In subsequent experiments, using the same conditions, Luben *et al.* (1994) demonstrated a transient increase of the enzyme activity of PKC and a progressive down-regulation of PKC activity that is often seen after treatment of cells with tumor promoting phorbol diesters.

## II. Rationale for Proposed Research

A signal transduction mechanism provides a nonlinear mechanism that is able to explain the bioeffects of elf-EM fields through amplification. This approach is to investigate whether signal transduction processes are held in common between events leading to neoplastic progression and those observed in induced cell differentiation following EM field exposure.

### Aim I. Magnetic fields and HL-60 cell differentiation

Tumorigenesis is a multi-step and multifactorial process, which includes initiation, promotion and progression. Initiation involves a genotoxic event in which the carcinogen interacts with target cells to directly affect DNA. However, on an experimental level, no direct genotoxic effects of EM field have been confirmed (McCann *et al.*, 1993). Therefore, if elf-EM field plays a role in tumorogenesis, it may either promote a process that is already initiated and/or modify cell regulatory processes associated with a number of subcellular events that are generally

nongenotoxic.

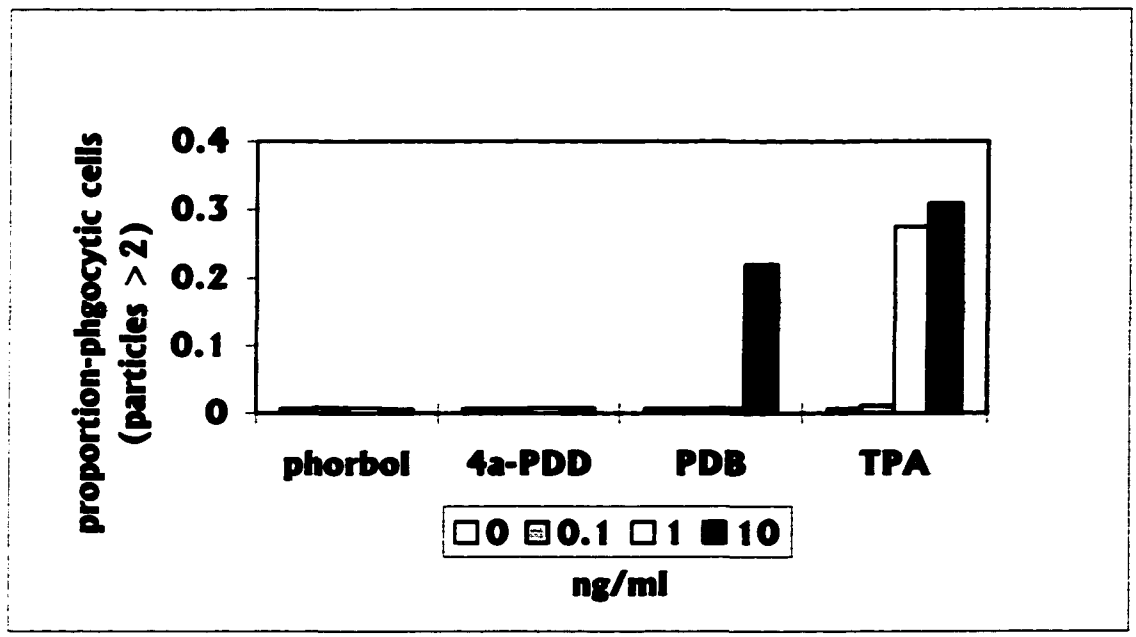
The present study is based on the observation that the known tumor promoter, TPA, can induce human promyelocytic leukemia cells (HL-60) to differentiate into macrophage-like cells. Dertinger *et al.* (1995) devised an *in vitro* method for identifying tumor promoting activity of TPA using HL-60 cell differentiation. Terminally differentiated HL-60 cells usually present morphological changes which are obvious upon microscopic inspection and functional changes; the cells can phagocytize fluorescent particles. Phagocytosis of fluorescent microspheres by HL-60 cells in the presence of TPA can be monitored using flow cytometry (Blair *et al.*, 1986). Phagocytized fluorescent latex particles are used as sensors to highlight the phagocytosis positive cells and to automatically quantitate particle internalization. The degree of phagocytic activity is dependent on parameters such as microsphere size, microsphere number and exposure time.

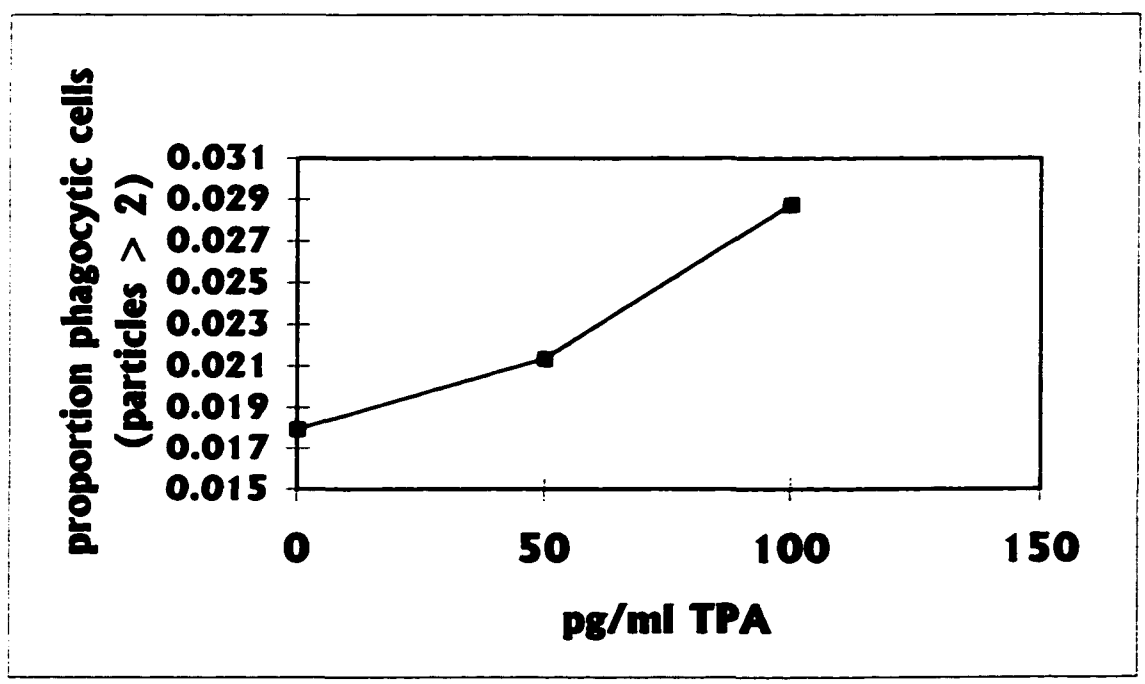
The inherent tumor promoting activity of TPA is reflected in its induction in HL-60 cells. HL-60 cell differentiation provides a mean of testing the tumor promoter's specificity and sensitivity on this cell model (Figure 3). HL-60 cells treated with phorbol and a series of phorbol esters comprised of 4α-phorbol 12,13-didecanoate (4α-PDD), phorbol 12,13-dibutyrate (PDB) and TPA were evaluated relative to their induction capacity. TPA was the most potent inducer. HL-60 cells responded to picogram levels of TPA which indicated that this is a sensitive method to test TPA in vivo tumor promoting activity. The results showed that the number of phagocytized particles in the induced cells was proportional to the in vivo induction activity at very low levels of TPA. This series of experiments indicated that an extremely sensitive assay was available for testing the end point effects of EM field.

Research from several laboratories suggested that part of the mechanisms of EM field-cell

Figure 3. Specificity and sensitivity of phorbol esters on HL-60 cell differentiation

The figures are from the original data of Dertinger *et al.* (1995). Plot 1. Comparison of the effects of phorbol esters. Bars represent the proportion of cells with phagocytic activity (percentage of induced HL-60 cells). Phorbol does not induce HL-60 cell differentiation. 4α-PDD has a slight potential to induce HL-60 cell differentiation. PDB induce cell differentiation at concentrations above 10ng/ml. TPA is a most potent cell differentiation inducer in this study. Plot 2. Dose-dependence of TPA on HL-60 cell differentiation. TPA at pg/ml level can induce differentiation. The curve indicates a linear response of HL-60 cells to TPA induction. Abbreviations: 4α-PDD, 4α-phorbol 12, 13- didecanoate; PDB, phorbol 12,13- dibutyrate; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.



interaction could be similar to that of TPA tumor promotion. TPA affects HL-60 cell differentiation via cellular regulatory devices associated with tumor promotion (Figure 4). The background for making this comparison is based, in part, on experiments that compared the effects of EM field and TPA on regulation of *c-fos* gene expression and signaling transduction pathways (Karabakhtsian *et al.*, 1994; Rao and Henderson, 1996).

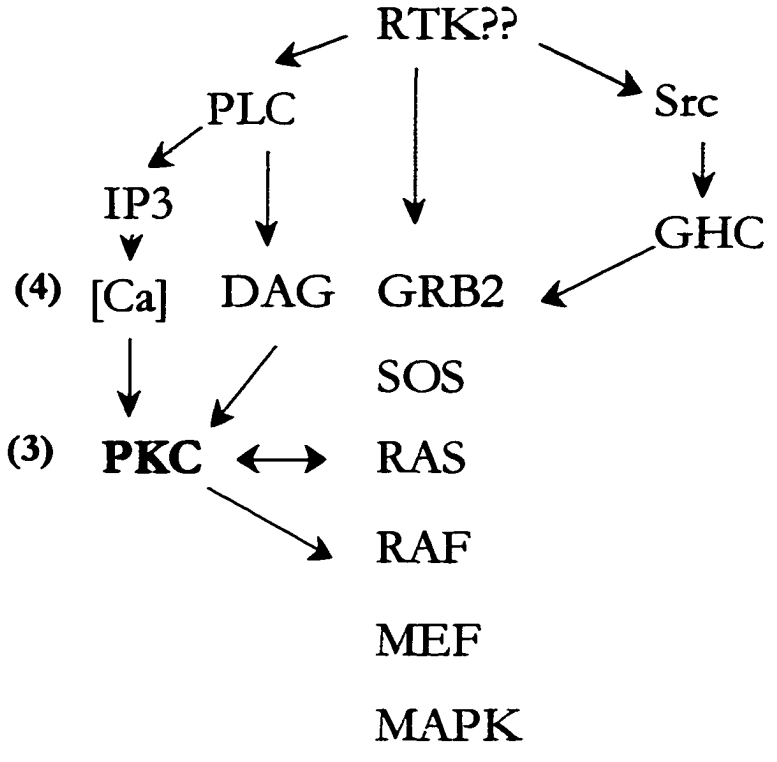
The *c-fos* gene is of particular interest because it is an early-response gene and the Fos protein is an early nuclear transcription factor (Doucet *et al.*, 1990) which involves gene expression, cell maturation, proliferation and differentiation. Experiments have shown that EM fields exposure causes an increase in expression of the steady state levels of *c-fos* mRNA expression in HL-60 cells. This effect is abolished when the cells were treated with EGTA to deplete calcium (Karabakhtsian et al., 1994). The increased steady state levels of transcripts of *c-fos* expression are transient in human HL-60 cells exposed to short periods to EM fields. (Philips *et al.*, 1992).

Experiments of Rao and Henderson (1996) supported the effect of EM field regulation on the expression of the *c-fos* gene. An upstream promoter regulatory region of c-fos has been delineated (Prywes *et al.*, 1988). This promoter's region includes a TPA response element. HeLa cells were transfected with a series of c-fos gene promoter constructs upstream of the bacterial CAT gene in a reporter gene assay system. The positive control, TPA, increased CAT activity by 20-40% above control, while 20 minute exposures to 60 Hz, 60 mG field increased the activity5-20% above the control. The specific segment of *c-fos* gene promoter affected by EM field contains the SRE/AP-1 site, which is regulated by TPA. This experiment demonstrated the response of c-fos upstream regulatory region to EM field exposure was time dependent, similar to the timed

Figure 4. Studies of signal transduction pathways involving TPA and EM field exposures in HL-60 cells

The figure is a generalized signaling pathways in cells. The numbers in the figure represent the previous studies of signaling pathways in HL-60 cells. ① an increase of steady state transcript levels of *c-fos, c-jun* and *c-myc* (Broude *et al.*, 1994)). ② AP-1 site. *C-fos* promoter which contains AP-1 site (transcription factor binding site) responses to EM field exposure (Rao and Henderson, 1996). ③ Inhibition of PKC by chelerythrine negates the response of *c-fos* gene to EM field exposure (Kochlati *et al.*, 1999). ④Calcium. An increase of the steady state levels of c-myc and c-fos mRNA to EM field exposure could be blocked by the calcium channel blocker, Verapamil (Karabakhtsian *et al.*, 1994).

**EMF**




(2) **AP-1** ;NFKB; EL1

primary

c-fos

(1) c-jun

c-myc

secondary

ODC

Cyclin D1

stromelysin

expression elicited by mitogenic and other inducing factors.

This research compared the induced phagocytic activity in the presence of TPA and/or EM field exposure in HL-60 cells. Other changes such as cell aggregation, cell spreading and adherence to plastic plates as well as the presence of internalized flourescent latex beads can be observed under phase-control microscopy. The measurement of phagocytosis by flow cytometry proved to be a direct and sensitive method to detect the effects of EM field on cells. The fact that EM field could be related to differentiation helped provide a clue as to mechanisms of EM field-cell interaction.

**Aim II. Magnetic Fields and Protein Kinase C**

This portion of research was concerned with PKC translocation in HL-60 cells exposed to 60 Hz, 60 mG and 1 G magnetic field in order to confirm or disprove proposed mechanisms of EM field effects mediated by interactive signal transduction.

Protein kinase C, a calcium-activated, phospholipid-dependent enzyme, is a critical component in the membrane signaling pathways. Its activation is initiated by the interaction of hormones, growth factors, mitogens, and neurotransmitters with their cell surface receptors, leading to phospholipase C mediated hydrolysis of inositol phospholipids into diacylglycerols and inositol triphosphate. Diacylglycerols and $Ca^{++}$, together with membrane phospholipids, like phosphatidylserine, activate PKC. Once activated, PKC phosphorylates other proteins at serine and threonine residues which, in turn, triggers many cellular responses, including cell proliferation, differentiation, membrane transport and gene expression.

PKC is not a single enzyme, but a family of related isozymes. PKC superfamily consists

of at least 11 distinct isoforms (Transduction Laboratories). This superfamily has been divided into three categories on the basis of $Ca^{++}$ and lipid requirements. The conventional PKCs (cPKC) consist of four members (α, βI, βII, γ), this group of PKCs requires both $Ca^{++}$ and diacylglycerols. The second group of PKCs, the novel PKC (nPKC), includes δ, ϵ, η and θ, requires DAG but are independent of $Ca^{++}$. The third group of PKCs, atypical PKC (aPKCs) includes ζ, λ and μ, do not require $Ca^{++}$ and are insensitive to DAG. The members of the PKC families exhibit a distinct pattern of tissue-specific expression and intracellular localization (Nishizuka, 1988) (Table 1). The functional significance for the existence of various isoforms remains to be elucidated. It is plausible that different isoforms may be selectively stimulated in response to external stimuli and carry out different functions.

Table 1. PKC subspecies in mammalian tissues.

| **Subspecies** | **Activators** | **Tissue** |
|---|---|---|
| α | PS, $Ca^{2+}$, DG, FFA | Universal |
| βI | PS, $Ca^{2+}$, DG, FFA | Some tissues |
| βII | PS, $Ca^{2+}$, DG, FFA | Many tissues |
| γ | PS, $Ca^{2+}$, DG, FFA | Brain only |
| δ | PS, DG | Universal |
| ϵ | PS, DG, FFA | Brain and others |
| η | ? | Lung, skin, heart |
| θ | ? | Skeletal muscle |
| ζ | PS, FFA | Universal |
| λ | ? | Ovary, testis, etc. |

The mechanism of TPA action in cells has been studied intensively. PKC acts as a TPA receptor (Niedel *et al.*, 1983). Similar to DAG, TPA binds to PKC and mediates a series of signal transduction events leading to cellular responses (Nishizuka, 1986). When binding to PKC, TPA activates PKC persistently. In contrast to the normal interaction with DAG, short-term treatment with TPA leads to the activation of PKC, while prolonged treatment down regulates PKC (Rozengurt *et al.*, 1984; Blumberg *et al.*, 1986). Diacylglycrol activates PKC transiently but has little or no effect on HL-60 cell differentiation (Kreutter *et al.*, 1985; Yamanoto *et al.*, 1985; Morin *et al.*, 1987). Phorbol ester-induced HL-60 cell differentiation is associated with PKC down-regulation (Solanki *et al.*, 1981; Weinberg *et al.*, 1984).

It is assumed that transducing properties of PKC resides in its ability to phosphorylate proteins (Hishizuka, 1986). In intact cells, phosphorylation may be directed by topological location of PKC following activation (Nishizuka, 1989). TPA causes a rapid translocation (within 10 minutes) of PKC from the cytosolic (soluble) fraction to the particulate (membrane) fraction. This was determined experimentally by immunoblotting of the enzyme protein in the isolated subcellular fractions and by immunocytochemical localization of the enzyme during HL-60 cell differentiation (Shoji *et al.*, 1986). The translocation to the plasma membrane of the enzyme may represent initial events related to the TPA effect on terminal differentiation of HL-60 cells to a monocyte/macrophage pathway.

The present study is also based on a postulated mechanism for EM field cell interaction that a conformational change at the cell membrane induces signal transduction via second messengers which ultimately result in cellular changes (Luben, 1991; Adey, 1993; Liburdy, 1994). Some investigations have suggested the effects of EM field exposure could be related to PKC

activation and subsequent activation of associated signaling pathways. The known PKC activation by TPA in HL-60 cell differentiation is also an important predicted mechanism that explains EM field-cell interaction.

Previous studies suggested that PKC is involved in EM field-induced cellular responses in different cell lines (Wallaczek, 1992; Uckun *et al.*, 1995; Dibirdik *et al.*, 1998; Kristupaitis *et al.*, 1998). For example, HL-60 cells exposed to a 50 Hz, 8 G magnetic fields have increased binding of the PKC-specific phorbol ester, PDBu, suggesting that these relatively strong magnetic fields may modify the cellular response to tumor promoters (Monti *et al.*, 1991). Exposure of bone cells to a 1 G, 60 Hz sinusoidal magnetic field also showed a rapid transient increase of PKC activity in the cell membrane, followed by a progressive down-regulation of PKC activity (Luben, 1994). Down-regulation of PKC activity is often seen after treatment of cells with agents such as hormones and phorbol esters which activate it. Holian *et al.* (1996) showed exposure of HL-60 cells to 60 Hz electric fields at 100-1000 mV/cm, caused the PKC activity in the cytosol to be decreased, but there was no concomitant change seen in particulate PKC activity.

## III. Data Summary

HL-60 cells were responsive to elf-EM field exposure. This research showed that HL-60 cells can be driven into terminally differentiated macrophage-like cells with increased phagocytic activity following exposure to EM fields (Tao and Henderson, 1999). The study also demonstrated that there exists a additive effect in HL-60 cell differentiation with TPA and EM field exposure. The PKC translocation shows a different response of cells to EM field exposure and TPA treatment.

## I. Magnetic field causes HL-60 cells differentiation

A sinusoidal 60 Hz EM field drives the differentiation of cultured hematopoietic progenitor cells similar to TPA. The results using microscopic analyses demonstrated that similar morphological changes appeared following EM field exposure, and/or TPA treatment. Cells subjected to 60 mG EM field showed an increase in cell differentiation. The results using flow cytometry analyses showed a significant increase in the number of phagocytic cells was observed after HL-60 cells exposed to 1 G and 2G EM field. The earliest significant induction was at 24 hours. There is also a linear dose-response of HL-60 cells to low concentrations of TPA at 50-250 pg/ml. The relative effect of a 1 G EM field on HL-60 cells at 24 hours is approximately equivalent to that of low concentrations of TPA. (250-500pg/ml) TPA induced phagocytic cells appear as early as 12 hours at 500 pg/ml of TPA. The data also showed that there was an additive effect when EM field and TPA were used together at lower concentrations of TPA (50-500 pg/ml). The additive effect declined as the TPA concentration was increased, *i.e.*, the effects were swamped out by TPA at concentration of 1 ng/ml and above. This study suggests that EM field act as a factor or cofactor to cause biological effects.

## II. Magnetic Fields and PKC-α Translocation

These experiments were to determine if PKC is involved in EM field induced HL-60 cell differentiation. TPA can cause a significant PKC-α translocation and total PKC-α is down-regulated. Data from cells exposed to 60 Hz magnetic fields (60 mG and 1 G) is controversial. Cells exposed to 60 mG show changes in PKC translocation but the difference is not statistically significant. Cells exposed to 1 G EM field exposure showed an increased trend for PKC translocation. In most time points, however, this increase is not significant, and the total PKC-α

is not down-regulated. The effect of TPA on PKC translocation demonstrates a different response. The results from EM field exposure related PKC translocation on HL-60 cells suggested that either there is PKC indirect involvement of EM fields or other alternative mechanism which may relate to EM field exposures.

## Materials and Methods

### Abbreviations

BSA (bovine serum albumin)

EDTA (Ethylenediamine TetraAcidic Disodium Salt)

PMSF (Phenylmethylsulfonic Fluoride)

DTT (dithiothreitol)

PBS (Phosphate Buffer Saline)

TEMED (NNNN-tetramethylethylenediamine)

SDS (Sodium Dodecyl Sulfate)

TPA (12-tetradecanoylphorbol-13-acetate)

### Reagents and Solutions

Growth Media: RPMI Medium 1640, 10% fetal bovine serum, 1% penicillin/ streptomycin.

SDS (20%): 20 g SDS in 100 ml distilled water.

Protein Lysis Buffer: 10 mM Tris/HCl (pH 7.5); 5 mM NaCl; 1 mM EDTA; 2 mM DTT; 0.5 mM PMSF; 10 ug/ml Leupeptin and 10 ug/ml Aprotinin.

PMSF (0.1 M stock): 0.174 g in 10 ml isopropanol alcohol.

PBS (10X): 1.5 M sodium phosphate pH 7.2; 1.5 M NaCl.

1X trypsin-EDTA: 0.25% trypsin; 1 mM EDTA (Gibco).

DTT (1 M): 3.09 gms in 20 mls of sodium acetate, pH 5.2.

SDS gel loading buffer: 50 mM Tris-Cl, pH 6.5; 100 mM dithiothretiol; 2% SDS; 0.1%

Bromophenol blue; 10% glycerol.

Gas mixture: 5.0% $CO_2$; 20% $O_2$; 75% $N_2$.

**Cell Lines**

HL-60 cell, derived from a patient with acute promyelocytic leukemia, provides a unique *in vitro* model system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells of the granulocyte/monocyte/macrophage lineage. The subline used was obtained from Dr. I. B. Weinstein, Columbia University Health Sciences. The karyotype of this line is consistent with known characteristics of HL-60 cells (Wolman *et al.*, 1995)

**Tissue Culture**

Stored cells are thawed at regular intervals (once every two months). They are kept in culture for about two weeks before they are used for experimental procedures. The cells are maintained in RPMI 1640 (Gibco) with 10% fetal calf serum and 1% Pen-Strep. The medium is changed on a regular schedule (normally 3 times weekly). Calf serum is purchased from Sigma on an annual basis to ensure that the serum lot is uniform; new batches are tested relative to cell growth. The viability of cells is determined by trypan blue dye exclusion. Cells are maintained in exponential growth; cell density is determined with a hemocytometer

**EM Field Exposure Conditions**

Cells are exposed to continuous sinusoidal 60 Hz electromagnetic field at 60, 500, 1000

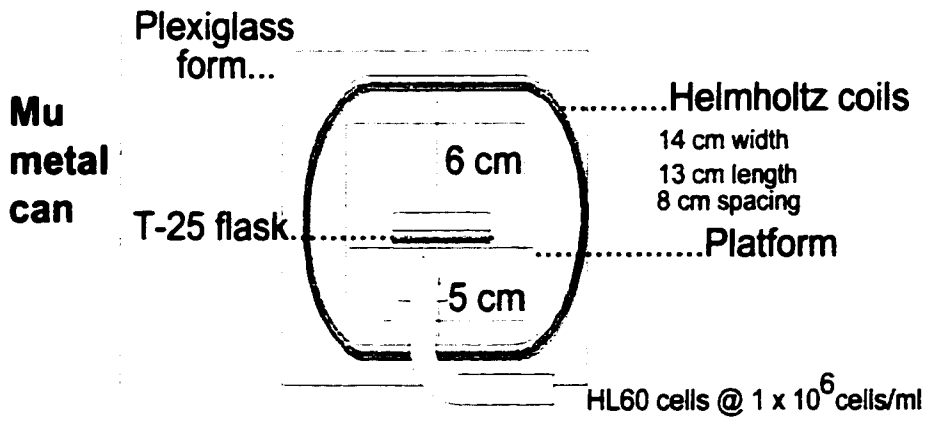
and 2000 mG for periods up to forty-eight hours in a Helmholtz Coil exposure system designed by Electric Research and Management, Inc. (ERM). The exposure conditions were selected as those typical of other experiments which have tested the effects of EM fields on the signaling pathways. The ERM exposure system provides a graded series of field settings, which are then maximized using the function generator. A sine wave generator (Wavetek 11 MHZ function generator-model 21) with variable frequency control is used. The ERM system has the capacity to conduct simultaneous sham exposure under blind conditions. The exposure coils consist of two double wound coils in an approximate Helmholtz configuration (164 turn rectangular windings of gauge 19 magnet wire measuring 13 x 14 cm with 8 cm spacing; the coils are 1 cm in diameter wound around an approximate square form). The coils are supported by an acrylic frame in which the test samples are placed. The coils are placed in two μ metal cans each inside a separated compartment of a double-door incubator. To minimize the effect of extraneous EM fields, the exposure apparatus is place in a μ metal can during exposure. Flasks or dishes are placed on a plexiglass stand in the horizontal plane in an area shown to have a uniform magnetic field. Field characteristics were measured using a Tektronix 2245A oscilloscope and a Metex Digital Multimeter (Figure 5).

To operate the exposure system in the double blind mode, someone other than the person conducting the experiment must initially set the mapping switches on the inside of the control box to either map coil "A" to coil "1" and "B" to coil "2" or coil "B" to coil "1" and coil "A" to "2". Once this is done, the control box door can be locked and the information about the mapping can be kept confidential until the experiment is over. In this way the experimenter does not know which of the two exposure chambers produces the real field at any given time throughout the

Figure 5. EM field exposure system

Helmholtz coil configurations: The Helmholtz-aiding coils (13x 14 cm and 8 cm spacing) are composed of 19 G wire bundles wound 164 times around a plexiglass form. T-25 flasks or 12 well plates (which approximate at T-25 flask) containing cells are placed horizontally on a plexiglass stand. The bottom of the flask is 2 cm below the axis level. The height from the flask bottom to the top surface of liquid is about 1.1 cm. The height of the liquid is 0.6 cm. Helmholtz coils are shielded in a μ metal container within $37.5^0$ C in an incubator. A μ metal can is used as precautionary measure to minimize the extraneous EM fields. The container also prevents other cells in the incubator from possible exposure.

## EXPOSURE CONDITIONS




**60 Hz; 1G (rms)**

**Temperature is monitored with a Physitemp thermocouple probe sensitive to 0.1°C**

experiment.

Cells are taken from a single "parental" flask and placed into six wells of a 12-well (2 mls/well) plate. The plate is obtained by dividing a 24-well plate [this approximates the exposure geometry using a T-25 flask]. The concentration of the cells is adjusted to about 1 x $10^6$ cells/ml before exposures using fresh medium and cells were settled down for 30 minutes. The coil is turned on at least 30 minutes before experimental procedures are initiated, and the cells are removed from the coil while the signal is still on. The signal generator is placed outside the incubator. Media temperatures are monitored under conditions equivalent to exposures (Physiotemp thermocouple probe sensitive to 0.1$^0$C). The magnetic fields in the current incubator have been measured over a period of five years at about 2 mG.

**Assaying Phagocytic Activity Using Microscopy**

Fluorescent microspheres (3 μm, Polyscience Fluoresbrite) were sterilized in methanol for 10 minutes. The spheres were collected by centrifugation, sonicated for one minute in RPMI medium and added to a T-25 flask containing the cells. The spheres and cells were then placed in wells for exposure. Cells will be exposed for 0, 4, 12, 18, 24 and 48 hours. Before experiments, cells are adjusted to a density of approximately 1 x $10^6$ cells/ml. Polyscience Fluoresbrite microspheres are added to the cells at a cell:sphere ratio of 1:10. The cell-microsphere suspension is then aliquoted into the middle wells of a 24-well plate (2 mls/well). One 12-well plate cells is exposed to the sinusoidal 60 Hz field. One 12- well plate cells is treated with TPA (from 50 pg-1 ng/ml) in a volume of 20 ul diluted solution in 2 ml cell medium. The level of DMSO (TPA is dissolved in DMSO) is kept to less than 0.1 ul/ml. No DMSO was added into control and EM field

exposure group to determine the effect of EM field after adding TPA, the cells are incubated at $37^0$C with 10% $CO_2$ for different time period as used in EM field exposure. After exposure, the medium was removed and replaced with RPMI containing 0.25 % trypsin for five minutes to remove adherent spheres and disaggregate cells. The cells were then pipetted vigorously to further reduce aggregates and remove spheres attached to the cell membrane. The cells were washed several times and to reduce free-floating fluorescent particles This was monitored by microscopy. The cells are collected into an Eppendorf tube, centrifuged and washed with 3 times with 1 X PBS, 300 ul of 4% paraformaldehyde (Electron Microscopy Sciences) is added, and the cells are placed at $4^0$C overnight. The cells are spun out of the fixative and one drop of permafluor aqueous mountant (Lipshaw Immunon) is added into the Eppendorf with gentle shaking. 30 ul of solution is placed onto a glass slide and covered with a coverslip. Cells that are phagocytic are identified as fluorescent under 10x microscopy. The percentage of cells containing particles is calculated. The TPA dilution series is used to construct concentration curves for comparison to the effects of EM field exposures.

**Measuring Phagocytic Activity Using Flow Cytometry**

Flow cytometry is used as the primary method for determination of the phagocytic uptake of fluorescent particles. The analysis used conditions essentially as described by Dertinger *et al*, (1995). Free microspheres and fluorescent cells were distinguished with the forward scatter parameter (particle size). Cells are sorted at various gates to confirm the mean number of fluorescent beads/cell. Essentially, gating (in these experiments, one or more particles per cell) is used to provide the measurement of increases in fluorescent particle number over the pattern

exhibited by unexposed cells. Excitation of fluorescence used the Innova Enterprise (TM) laser (488 nM beam at 135 mW). Imaging microscopy was used to set conditions and to confirm the differentiation state based on morphology. Microscopy was also used to assess possible anomalous binding of the fluorescent particles to cells. Internalized Fluoresbrite particles are large and bright enough to be recognized under 10x phase; image magnification is used to confirm internalization.

**Protein Fractionation**

HL-60 cells ($1.5 \times 10^7$ cells) are placed on ice immediately following TPA treatment or EM field exposure. After 10 minutes incubation on ice, the cells are centrifuged and the medium is discarded. The cells are washed 3 times in 1 x PBS, and spin down after each washing. The collected cells are placed in 2 ml homogenizing buffer [10 mM Tris/HCl (pH 7.5); 5 mM NaCl; 1 mM EDTA; 2 mM DTT; 0.5 mM PMSF; 10 ug/ml Leupeptin and 10 ug/ml Aprotinin]. The cells are lysed using a 21 G 1½- gauge needle 15 times, then centrifuged at 100,000 xg for 1 hour at $4^0$C. The supernatant is saved as the cytosol portion. The pellet is placed in 2 ml homogenizing buffer (with 1% Triton-100), and incubated on ice for 30 minutes. The suspension is centrifuged at 100,000 xg for 1 hour at $4^0$ C, and the supernatant is collected as the membrane portion. The samples are stored at $-70^0$ C until used for electrophoresis and immunoblotting.

**Measuring Protein Concentration**

This uses the Bio-Rad *DC* Protein Assay. This assay is a colorimetric assay for protein concentration following detergent solubilization. The reaction is similar to the well-documented Lowry assay. The reagent package includes: reagent A, an alkaline copper tartrate solution, reagent

B, a dilute Folin reagent, reagent S. Add 20 ul or reagent S to each ml of reagent A. Prepare 7 dilutions of a protein standard bovine serum albumin containing from 1 ng/ul to about 7 ng/ul. A standard curve is prepared each time the assay is performed. Pipet 10 ul of protein standard and sample into a clean, dry cuvatte. Add 25 ul of reagent A' (reagent A + reagent S) in each well. Add 200 ul reagent B into each well. After 15 minutes, absorbances can be read at 710 nM. A standard protein curve was delineated. The sample protein concentration is determined based on the standard curve (Figure 6).

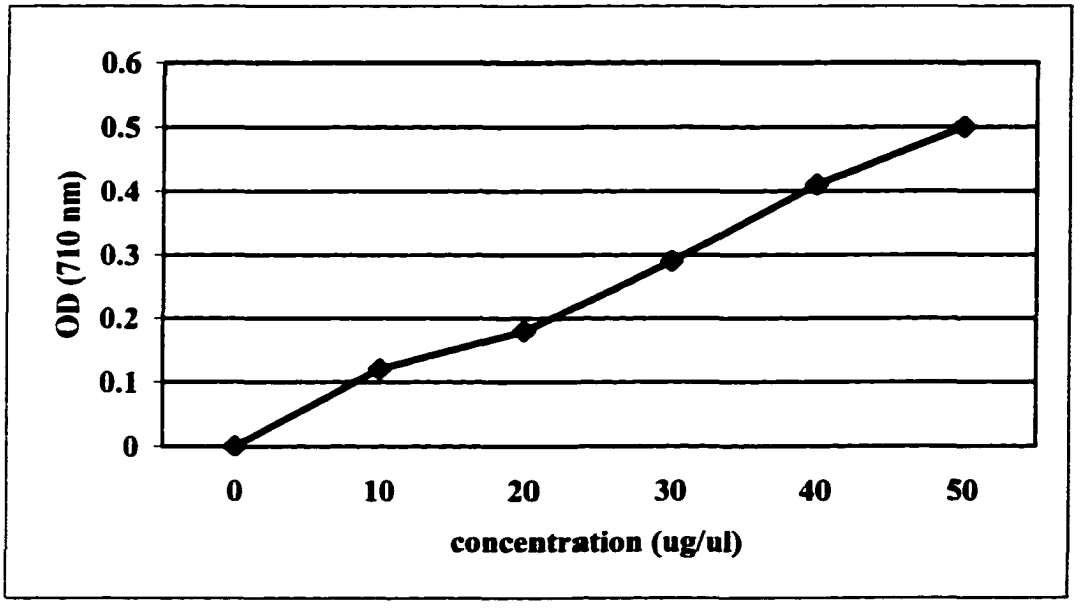
**Electrophoresis and Immunoblotting**

The assembled glass plate sandwich uses 0.75-mm spacers locked to a casting stand. Five ml of separating gel is prepared [8% SDS-polyacrylamide gel: 1.33 ml of 30% acrylamide/Bis solution (29:1 acrylamide: N,N'-Methylene-bis-acrylamide) 1.25 ml of 4 X stock solution [0.12 M Tris.Cl (pH 8.8); SDS (0.5%)] and 2.42 ml of $H_2O$. The solution is degassed for 15 minutes and 15 ul of 10% ammonium persulfate and 5 ul of TEMED is added with gentle mixing. The separating gel solution is applied to the sandwich along one edge of the spacer, and the gel is allowed to polymerize for 30 minutes at room temperature.

The 5 ml stacking gel is prepared by mixing 0.65 ml of 30% acrylamide/Bis solution (29:1), 1.25 ml of 4x stock solution (0.12 M Tris.Cl/0.5% SDS, pH 6.8) and 3.05 ml of $H_2O$. This solution is degassed for 15 minutes and 15 ul of 10% ammonium persulfate and 5 ul of TEMED is added and swirled gently to mix the gel solution. The stacking gel is applied to the top of separating gel. A 0.75-mm Teflon comb is inserted into the layer of stacking gel solution. The stacking gel solution polymerizes in 30 minutes at room temperature.

Figure 6. Standard Protein Concentration Curve

Concentration was plotted by using regression analysis. The standard protein used was albumin. Optical density (at 710 nm) was plotted on the Y axis and concentration on the X axis (μg/μl). The protein samples were represented as means of triplicate readings. Concentrations of the protein samples were determined by correlating OD readings of the samples with standard curve and extrapolating it on to the X axis.



The protein sample (30 ug) is mixed with 2x SDS/sample buffer (sample buffer: 25 ml of 4 X stock solution (pH 6.8), 20 ml of glycerol, 4% SDS, 0.2 M DTT, 1 mg Bromphenol Blue/100 ml). This is heated 5 minutes at $100^0$ C to denature protein and then put on ice. The sandwich is attached to the electrophoresis buffer chamber. Using a 100 ul syringe with a flat-tipped needle, samples are loaded into wells. The gel is run at 10 mA (DC) until the bromphenol blue tracking dye enters the separating gel. Then the DC current is increased to 15 mA. After the bromphenol blue tracking dye has reached the bottom of the separating gel, the power supply is disconnected. The gel sandwich is disassembled, and the gel is removed from the lower plate and placed in an electroblot sandwich. The electroblot sandwich is placed into running a buffer chamber filled with transfer buffer (0.014 M Tris and 0.02 M glycine (4 liters); add 1.2 liters methanol). The proteins are transferred to nitrocellulose membranes using 200 mA overnight at $4^0$ C. The nitrocellulose membrane is placed in blocking buffer (5% nonfat milk in 1 X PBS) for 60 minutes at room temperature. The membrane is transferred to a "seal-a-meal" bag and the primary monoclonal isoform-specific PKC antibody diluted in blocking buffer is added to incubate with shaking overnight at $4^0$C. The primary antibody solution is discarded and nitrocellulose membrane is washed with 1 X PBS three times. The membrane is transferred to a "seal-a-meal" bag and the enzyme-conjugate anti-mouse IgG: horseradish peroxidase is added. It is diluted in blocking buffer (1:2000) and incubated with shaking for 60 minutes at room temperature. After discarding the solution, the membrane is washed in 1x PBS three times and the membrane surface is covered with western blotting detection reagents (ECL) for 5 minutes. The blot is ready to expose to X-ray film (Kodak, X-OMAT). The film is exposed for 2 and 10 minutes and is then developed. The film is stored for further densitometric measurement using Molecular Dynamic personal

Densitometer (PDSI-486). The program uses ImageQuant.

**Isolation of RNA** (Kochlati *et al.*, 1999)

Following exposure, cells were placed in an ethanol-ice slurry for 10 minutes, then washed with ice-cold 1 x PBS. Cells were lysed using SDS lysing buffer [100 mM Tris-HCl (pH 8.0); 100 mM NaCl; 20 mM EDTA; 1% SDS] using a 20 G needle. Total cellular RNA was isolated using the phenol-chloroform extraction method with lithium chloride/ethanol precipitation (Maniatis *et al.*, 1989). Contaminating DNA was digested with 80 units of DNase I, followed by two phenol-chloroform extractions. Following precipitation of RNA in ethanol, phenol-chloroform extractions were done and RNA was again precipitated in ethanol. Each RNA sample was tested by 1% agarose gel electrophoresis to determine the integrity of the RNA based on the 5S, 18S and 28S bands and to confirm the lack of DNA contamination, following ethidium bromide staining of the gel.

Reverse transcription (RT) was made using the Reverse Transcription System (Promega). Minus reverse transcriptase controls were not run. Rather, all samples were subjected to a DNAse digestion step. In addition, if there was DNA contamination it would be apparent on the PCR gel because of the difference in size of the products. To demonstrate quantitative ability of RT- PCR under the conditions used here, PCR was run using serial dilutions of total cDNA from a single RNA sample. B2-microglobulin levels were measured. The response to dilution was linear.

RT-PCR used AMV reverse transcriptase, oligo(dt) 15 and an internal positive control supplied by the manufacturer. 1 μg total cellular RNA in 10 μl of nuclease-free dd-water was added to the RT mixture and kept 15 minutes at room temperature, then placed in the thermal cycler at 42° C for 45 minutes, then at 95°C for 5 minutes for enzyme inactivation. Multiplex hot

start PCR was carried out with specific primers (c-*myc*, c-*fos* or β-2 microglobulin) from Oligos, Inc. at 15 pmol of each primer per reaction. The experiments used a Perkin Elmer DNA Thermal Cycler 480 with the following protocol: 94°C (5 min); then 94°C (1 min), 60°C (1 min), 72°C (1 min) for 35 cycles; and 72° C (8 min). Hot-start PCR was done with AmpliWax PCR Gem 100 (Perkin Elmer). The total volume of each reaction was 75 µl. Each reaction used 5 units of AmpliTaq DNA polymerase, Stofell fragment (Perkin Elmer), 1x Stofell Buffer [10 mM Tris-HCl, (pH 8.3); 50 mM KCl], 200 µM nTPs and 2.5 mM $MgCl_2$ ]. The amplification products were analyzed by gel electrophoresis on 2% agarose gels containing 0.05 mg/ml ethidium bromide. Gels were photographed in UV-light and the negatives scanned and quantitated using a laser densitometer (Molecular Dynamics, Inc.). RNA samples from each experiment were analyzed by RT-PCR simultaneously. Values are expressed as the ratio of density resulting from RNA extracted from exposed (E) and control (C) cells, as extrapolated from steady state β-2 microglobulin levels. Each sample from a single exposure was analyzed by RT-PCR at least twice. The mean was used for analysis.

The following primers were used:

for *c-fos* (*22*):

5'-GGC-TTC-AAC-GCA-GAC-TAC-GAG-GCG-T-3' (901-925)

5'-CCT-CCT-GCC-AAT-GCT-CTG-CGC-TCG-3' (1996-1973)

for c-*myc* (*23*):

5'-TGG-TGC-TCC-ATG-AGG-AGA-CA-3' (1727-1746)

5'-GTG-GCA-CCT-CTT-GAG-GAC-CA-3' (3293-3274)

for beta-2microglobulin (*24*):

5'-ATC-CAG-CGT-ACT-CCA-AAG-ATT-3' (943-949; 1340-1353)

5'-CAT-GTC-TCG-ATC-CCA-CTT-AAC-TAT-3' (1606-1618; 2235-2245)

**Inhibition studies; Positive controls**

Chelerythrine (LC Laboratories) was used as an inhibitor of protein kinase C (PKC) at a concentration of 1μM (Herbert *et al.*, 1990). Cells were incubated with Chelerythrine for 1 hour prior to exposure. Verapamil (ICN) was used as an inhibitor of active L-type calcium channels at a concentration of 100 μM. Cells were incubated with Verapamil for 10 minutes prior to exposures. TPA (10 μM) stimulation of *c-fos* mRNA was used as a positive control in some experiments. TPA was added to the cultures for 20 minutes, or the equivalent of the EMF exposure period. Where DMSO was used as a solvent, both exposed and sham-exposed cells contained the same concentration of DMSO.

**Statistical Analysis**

Data was analyzed statistically by using the Microsoft Excel Description Analysis program. Means were calculated by using the standard formula; Mean = $\sum X/n$, where $\sum X$ is the sum of sample values and n is the number of samples. The standard error of the means (SE) was obtained by dividing the standard deviation of the population by the square root of the number of items in the population ($SE = \sigma/\sqrt{n}$), where $\sigma$ is the standard deviation of the population and n is the number of items in the sample. The SE value was used to determine the reliability of the sample mean in estimating the true mean.

The student $\tau$-test assumes that the means of both data sets are equal and is referred to as

a homoscedastic $\tau$-test. The $\tau$-test was used to determine if the differences between the two samples (the population exposed to the fields and those not exposed) was significant. The test is based on the Null Hypothesis which assumes that no difference exists between the two samples. If the probability of an outcome or the $\rho$ values obtained is greater than 0.05, then, by convention, the hypothesis is true. The $\tau$-test is calculated from $t = x - \mu/\sigma/\sqrt{n}$, where x is the mean for one the data sets and $\mu$ is the mean from the other data set, $\sigma$ is the sample variation and n is the sample size.

## Research Results

### Overview

The aim of this study was to determine if EM field exposure could induce HL-60 cell differentiation and if changes in signal transduction which play a role in this process can be recognized. The ultimate aim was to develop a sensitive method to determine the end-point effect of EM field exposure on living systems.

HL-60 cells were responsive to EM field exposure and TPA treatment. There was a significant increase of phagocytic cells observed in cells exposed to 1 G and 2 G EM fields for 24 hours. There exists an additive effect on cell differentiation after cells were exposed to EM field and TPA together. HL-60 cell differentiation and activation of PKC by TPA was significant. TPA can cause PKC α down-regulation. EM field exposure do not cause PKC down-regulation and the PKC translocation caused by EM field exposure is not statistically significant. This study demonstrated that the terminal changes of these cells in response to EM fields and TPA are similar. On the other hand, there was no definitive proof that PKC translocation is related to EM field induced cell differentiation.

### Part One: Results of gene expression

Our data show a formally statistically significant increase in steady state transcript levels of c-*fos* following exposure to 60 mG EM field. There is substantiating evidence supporting increased steady state transcript measurements in exposed cells, particularly in the measurement of *c-fos* transcripts. The increase can be wiped out by inhibition of transduction pathways proposed as important in cellular-EMF interactions. The results confirmed that 20 minutes was

an adequate point for measuring *c-fos* following treatment with TPA or EMF exposure (Table 2). The results for RT-PCR of *c-fos* gave a sham1/sham2 value of 0.98. When this was corrected to $\beta$2-M levels; the value was 1. A series of experiments confirmed a mean increase of about 20% in cells exposed to EMF as compared with unexposed cells. The same values were obtained under normal or multiplexing conditions. The increase was wiped out by PKC inhibition, as well as inhibition of calcium channels by verapamil.

Table 2. Summary of results: comparing steady state transcript level of *c-fos* using RT-PCR

| | 60 mG 20 min | chelerythrine | verapamil | TPA |
|---|---|---|---|---|
| determinations | 42 | 20 | 8 | 42 |
| exposures | 17 | 7 | 4 | 17 |
| E/C: mean±SE | 1.2 ± 0.02 | 0.98 ± 0.04 | 0.82 ± 0.07 | 2.36 ± 0.1 |
| p (differ from 1) | $1.7 \times 10^{-10}$ | 0.006 | 0.04 | |

The fact that *c-fos* could be induced by EM field exposure was the first strong proof that signaling processes could be involved. This was a corner stone in planning other experiments to determine the role of signaling in the response to EM fields

**Part Two: Magnetic Field Exposure and Cell Differentiation**

This experimental series was designed to develop a direct and unequivocal test to determine the end point effects of electromagnetic fields on cells. Several factors were of importance to the experimental design. The first was that the tests had to be very sensitive since

the effects of EM field on cells are expected to be low. Associated with sensitivity was the ability to find the end point at which the smallest affected cell population could be determined. Secondly, the tests should allow a comparison with known tumor promotion systems. Finally, the experimental test system should be amenable to analysis of signaling systems. The system chosen was a modification of an experimental protocol originally described in Dertinger *et al.* (1995) where the relative ability of TPA to induce differentiation in HL-60 cells was related to the known tumor promotion efficacy.

**Differentiation Studies Using Microscopic Analysis**

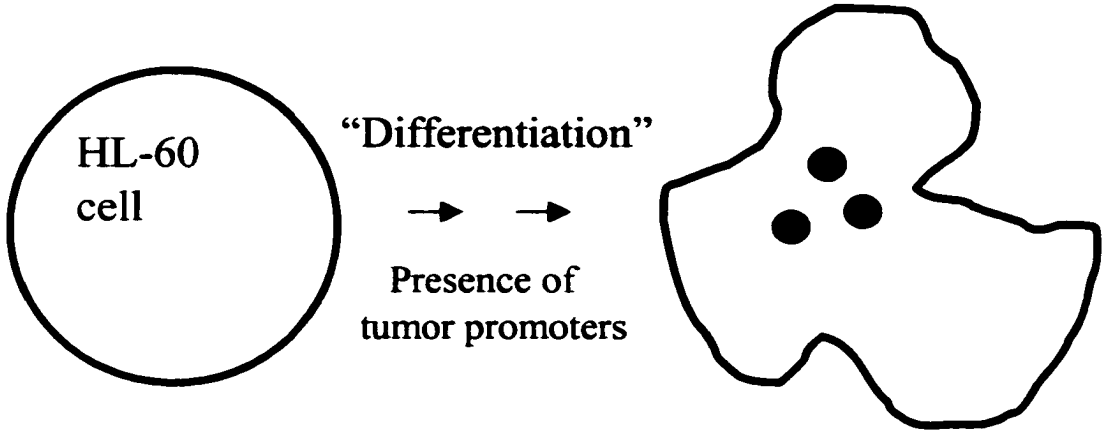
This group of studies was to determine the exposure conditions for testing if HL-60 cells would respond to EM field exposure and TPA treatment. HL-60 cells, following TPA treatment, can be induced from a suspension culture into fibroblastic-like phagocytic cells with several characteristics of macrophages, such as adherence to plastic plates and the ability for phagocytosis (Collins, 1987) (Figure 7). The advantage of using phase-control microscopic analysis was to observe the morphological changes and to distinguish cells with or without internal fluorescent particles.

As shown in Figure 8, HL-60 cells with internalized fluorescent particles can be analyzed using phase-contrast microscopy. The increased number of induced cells with fluorescent particles marks the *in vivo* phagocytic activity stimulated by external stimuli. The presence of microsphere-loaded cells was scored by microscopic inspection. By observing the microscopic depth of field, the particles inside the cells can be distinguished from those that stick on the cell surface.

Figure 7. HL-60 cell differentiation induced by TPA

This figure summarized the general process of HL-60 cells differentiation induced by TPA into terminally differentiated fibroblastic-like phagocytic cells. Both morphological changes and functional changes during differentiation can be monitored under microscopy and measured by flow cytometry using fluorescent particles as a marker.

## Phorbol esters induce HL-60 differentiation to the phagocytic endpoint in proportion to their *in vivo* promoting activity

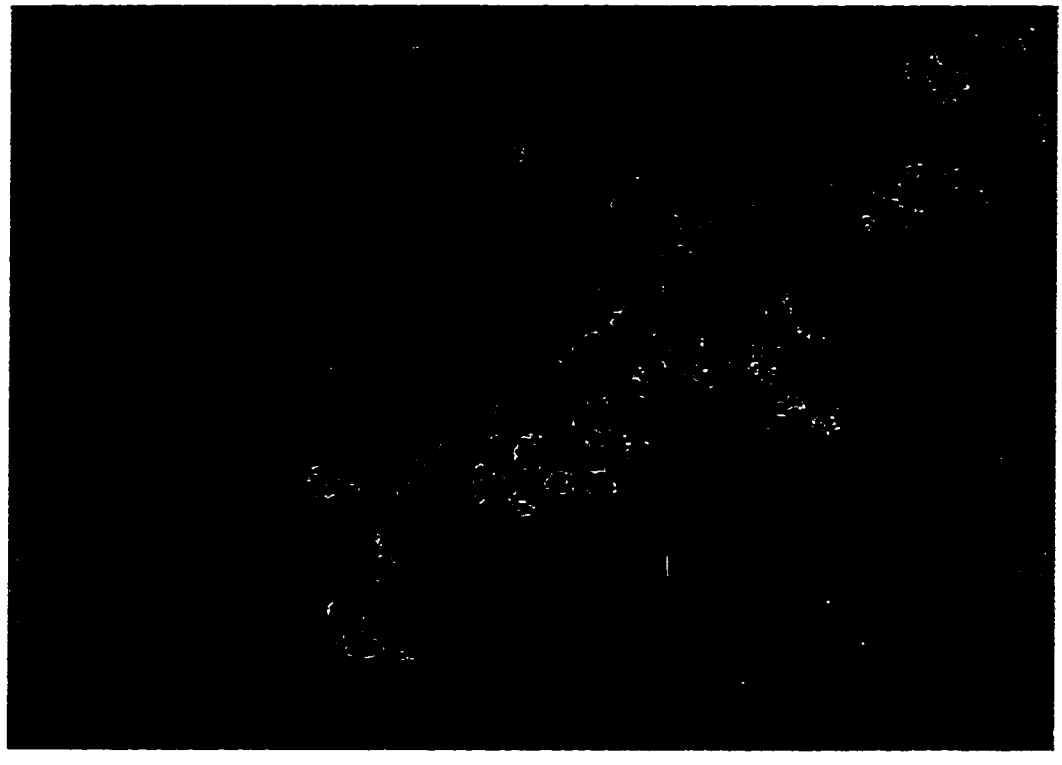



Non-phagocytic suspension culture

Attached fibroblastic-like culture with high phagocytic activity

Figure 8. Photomicrograph of phagocytic cells.

The illustration shows the terminal differentiated HL-60 cells exposed to TPA and/or EM fields for 24 hours. The differentiated cells are fluorescent particle containing cells.



The quantitation of microscopically examined cells showed that a 60 Hz, 60 mG EM field exposure for 24 hours can drive HL-60 cells toward fibroblastic-like macrophage cells with sham increased phagocytic activity (Figure 9). The data showed that the mean of induced HL-60 cells with phagocytic activity in a series of 9 experiments was 7.3% of total cells, while sham-exposure control was 2.5% of total cells. The difference is statistically significant. The relative effect of 60 mG EM field on phagocytic activity in exposed HL-60 cells to sham-exposed cells in the same 9 experiments (E/C ratio) was 3.0 using microscopic analysis (Figure 10). This study suggested that functional and morphological changes of differentiated cells can be examined by the simple method of microscopy.

This group of experiments was also designed to set up a positive control to be compared with EM field exposure. Microscopic examination demonstrated that TPA treated HL-60 cells (24 hours) were aggregated in small clumps and attached to the bottom of the wells while control cells were uniformly round and remained in suspension. As shown in Figure 11, HL-60 cells were induced into terminal differentiation with high phagocytic activity in the present of TPA at 6 ng/ml. This should be compared to the 2.5% induced phagocytic cells in sham-control, 7.3 % of induced phagocytic cells in EM field exposure and TPA induced 34% phagocytic cells at 24 hours. This result suggested that both TPA and EM field exposure can cause cell differentiation and TPA is a stronger inducer.

Cells exposed to a 500 mG magnetic field for 24 hours were also analyzed by microscopy. The results showed increased phagocytic activity. The mean of increase phagocytic activity in exposed cells of a series of 3 experiments was 2.0%, while that of the sham-exposure control was 1.7% (Figure 12). The relative effect of induction by 500 mG EM field (E/C ratio) was 1.2.

Figure 9. Microscopic analysis of the effect of a 60 mG EM field on phagocytic activity in HL-60 cells. The bars represent the percentage of induced differentiated HL-60 cells in total cell population in 9 experiments following exposure to a 60 mG EM field at 24 hours. The induced cells are fibroblastic-like cells with phagocytic activity. This analysis counts phagocytic cells which contain fluorescent beads under microscopic observation. The gray bars are sham-exposure controls. The dark bars are EM field exposures. The last set of bars is the mean ± SE of 9 experiments.

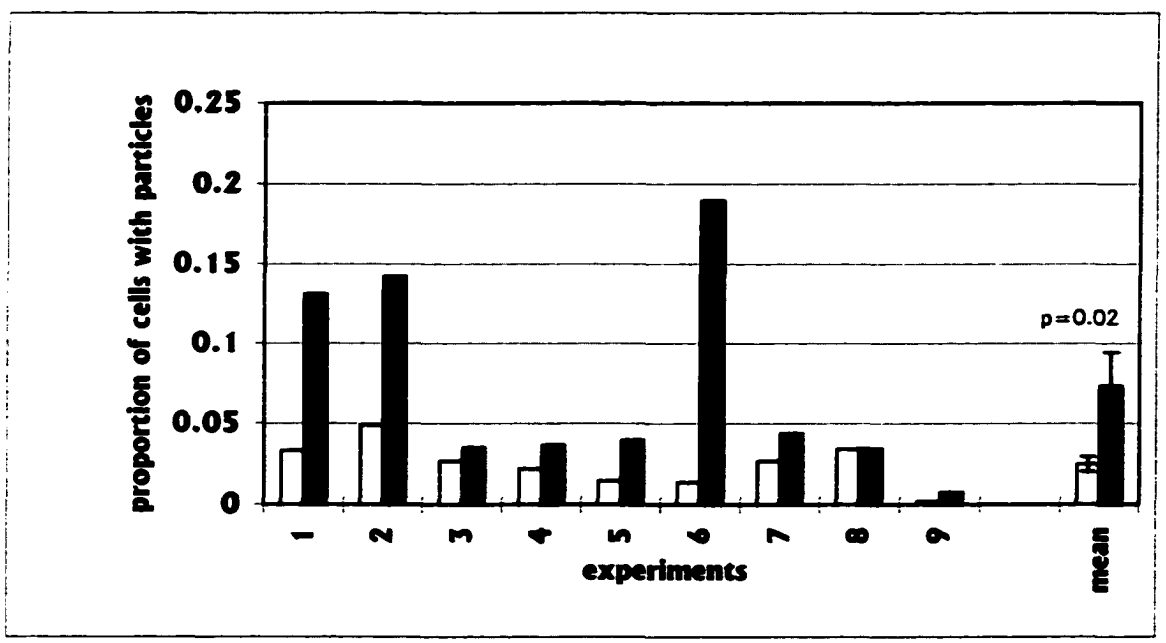


Figure 10. The relative effect of a 60 mG EM field on phagocytic activity in HL-60 cells

The experiments in this graph are the same as in Figure 9 using microscopic analysis. The bars represent the E/C ratio, which is the mean of the proportion of cells with particles in EM field exposure over sham-exposure in a series of experiments. The average mean of this series of experiments is 3.0. The difference of EM exposure over sham-exposure is statistically significant.

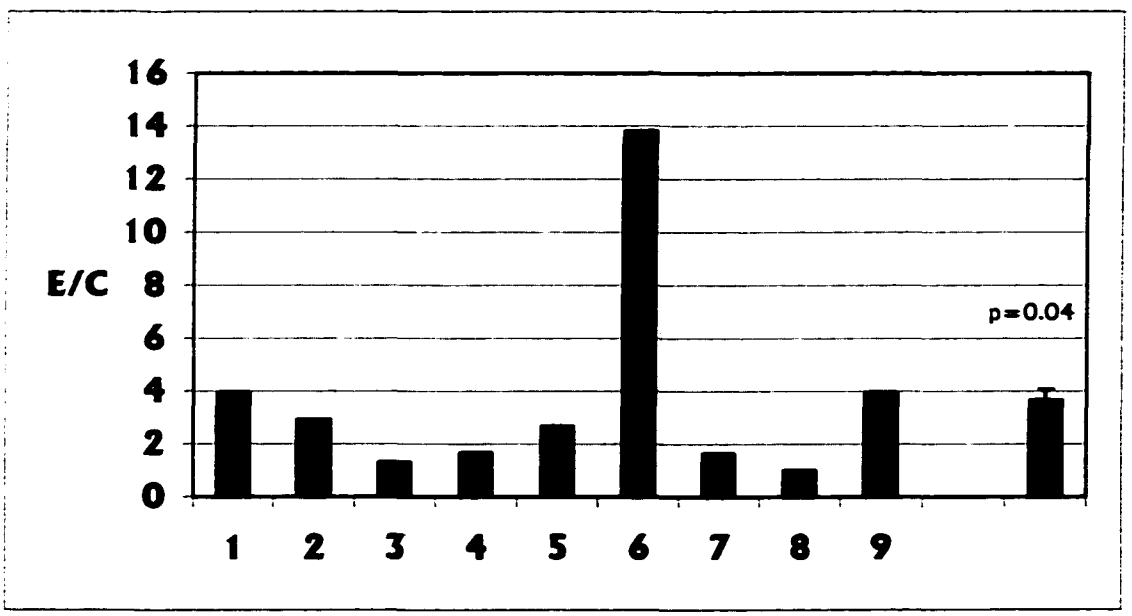


Figure 11. A comparison of the effects of TPA and 60 mG EM fields

The cells were counted under microscope. The bars represents the proportion of cells with particles. Cells were treated with either 60 mG EM field exposure or 6 ng/ml TPA for 24 hours. The data shows both EM field and TPA cause HL-60 cell differentiation and that TPA is a stronger inducer.

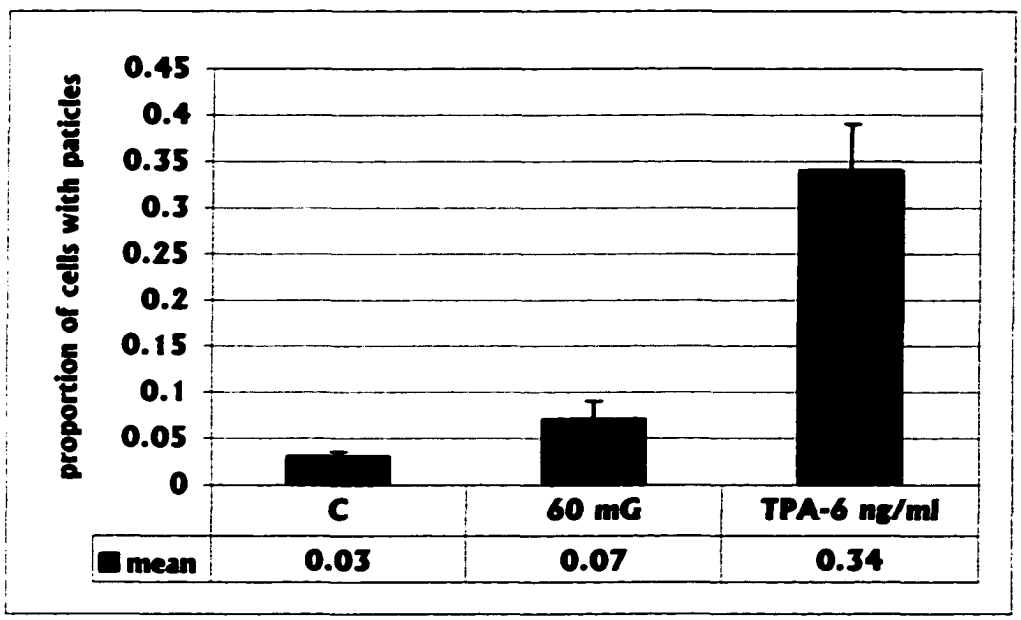
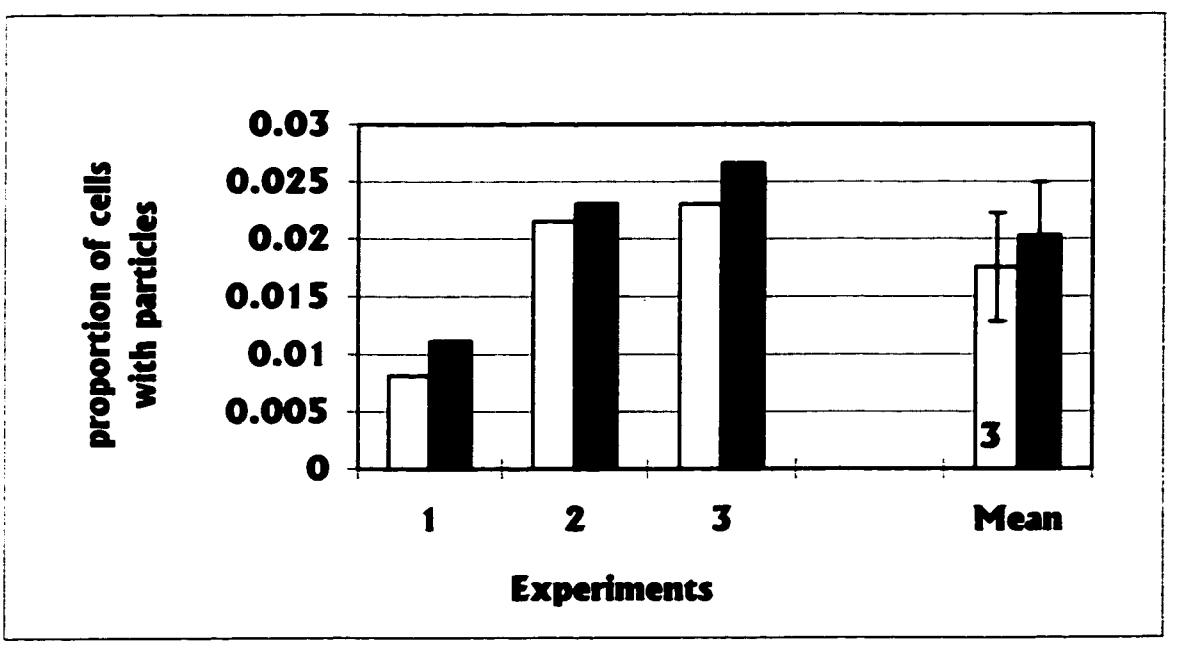


Figure 12. Microscopic analysis of the effect of a 500 mG EM field on the phagocytic activity in HL-60 cells. The bars represent the proportion of cells with particles (percentage of induced HL-60 cells ) in a series of experiments following exposure to a 500 mG EM field for 24 hours. The gray bars represent the sham-exposure controls. The dark bars represent the EM field exposure. The last set of bars is the mean ± SE in this series of experiments.



The difference is not statistically significant (Figure 13).

This study showed a differential response of HL-60 cells to 60 mG and 500 mG exposure conditions and also demonstrated that both EM field and TPA both cause cell differentiation. There are some experimental variations can be observed in 60 mG field exposure. And the base line levels of phagocytic cells of sham-exposure in 60 mG and 500 mG fields are different. This microscopic analysis data suggested a more sensitive and objective error-free method should be used to measure the effects of EM fields and the effect of cell status should also be considered.

**Flow Cytometry Analysis: The effects of TPA**

The use of flow cytometry was to set the parameters precisely to measure the functional changes of induced cells and to eliminate the subjective error while analyzing large populations of cells. Cells with internalized latex particles were clearly resolved from cells that were not associated with microspheres in green fluorescence using flow cytometry (Figure 14). Free microspheres and cells with phagocytosed particles were resolved with the forward scatter parameter (FSC) which corresponds to particle size. The forward scatter and green fluorescence (FLC) information was acquired in the dual parameter model. Analysis gates were set to quantitate cells with and without internalized spheres (R1), one or more internalized spheres (R2) and two or more internalized spheres (R3).

This experiment was performed using a series of concentrations of TPA at 0, 0.05, 0.10, 0.25, 0.50, and 1.0 ng/ml (Figure 15). The dosage conditions used in the present study tested the sensitivity of flow cytometric analysis. Flow cytometry imaging showed that the phagocytic activity of terminal differentiated HL-60 cells clearly delineated the potential of TPA induction

Figure 13. The relative effect of a 500 mG EM field on phagocytic activity in HL-60 cells The bars represent the E/C ratio, which is the mean of the proportion of cells with particles in EM exposure over sham-exposure in a series of experiments. The average mean of this series experiments is 1.2. The difference between sham-exposure and exposure is not statistically significant.

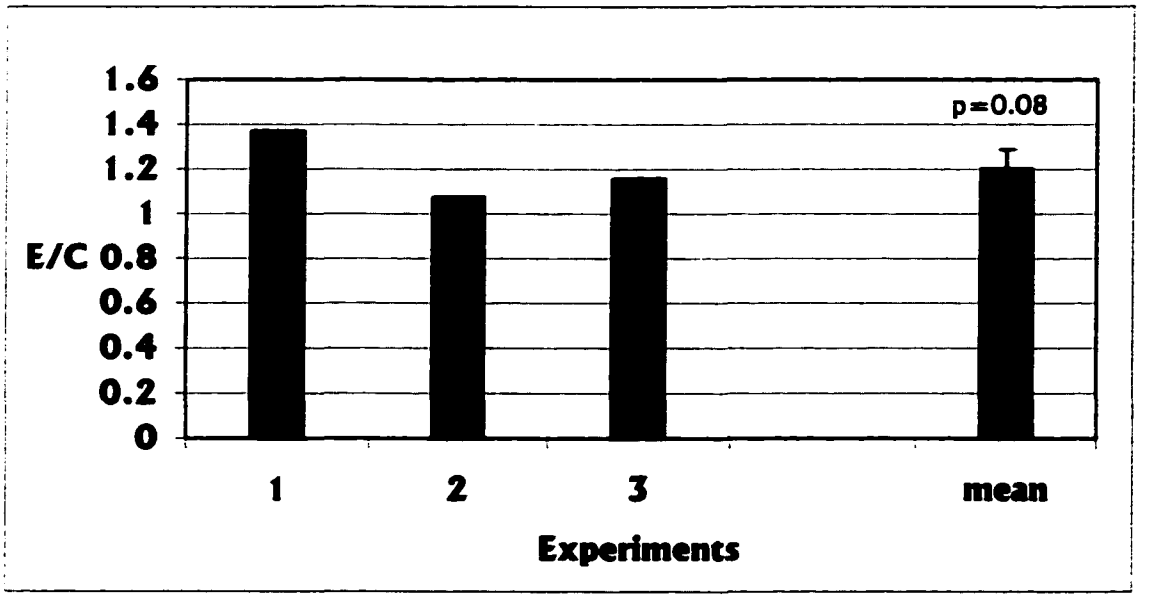


Figure 14. Diagram of flow cytometry analysis

The graph represents one example of flow cytometric analysis of TPA induced increased phagocytic activity in HL-60 cells. The statistics represent the counted particles and cells from a total 20,000. FSC is forward size scatter, representing the size of particles. FL represents the fluorescence intensity. R1 is the total cell particles. R2 is the cells containing one or more fluorescent particles. R3 is the cells containing two or more fluorescent particles

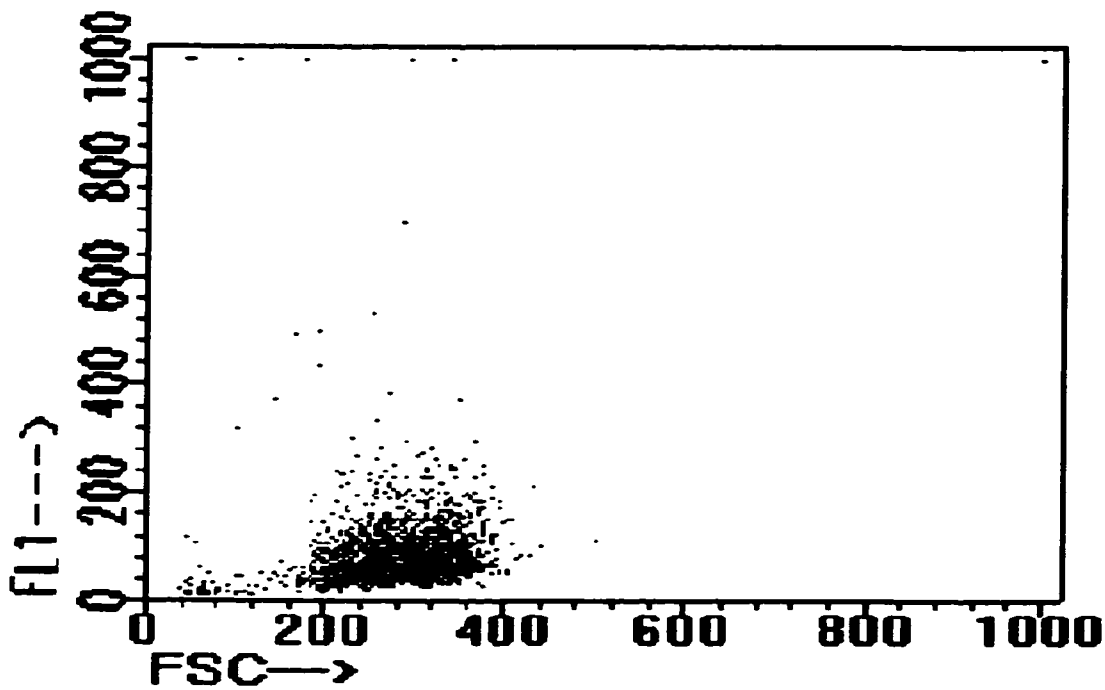
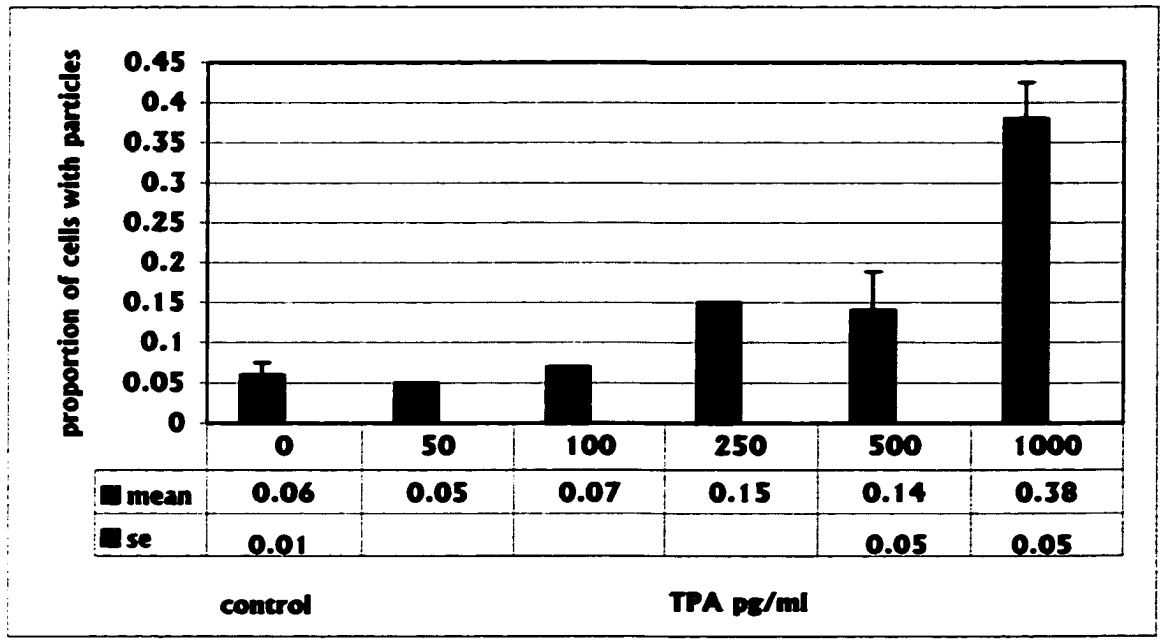


Figure 15. TPA dose-dependence of phagocytic activity in HL-60 cells

Cells were treated with a series of concentrations of TPA for 24 hours. Bars represent the mean of proportion of cells with fluorescent particles of total cell population. Bars with SE markers represent three or more exposures. Other experimental points were done in duplicate



in this experiment. The number of induced microphage-like cells increases with the increase of TPA concentrations. The internalized particles increase with the increment of TPA concentrations. At the low concentrations of TPA (50-500 pg/ml), most induced cells were gated as containing one fluorescent particle, while at higher concentrations of TPA (1 ng/ml and above), the induced cell contains two or more particles. The number of internalized particles and the number of increased induced cells reflect the potential of TPA induction. This study demonstrated that HL-60 cell differentiation is TPA dose-dependent. HL-60 cells treated with TPA (500 pg/ml) also showed that the increased phagocytic cells appeared significantly at12 hours (Figure 16). This study indicated that HL-60 cell differentiation is TPA time-dependent.

This study built up a dose-and time dependence between low concentrations of TPA and HL-60 differentiation including morphological and functional changes. This provided a comparable measuring system to identify the intensity and time effects of EM field and to determine the end-point effect of EM field effects on cells.

**Flow Cytometry Analysis: The Effect of EM Field Exposure**

The following experiments used flow cytometric analysis to show that a differential response was observed after HL-60 cells were exposed to 500 mG, 1 G and 2 G magnetic fields for up to 24 and/or 48 hours.

As shown in Figure 17, 500 mG EM field exposure for 24 hours did not result in significant changes in the number of differentiated cells. The proportion of the number of induced cells in eight exposures is 0.54% while that of sham-exposure is 0.62%. The difference is not statistically significant. The result is consistent with the data from microscopic analysis in

Figure 16 Time course of HL-60 cell differentiation as induced by TPA treatment

HL-60 cells were treated with TPA (500 pg/ml) for 24 hours. The data indicate that an increase in phagocytic activity appears as early as 12 hours as compared to sham controls. The triangles represent the HL-60 cells treated with TPA (500 pg/ml). The squares are sham controls.

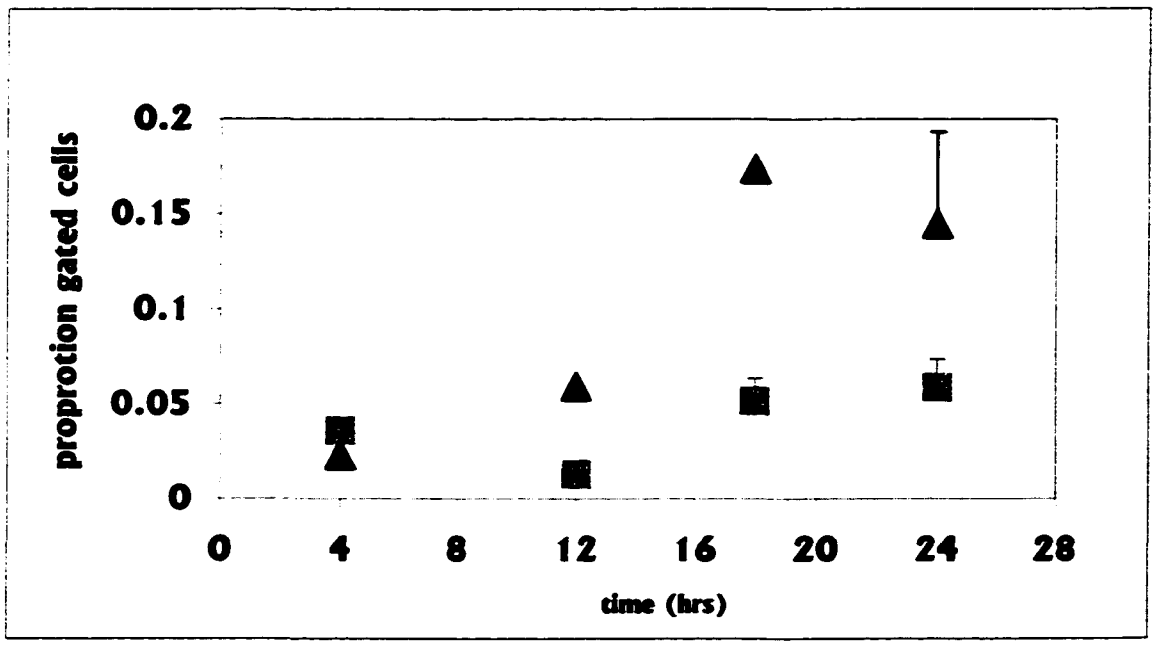
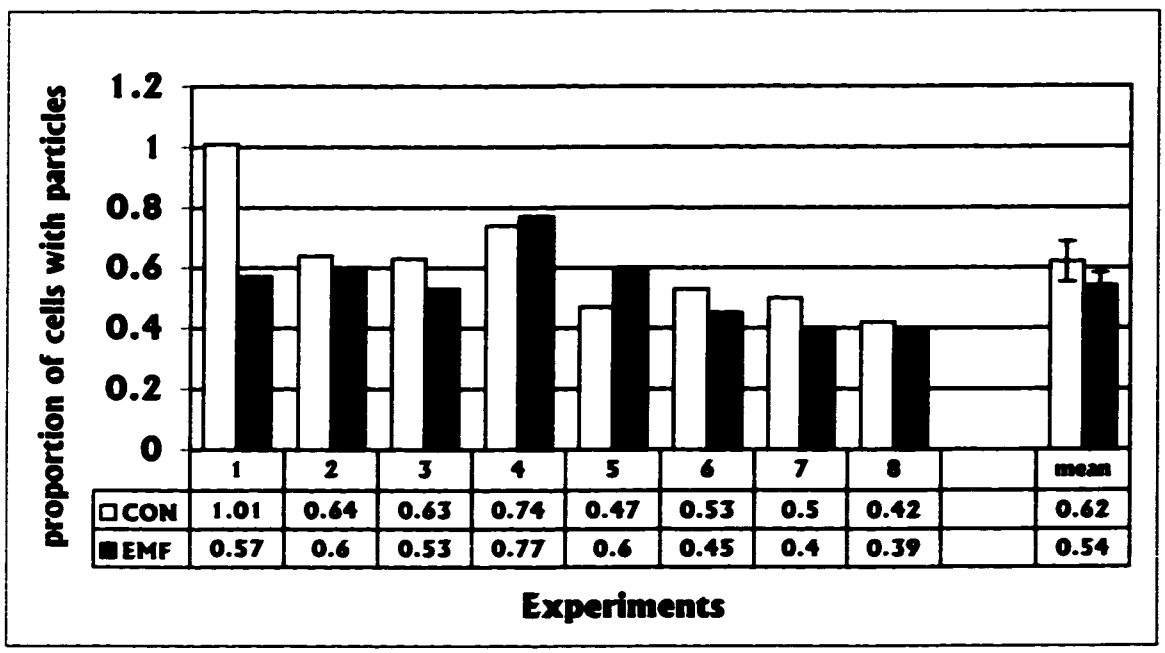


Figure 17. Effect of 500 mG exposure on phagocytic activity in HL-60 cells

The figure summarizes a series of 8 exposures of HL-60 cells to 500 mG EM field at 24 hours. The bars represent the mean of the proportion of induced cells in total cell population. The data obtained from flow cytometric analyses shows a non-significant change.




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | | mean |
|---|---|---|---|---|---|---|---|---|---|---|
| □CON | 1.01 | 0.64 | 0.63 | 0.74 | 0.47 | 0.53 | 0.5 | 0.42 | | 0.62 |
| ■EMF | 0.57 | 0.6 | 0.53 | 0.77 | 0.6 | 0.45 | 0.4 | 0.39 | | 0.54 |

which no statistically significant increase of differentiated cells was observed in HL-60 cells exposed to 500 mG EM field exposure.

A significant increase in the number of phagocytic cells was observed in cells exposed to a 60 Hz, 1 G EM field for 24 hours. The proportion of induced cells in five exposures is 10% while that in sham-exposure is 6% (Figure 18). The significant increase of phagocytic cells appears at 24 hours and this induction lasts 48 hours. The relative effects of 1 G EM fields on induction (E/C ratio) at 24 and 48 hours is 2.5 and 1.5, respectively (Figure 19). In each experiment, flow cytometry counted 20,000 particles, the reason for the reduced E/C ratio at 48 hours could be due to the less number of experiments to overcome the experimental variation or the increased induced cells appeared in sham-exposure group at 48 hours and the cell growth continuously during elongated exposure (Table 3). The result also suggests that under the same intensities of exposure, the time of EM field exposure, cells status would affect differently in a temporal manner.

Table 3. Effect of 1 G EM field exposure on HL-60 cell differentiation (% gated induced cells)

| | 4 hr | 12 hr | 18 hr | 24 hr | 48 hr |
|---|---|---|---|---|---|
| control | 1.7% | 1.2% | 7.3% | 9.5% | 13.7% |
| 1 G EMF | 1.8% | 1.3% | 7.6% | 10.2% | 21.9% |

Figure 18. Effect of 1 G EM field exposures on phagocytic activity of HL-60 cells

The figure illustrates the proportion of cells with particles following exposure to 1 G EM field for 24 hours in 5 exposures. The last set of bars is mean ± SE. The graph shows a significant increase in phagocytic activity that is observed at 24 hours.

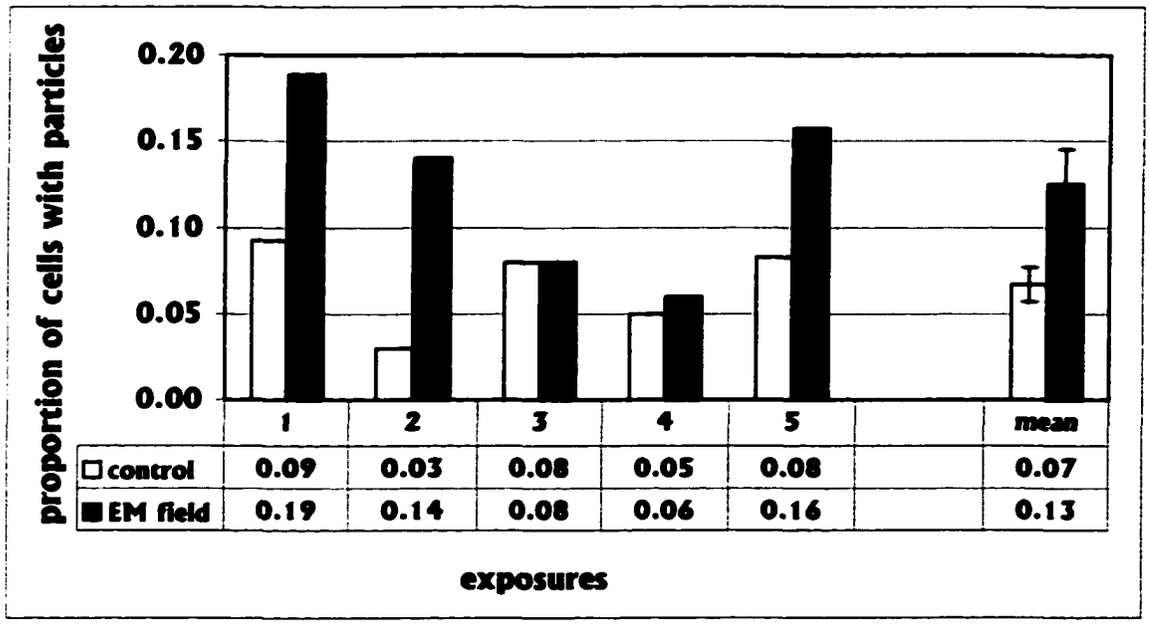
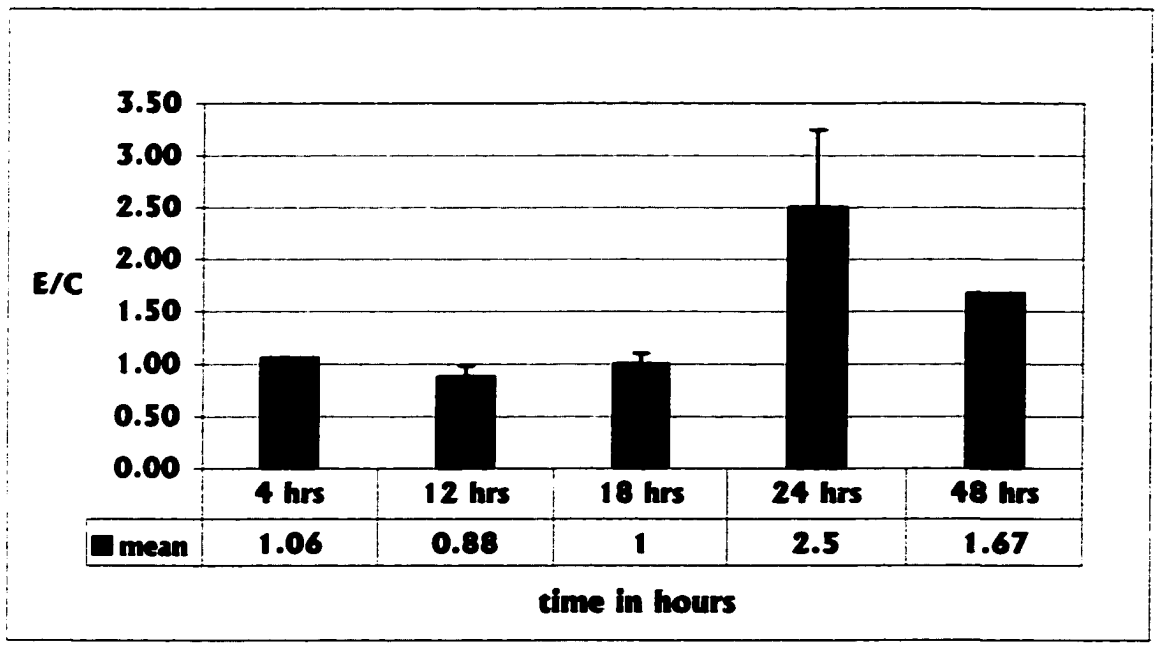


Figure 19. Time effect of 1 G EM field exposure on phagocytic activity in HL-60 cells

The figure shows the effect of time of exposure to a 1 G EM field on HL-60 cells. E/C represent the ratio of means of induced cells in EM exposure over sham-exposure. Number in bars are number of exposures. The difference is statistically significant.



Interestingly, the effect of 1 G EM field exposure affecting HL-60 cell differentiation at 24 hours is equivalent to 250-500 pg/ml TPA (Figure 20). This indicated that EM field has an end-point effect comparable to a potent tumor promoter, TPA at low concentrations.

Cells exposed to a 60 Hz, 2 G magnetic field for 24 hours also showed an increased number of induced phagocytic cells. Similar to the effect of 1 G EM field, the earliest significant maximum effect appear at 24 hours and the E/C ratio is 1.5, which was lower than that in cells exposed to 1 G (Figure 21) (Table 4).

Table 4. The effect of exposure of 2 G EM field on HL-60 cell differentiation (% gated cells)

| | 4 hr | 12 hr | 18 hr | 24 hr |
|---|---|---|---|---|
| Control | 1.1% | 1.8% | 2.8% | 8.8% |
| 2 G EMF | 1.2% | 1.3% | 3.0% | 8.4% |

The data from using flow cytometry and microscopic analysis suggested both time and intensity of EM field exposure would affect HL-60 cells differentiation differently. In this experiment, 500 mG exposure may cause insignificant changes while 1 G and 2 G EM fields induced apparent terminal differentiation.

**Cell Differentiation and TPA/EMF In Concert**

The previous experiments showed that HL-60 cells were responsive to TPA or EM field as measured by induced differentiation. The data also demonstrated that, at concentrations of TPA between 50 and 500 pg/ml, HL-60 cells showed a linear differentiative response (Figure 15).

Figure 20. Comparison of the effect of 1 G EM field and TPA on HL-60 cell differentiation The graph shows the effect of 1 G EM field and that of a TPA in a series of concentrations for 24 hours. The data shows that the induction by a 1 G EM field is equivalent to that of 250-500 pg/ml TPA. The bars represent the means of proportion of induced cells with particles. Bars with SE markers represent three or more exposures. Other experimental points were done in duplicate

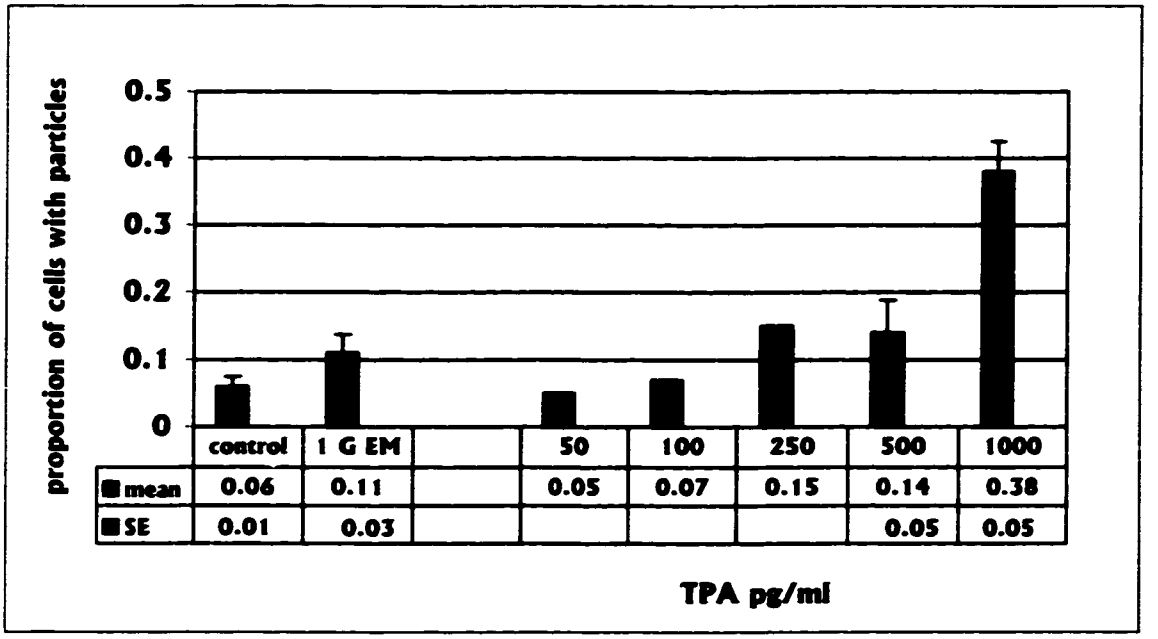
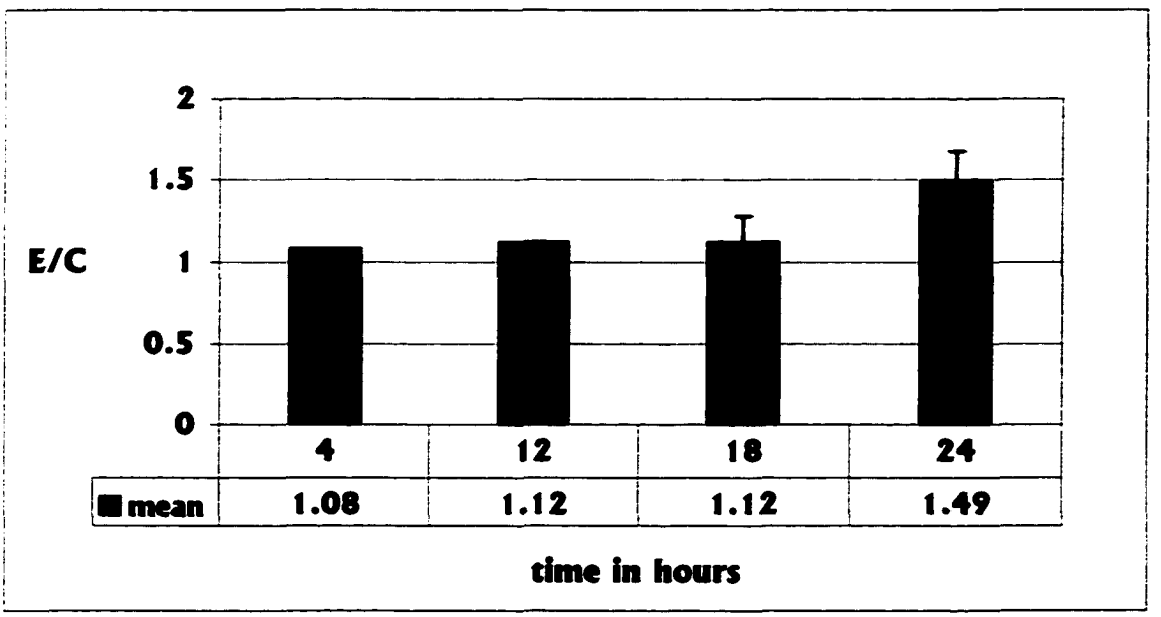


Figure 21. Effect of 2 G EM Field on phagocytic activity of HL-60 cells

The figure shows the results of a series of exposures of HL-60 cells to 2 G EM field for 24 hours. The E/C ratio is used to summarize the data after flow cytometric analyses. It is expressed as means ± SE. Numbers in bars are number of exposures. The increase is significant ($p=0.04$).



Within this range of TPA concentrations, cells exposed to a 1 G EM field and TPA in concert for 24 hours demonstrated an additive effect (Figure 22). This additive effect is swamped if TPA is at 1 ng/ml or above. The additive effect of EM field exposure declines as the increment of TPA concentrations (50-500 pg/ml) (Figure 23). A similar additive effect was also observed in cells exposed to 2 G EM field with TPA (Figure 24).

The present data demonstrates that the 1 G EM field result in HL-60 cell differentiation and the existence of additive effect with TPA indicates that EM field exposure and TPA treatment could share a common induced signaling pathway.

Figure 22. EM field exposure and TPA treatment together are additive.

Cells were exposed to either TPA or TPA with a 1 G EM field. The data is from a single experimental series, but other experiments gave comparable data. Gray bars, data from TPA treatments, dark bars, data from cells exposed to TPA and 1 G EM fields. TPA is at concentrations of 50-2,500 pg/ml). The first set of bars are sham-exposure in the absence of TPA and EM field exposure.

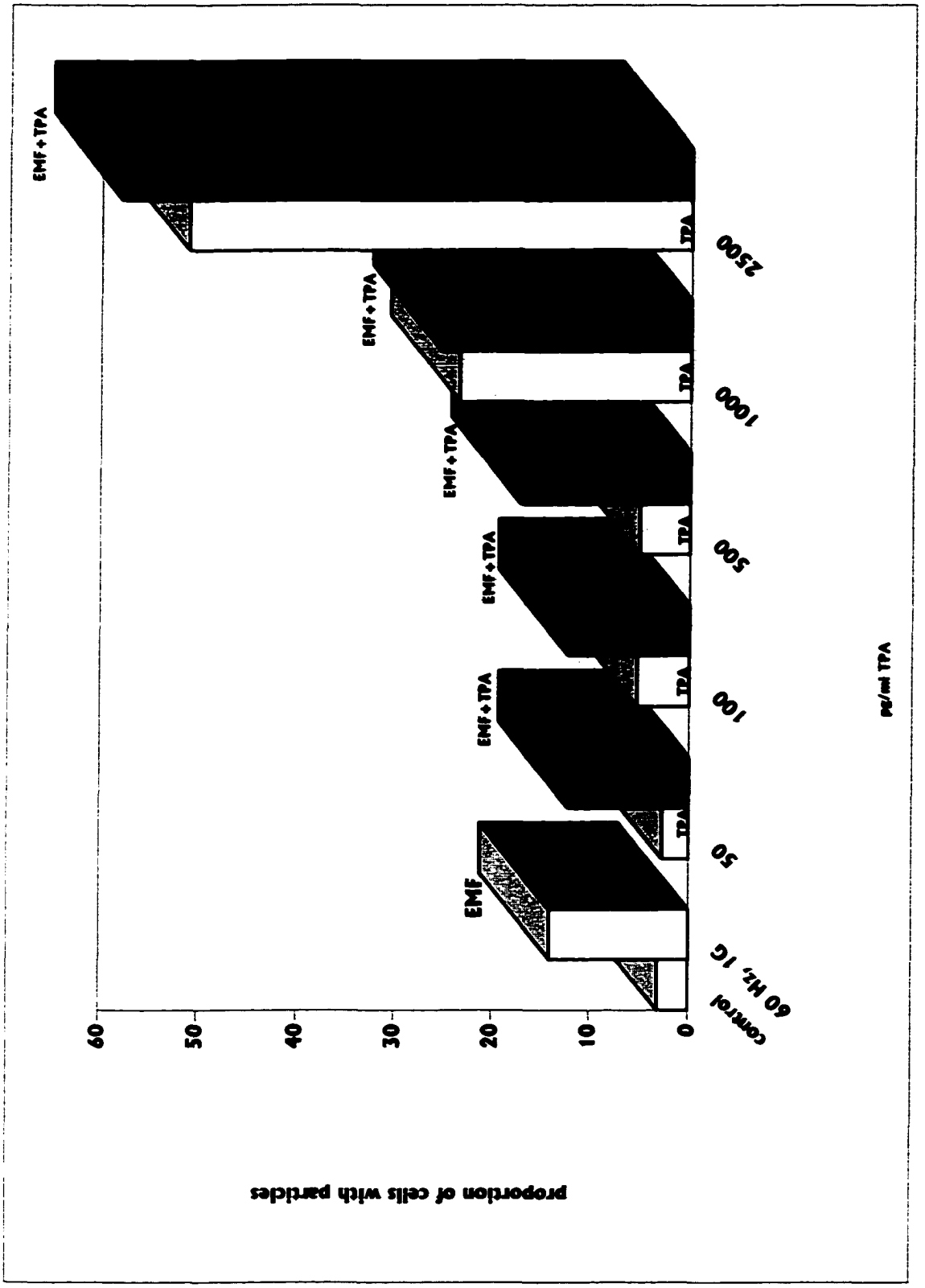


Figure 23. Additive effect of 1 G EM field with TPA on phagocytosis

Graphic representation of the results of exposure of HL-60 cells to a 1 G EM field and TPA with EM field for 24 hours. Measurements are phagocytized fluorescent particles in cells isolated from sham-exposure or EM field exposure. E/C is expressed as mean ± SE. Number in bars are number of experiments.

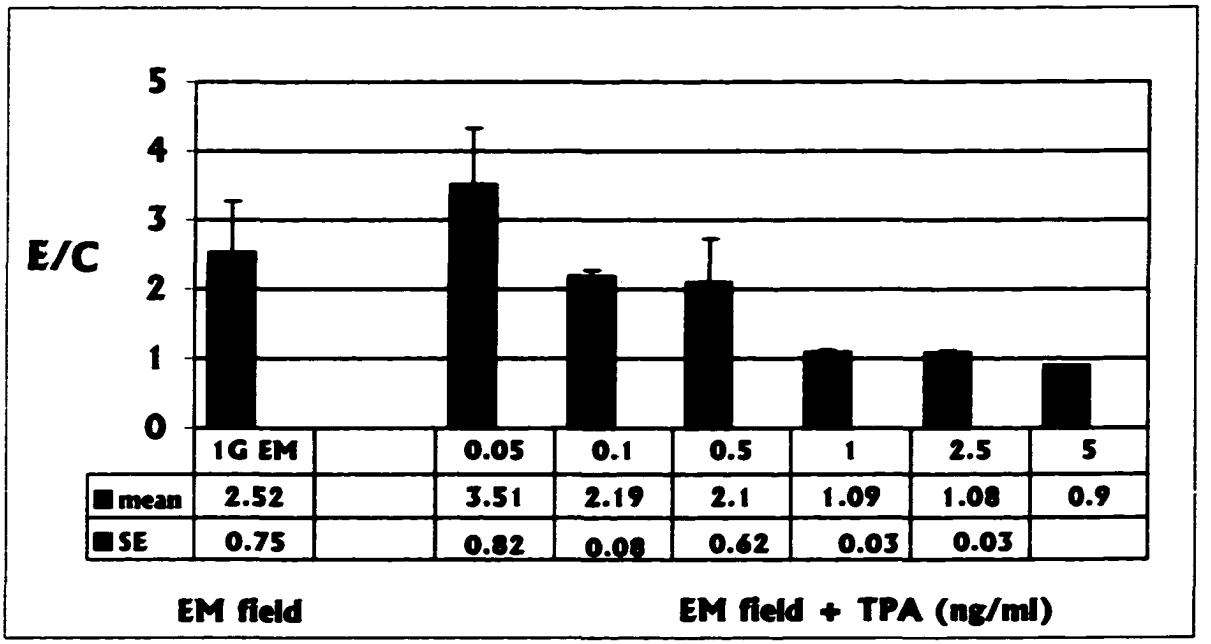
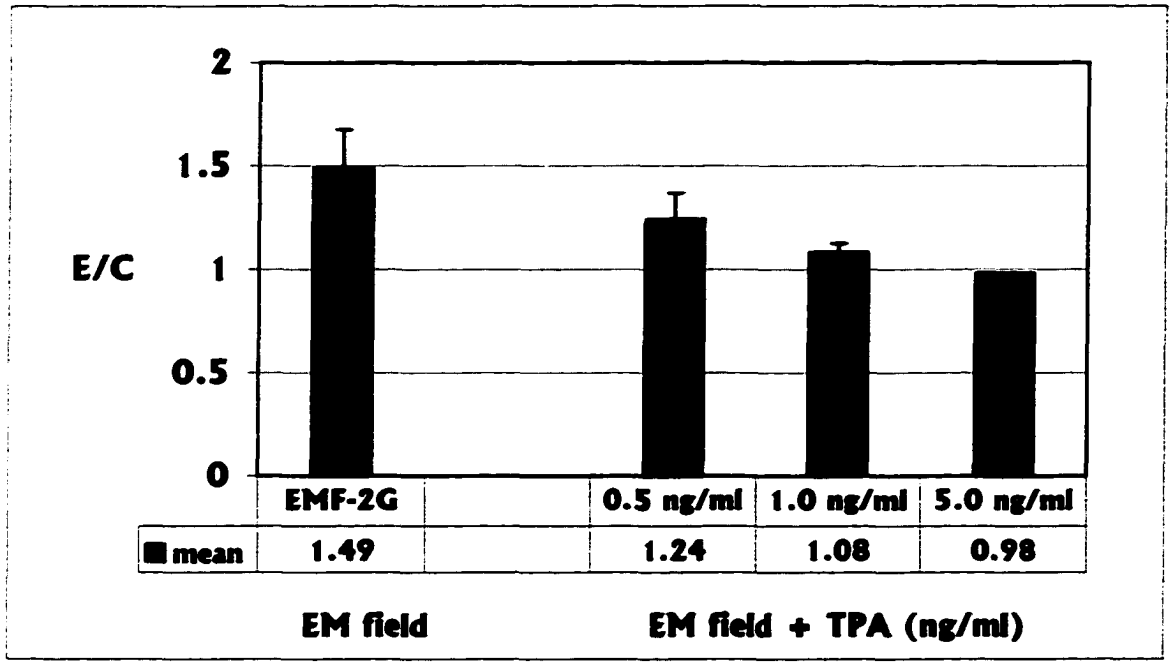


Figure 24. Additive effect of 2 G EM field with TPA on phagocytosis

Graph represent the results of exposures of HL-60 cells to 2 G EM field with TPA for 24 hours. E/C is expressed as mean ± SE. Number in bars are number of experiments.



**Part Three: Magnetic field exposure and Protein Kinase C**

PKC is involved in TPA induced HL-60 cell differentiation. If PKC is affected by EM field exposure, it would provide an opportunity to compare the effects of EM field and TPA on related transduction pathways.

This research measured the translocation of PKC-α under two exposure conditions, 60 Hz at 60 mG and 1 G magnetic fields. In each case, these were compared to TPA treatment. An increase in PKC translocation is significant following under treatment of TPA. Total PKC-α was down-regulated in HL-60 cell differentiation following TPA treatment of 24 hours. The down-regulation of PKC-α is not observed in HL-60 cells exposed to 1 G EM field for 24 hours. A significant change was observed in HL-60 cells exposed to 60 Hz, 1 G magnetic field at 5, 20 and 30 minutes. However, at most other time points the values for the PKC translocation were not significant (2.5, 10, 60 and 90 minutes, 18 hours). The data is therefore, inconclusive. The experiments also showed changes of PKC-α translocation in cells exposed to a 60 mG EM field. The changes of PKC-α translocation under 60 mG field are also not significant.

**Identifying PKC Subtypes**

Western blotting was used to determine the PKC translocation and specific PKC subtypes. Monoclonal anti-PKC subtypes were used to identity the PKC subtypes in HL-60 cells. The results demonstrate that PKC-α is the major subtype of PKC in HL-60 cells. PKC-β is detectable in both western blotting and immunoprecipitation, however, the signal is weak while using western blotting only. Neither of the two anti-PKC-δ recognized PKC-δ as an appropriate signal in this cell line using either western blotting or immunoprecipitation. One anti-PKC-ϵ showed

a weak PKC-ϵ signal with immunoprecipitation (Table 5). The PKC subtypes in this cell line is consistent with other studies suggested PKC-α, β, δ may affect HL-60 cells differentiation (Wooten *et al.*, 1993).

Having identified PKC subtypes in HL-60 cells, PKC-α was chosen to study the changes of PKC translocation after magnetic field exposure due to consistent signal. For each experiment, the cells were divided into sham-exposure, EM field exposure and TPA treatment.

Table 5. Identifying PKC subspecies in HL-60 cells

| | PKC-α | PKC-β | PKC-δ | PKC-ϵ |
|---|---|---|---|---|
| western blotting | strong | weak | weak | N/A |
| Immuno-precipitation & western blotting | strong | strong | weak | weak |

**PKC-α translocation under TPA treatment**

In these experiments, HL-60 cells were treated with TPA at 1.0 ng/ml. TPA can efficiently drive HL-60 cells into macrophage-like cell at this concentration. The data demonstrated that the translocation of PKC-α from cytosol to membrane is observed under TPA treatment (Figure 25) (Table 6). The total PKC-α was also measured at different time points (1, 6 and 24 hours) (Figure 26)(table 7). The total PKC was down-regulated significantly at 24 hours under 1 ng/ml TPA treatment. This observed down-regulation of PKC-α suggested involvement of PKC in cell differentiation. This down-regulation is also consistent with other observations that TPA induced

Figure 25. Effect of TPA treatment on PKC-α translocation.

The graph demonstrates the results of exposure to TPA at 5, 10, 15 and 20 minutes. The measurement used western blotting for determination of membrane translocated PKC in cells isolated from controls( C ) or (E) TPA. E/C is expressed as means ± SE. Experiments were done in duplicate. TPA significantly cause PKC translocation.

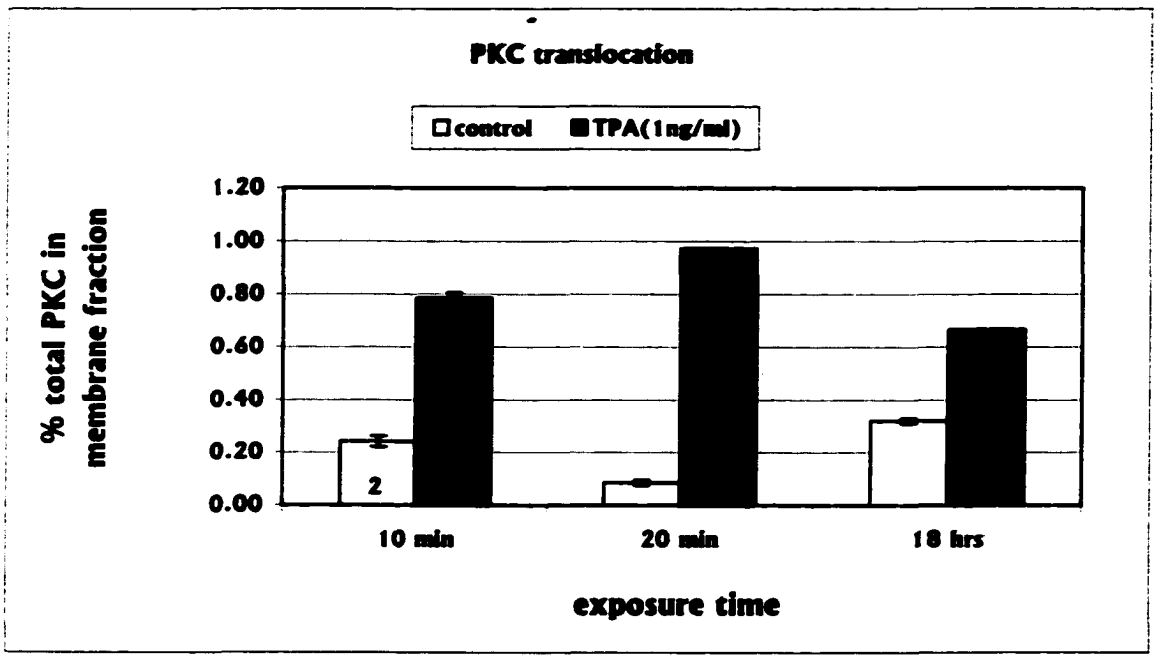


Table 6. Effect of TPA treatment on PKC translocation

| Time | 10 minutes | | 20 minutes | | 18 hours | |
|---|---|---|---|---|---|---|
| | control | TPA | control | TPA | control | TPA |
| experiments | 2 | 2 | 2 | 2 | 2 | 2 |
| mean(%mem) | 24% | 79% | 9% | 97% | 32% | 68% |
| SE | 0.1 | 0.02 | 0.01 | 0.00 | 0.01 | 0.00 |

Figure 26. Comparing the total PKC α measured in cells exposed to 1 G EM field and TPA treatment for 24 hours. The bars represent mean ± SE of the real value of total PKC α measured by densitometer. The number on bars represent the experiments. Cells are from the same parent flask, then exposed to 1 G EM field or 1 ng/ml TPA for 24 hours.

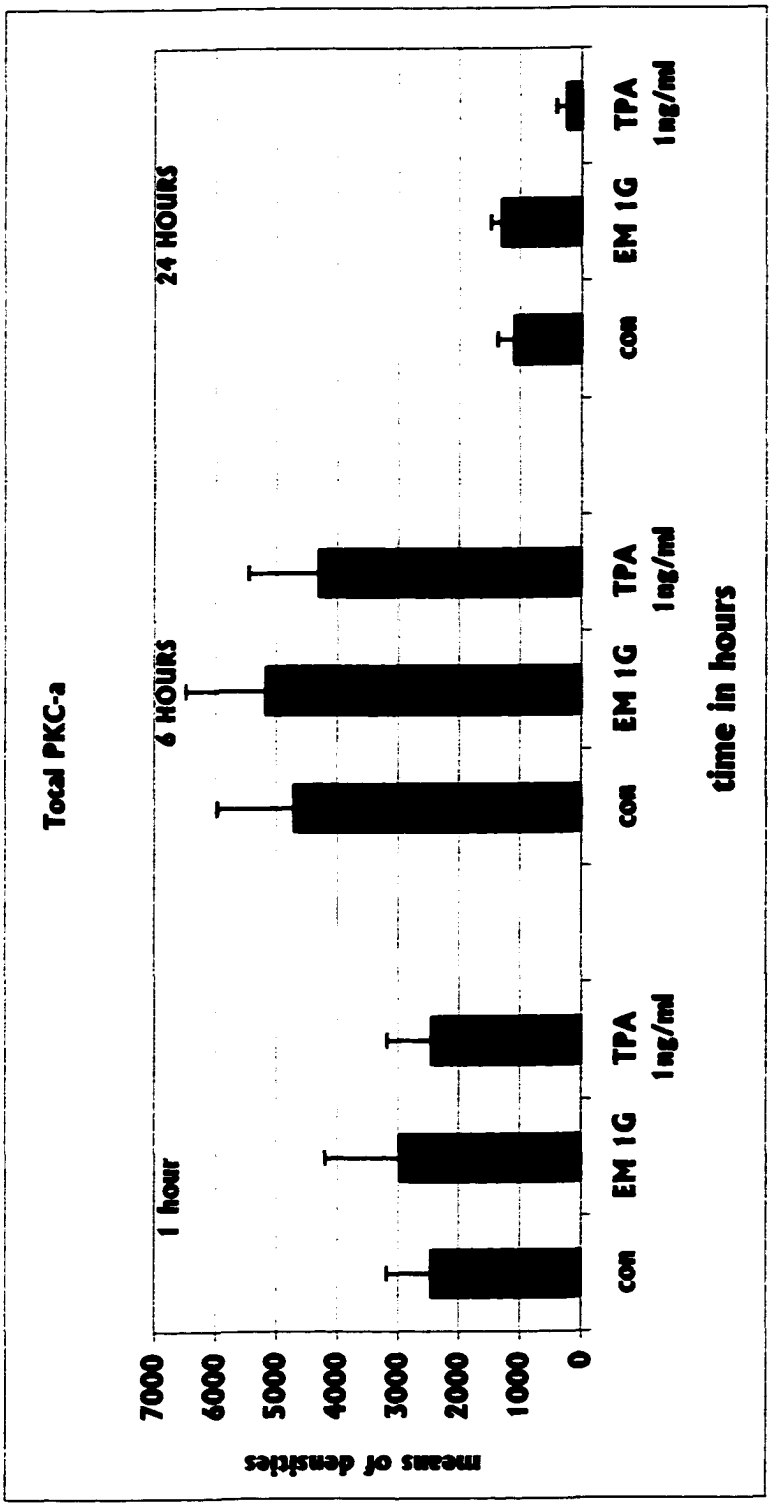


Table 7. Comparing the total PKC α measured in HL-60 cells exposed to 1 G EM field and TPA treatment.

| time | 1 hour | | | 6 hours | | | 24 hours | | |
|---|---|---|---|---|---|---|---|---|---|
| | CON | EM | TPA | CON | EM | TPA | CON | EM | TPA |
| exp | 10 | 10 | 10 | 6 | 6 | 6 | 4 | 2 | 4 |
| mean | 2445.2 | 2957.5 | 2438.5 | 4708.6 | 5170.8 | 4292.2 | 1086.5 | 1296.9 | 235.9 |
| SE | 742 | 1237.6 | 747.6 | 1262.1 | 1307.4 | 1164.8 | 285.5 | 187.2 | 177.7 |
| p(T≤t) | | 0.25 | 0.49 | | 0.08 | 0.16 | | | 0.03 |

HL-60 cell differentiation is associated with the inhibition of cell growth (Collins, 1987). Cells exposed to 1 G EM field were also measured the total PKC-α at 1, 6 and 24 hours. As shown in figure 26, there was no significant change of total PKC-α was observed. This is different from long term TPA treatment which cause significant down-regulation of PKCα.

**PKC-α Translocation under EM Fields Exposure**

As shown in Figure 27, 60 mG EM field exposure caused a change in membrane bound PKC-α at 5, 10, 15, and 20 minutes. The E/C ratio of the means of PKC translocation are 0.7, 1.3, 1.1 and 0.6 at 5, 10, 15 and 20 minutes, respectively. However, the difference between EM field exposure and sham-control is not statistically significant (Table 8). The nonsignificant changes of PKC-α translocation do not provide sufficient evidence to relate the long term changes of cell response to this PKC changes.

HL-60 cells were exposed to a 1 G EM field at 2.5, 5, 10, 20, 30, 60, 90 minutes and 18 hours (Figure 28) (Table 9). The data demonstrated that 1 G EM field exposure caused an increase of PKC-α translocation at 5, 20 and 30 minutes. However, in most other time points, such as, 2.5, 10, 60, 90 minutes and 18 hours, this increase of PKC-α is not statistically significant although the time course of PKC translocation shows a trend of long term persistent activation of PKC-α in the presence of the EM field. The early increase of PKC-α translocation induced by 1 G EM field is consistent with TPA activated PKC-α translocation, although the increase is not as steep as TPA. However, in the long term treatment, TPA cause total PKC down-regulation or depletion while EM fields do not (Figure 26).

This study demonstrated a differential response of PKC-α translocation at different EM

Figure 27. Summary of the effect of 60 mG EM field exposure on PKC translocation

A. Data from analysis of western blots of membrane translocated PKC-α in HL-60 cells exposed to 60 Hz, 60 mG EM field at 5, 10, 15 and 20 minutes. PKC measurements are from cells exposed to EM field or isolated from controls. E/C is repressed as mean ± SE. The increase in PKC translocation is not statistically significant.

B. The graph represent the means of real value of % total PKC α in membrane fraction of sham-exposure control and EM field exposure.

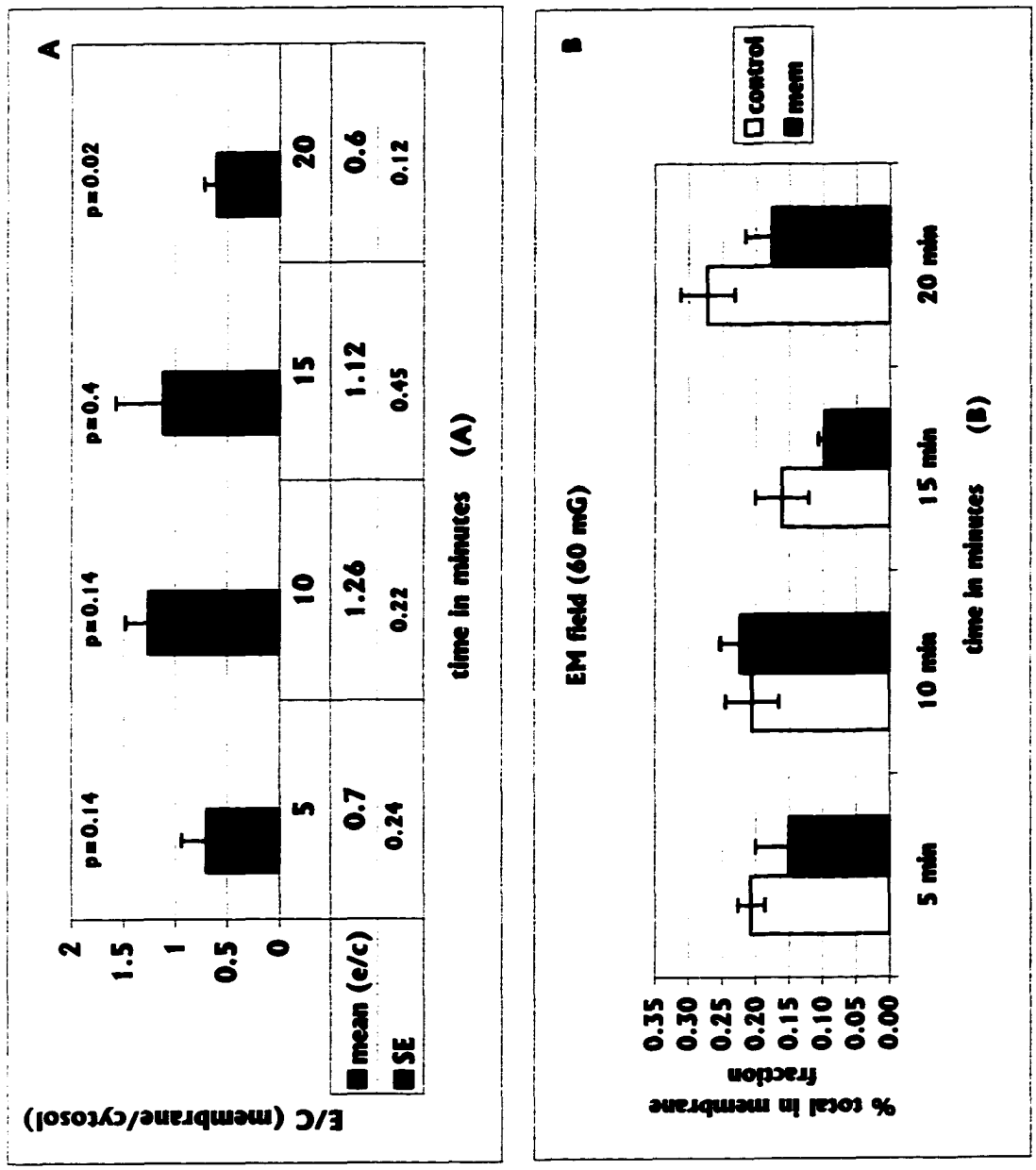


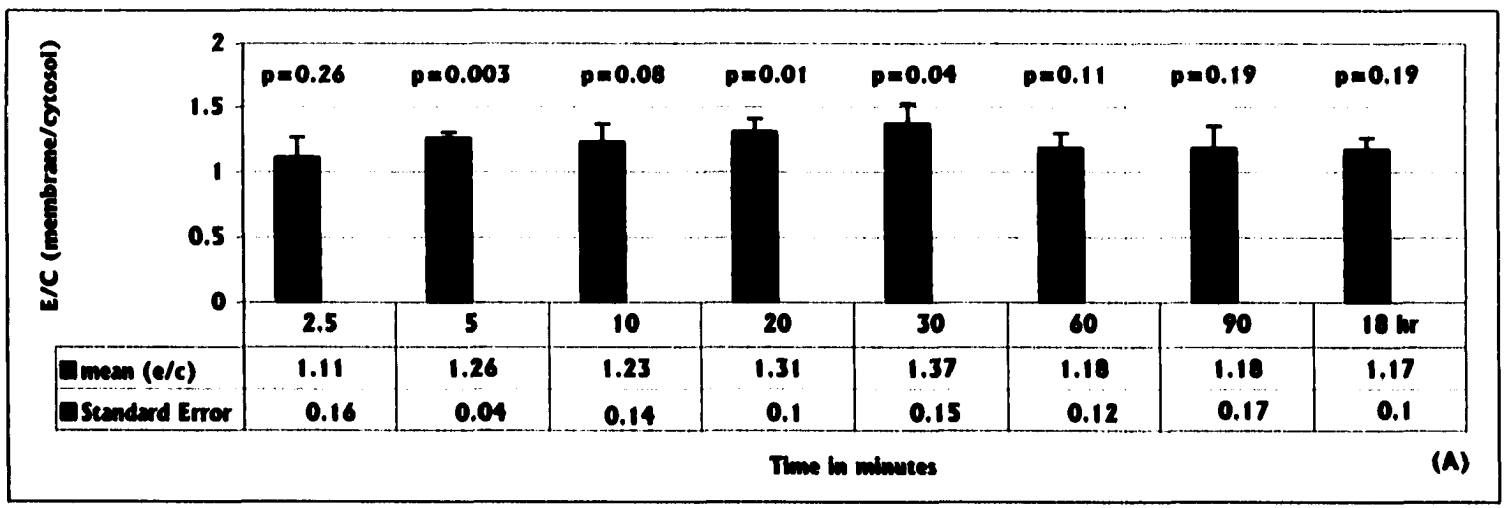
Table 8. Effect of 60 mG EM field on PKC translocation (measurement of % membrane PKC)

| Time | 5 min | | 10 min | | 15 min | | 20 min | |
|---|---|---|---|---|---|---|---|---|
| | Con | EM | Con | EM | Con | EM | Con | EM |
| exp | 5 | 5 | 6 | 6 | 7 | 7 | 4 | 4 |
| mean | 21% | 15% | 21% | 22% | 16% | 10% | 27% | 18% |
| SE | 0.02 | 0.05 | 0.04 | 0.03 | 0.04 | 0.01 | 0.04 | 0.04 |
| p(T≤t) | | 0.12 | | 0.29 | | 0.06 | | 0.03 |

Figure 28. Effect of 1 G EM field exposure on translocation of PKC α.

A. The relative ratio (E/C ratio) of EM field effect on PKC α translocation. Exposure at 5, 20 and 30 minutes, the increase of PKC α is small and statistically significant. However, exposure at most other time points the difference between sham-exposure and EM exposure is not significant. The calculation of E/C is the ratio of PKC in membrane/cytosol of EM field to the ratio of PKC in membrane/cytosol of sham-exposure.

B. Comparing the means of the real value of % total PKC α in membrane fraction of sham-exposure control and EM field exposure. The data demonstrate inter-experimental variation.



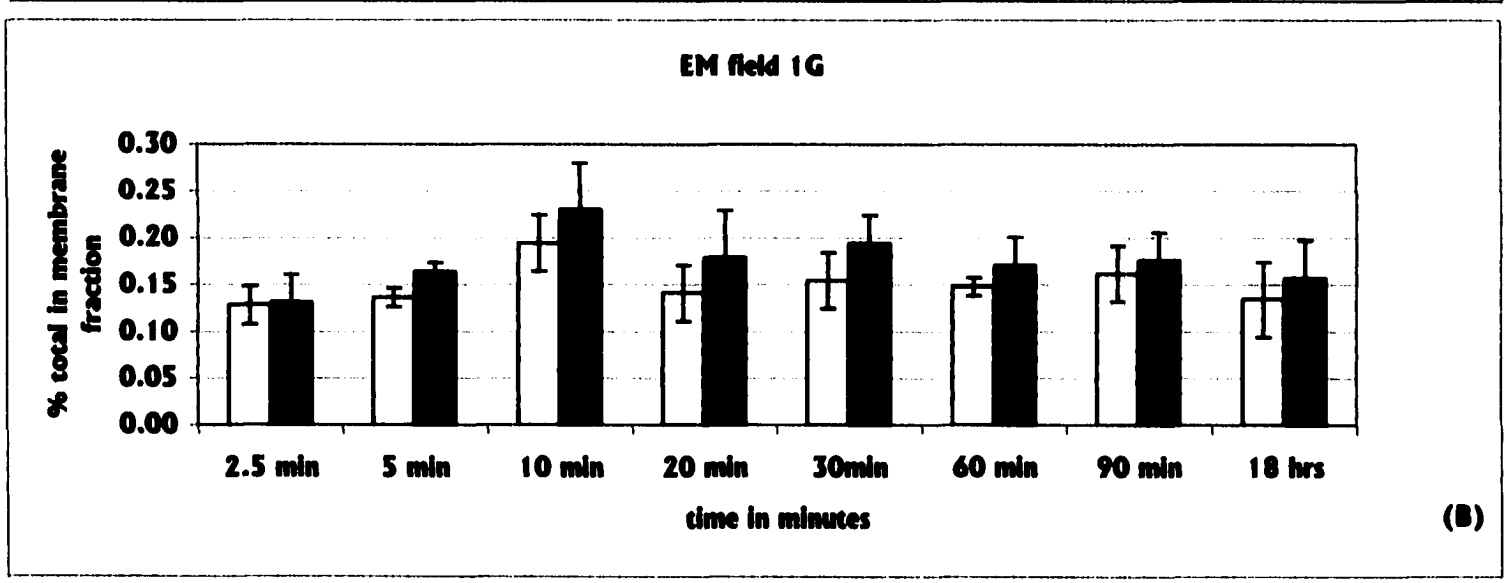



Table 9. Effect of 1 G EM field on PKC α translocation (measurement of % membrane PKC)

| Time | 2.5 min | | 5 min | | 10 min | | 20 min | |
|---|---|---|---|---|---|---|---|---|
| | Con | EM | Con | EM | Con | EM | Con | EM |
| exp | 6 | 6 | 5 | 5 | 6 | 6 | 8 | 8 |
| mean | 13% | 13% | 14% | 16% | 19% | 23% | 14% | 18% |
| SE | 0.02 | 0.03 | 0.01 | 0.01 | 0.03 | 0.05 | 0.03 | 0.05 |
| p(T≤t) | | 0.43 | | 0.001 | | 0.18 | | 0.03 |

| Time | 30 min | | 60 min | | 90 min | | 18 hours | 18 hours |
|---|---|---|---|---|---|---|---|---|
| | Con | EM | Con | EM | Con | EM | Con | EM |
| exp | 4 | 4 | 4 | 4 | 4 | 4 | 8 | 8 |
| mean | 15% | 19% | 15% | 17% | 16% | 18% | 13% | 16% |
| SE | 0.03 | 0.03 | 0.01 | 0.03 | 0.03 | 0.03 | 0.04 | 0.04 |
| p(T≤t) | | 0.04 | | 0.10 | | 0.27 | | 0.14 |

field exposure conditions as well as a different response of PKC-α to EM fields and TPA treatment. This study does not provide strong evidence that EM field induced HL-60 cell differentiation is associated with protein kinase C. The data on PKC translocation suggests that either EM field induced cell differentiation is not related to PKC signaling pathway or there are alternative signaling pathways.

## DISCUSSION

### Overview

The ultimate goal of studying HL-60 cell differentiation is to provide evidence that cell functions known to be altered during tumorogenesis can also be affected by electromagnetic field exposure. The present study suggests that HL-60 cell differentiation can be used as a sensitive method to find end point effects of EM field-cell interaction. The results from studies on PKC translocation suggests the existence of either a real lack of involvement of PKC, experimental deviation or a real difference between signaling pathways.

A 60 Hz electromagnetic field can induce differentiation in cultured hematopoietic progenitor cells. In addition, there is an additive effect between EM fields and the known tumor promoter, TPA. This suggests that EM field exposure could have a promotional or co-promotional effect on cell responses. Previous research suggest that TPA-induced HL-60 cell differentiation uses a predicted signaling pathway that has also been implicated in EM field exposure. PKC translocation and total PKC down-regulation was observed after cells were exposed to TPA. However, HL-60 cells exposed to EM fields do not cause similar changes. This study suggested that other alternative pathways could involve EM field induced HL-60 cell differentiation.

### Cell Differentiation and EM fields exposure

HL-60 cell differentiation was used to study tumor promotion. The rationale was based on prior observations. A known mouse skin cancer promoter, TPA, can significantly induce HL-60 cells into terminal fibroblastic macrophages. TPA affects many counterparts of cellular regulatory machine of tumorogenesis during its differentiation induction.

One of the most prominent characteristics of HL-60 cells is that these cells can be induced to differentiate into a number of different cell types of myelomonocytic lineage. Various agents induce HL-60 cells to differentiate into four general types of cells (Collins *et al.*, 1987). These include: (a) granulocytic differentiation induced by DMSO and retinoic acid; (b) monocytic differentiation induced by vitamin D3; (c) conversion to macrophage-like cells by the phorbol ester, TPA, and (d) eosinophil differentiation in alkaline media. The mechanism of action of HL-60 cell granulocyte induction is unknown. At least a 12 hour exposure to DMSO or retinoic acid is necessary before any differentiation occurs and continuous exposure to these compounds generally is required to induce maximum differentiation (Breitman *et al.*, 1980; Tarella *et al.*, 1982). Retinoic acid is believed to exert its biologic effect in many cells by interacting with a specific cytoplasmic retinoic-acid binding protein (RABP) (Sani *et al.*, 1974, Ong, *et al.*, 1975). However, HL-60 cells lack such a binding protein (Douer *et al.*, 1982). Retinoic acid-induced HL-60 differentiation is initiated at the cell surface (Yen *et al.*, 1984).

In the present experiments, TPA-treated HL-60 cells were markedly more adherent than untreated cells and associated with a rapid profound loss in proliferation capacity. Most of the treated cells were clumped and attached to plastic culture dishes. TPA increased the phagocytic activity of HL-60 cells in a dose dependent manner. Other investigators showed that at slightly higher concentrations (0.6 ng/ml) of TPA induced granulocytic differentiation of HL-60 in suspension culture. Concentrations of $\geq$ 0.84 ng/ml induced a macrophage-like phenotype. The linear increase in phagocytic activity under extreme low concentrations of TPA in this study supported previous results showing a dose-dependent response of this cell line. This is the rationale for using these procedures to measure the effects of EM field exposure. In these

experiments, flow cytometry measured the induction of TPA from treatment at concentrations as low as 50-100 pg/ml. The use of a sensitive assay circumvented some of the problems inherent in previous measurements The data showed 1 G EM field exposure causes cell differentiation equivalent to 250-500 pg/ml TPA, and 1 ng/ml TPA significantly swamps out the effect of the EM field. The result showed that the amplitude of the EM field could be analogous to a specific dose of TPA and it provides a possibility to measure the effects of much lower or much higher strengths of EM field exposure. The present results strongly indicate similarities between the effect of low concentrations of TPA treatment and EM field exposure.

More information have been obtained about the mechanism of TPA induced HL-60 cell differentiation. TPA exerts many of its effects through PKC, a calcium- and phospholipid-dependent enzyme that is widely distributed in numerous tissues. It plays a crucial role in transducing various extracellular signals across the cell membrane. The results of the present study showed that TPA can activate PKC translocation significantly and cause a total PKC-$\alpha$ down-regulation. other studies have suggested that TPA treatment of HL-60 cells abolished activity of PKC-$\alpha$,$\delta$ and $\zeta$ (Wooten *et al.*, 1993).

Another important feature of this cell line is that genes can be affected by TPA. It is known that HL-60 cells exhibit a 15-30 fold genomic amplification of c-*myc* as compared with normal cells.(Wolman *et al.*, 1985). HL-60 cells exhibit high steady state levels of c-*myc* mRNA (Blank *et al.*, 1992b). It is known that a rapid induction of c-*fos* expression has been observed when HL-60 cells are treated with TPA and EM field. EM field exposure can affect *c-fos* gene expression following either 60 mG or 1 G exposure (Phillips *et al.*, 1992). Other oncogenes related to HL-60 differentiation include the presence of increased activity levels of pp60c-src and

enhanced c-fms RNA levels following TPA treatment (Gee *et al.*, 1986, Sariban *et al.*, 1985). These results implied that both TPA and EM field exposure can affect similar cellular machine which could be involved in tumorogenesis, cell proliferation and differentiation.

The work of Byus *et al.* (1987) supported the hypothesis of cancer promotion by EM field by examining ornithine decarboxylase (ODC) activity in various cultured cells following exposure to 60 Hz fields and microwave field amplitude modulated at 16 Hz, and on synergistic effects of EM fields with TPA. Cain *et al.* (1993) showed that TPA and EM field increased co-foci density, which was not seen by exposed to EM field alone. McLean *et al.*(1991) showed a great number of tumors developed faster when treated with TPA and EM field together, compared with TPA alone on mice skin cancer promotion. The present study indicated that there exists an additive effects of EM field with TPA on cell differentiation. This result supports that promotion and co-promotion may play a role in tumorigenesis.

**EM Fields and "Window" Phenomena**

"Window effect" is described as a phenomena in which the effects of EM fields can be observed in certain range of frequency and/or intensity, outside of which the effects are not observable (Foster, 1988; Postow and Swicord, 1989). In this study, using flow cytometry, there are differential responses of HL-60 cell differentiation under different strengths of EM field exposure. For example, a 500 mG EM field did not result in significant cell differentiation in cells exposed for 24 hours. One G and 2G EM fields induced significant changes showing different efficiency. This suggested that there is an exposure window effect of exposure strength, or that different exposure strength may have different end-point effects.

A nonlinear dynamic system is hypothesized to explain the multiple-windowed, dose-response relation. Bawin *et al.* (1975) reported the 147 MHz radio-frequency (RF) EM field radiation, amplitude modulated (AM) at 16 Hz, could produce alterations in the association of calcium ions with isolated brain tissue and this effect occurred only at specific modulation frequencies between 6 and 20 Hz. Blackman *et al.* (1979) discovered that only within a certain range of power densities was the field capable of eliciting this response; power densities above and below this range, or "window", were not effective. Experiments demonstrated the persistence of windows (Blackman, *et al.*, 1980a). Further work with a modulated 50 MHz carrier revealed the existence of multiple power-density windows (Blackman *et al.*, 1980b). Further evidence of multiple power-density windows was supplied by Dutta *et al.* (1984; 1989), who reported that 915 MHz radiation and 147 MHz radiation, amplitude modulated at 16 Hz, could induce alterations in calcium-ion efflux from neuroblastoma cells in culture. These experiments diverge from the classic responses expected from most chemical and physical agents, *i.e.*, threshold and dose-rate-dependent responses. The observed macroscopic system responses, *i.e.*, calcium efflux phenomenon, result from a field-induced physicochemical change at transduction sites that are part of a given subsystem that directly responds to the EM field. There may be many subsystems that respond by different processes involving electron or nuclear magnetic resonance, or some other type of field-induced change. The coupling of these subsystems with nonlinear amplification processes would lead to a macroscopic (observed) response only when the particular combinations of frequency and intensity driving the coupled systems are part of the composite systems, that is, the macroscopic system will only respond when perturbed by specific set of driving forces and not others (Blackman *et al.*, 1989). The cell membrane is one possible site for the transduction and

amplification processes because of its dynamic and complex structure including surface molecules, ion channels, diverse metabolic and trans membrane signaling processes (Adey, 1981).

The temporal response of cells to EM field exposure is another important concern in this study. The present experiment demonstrated that 500 pg/ml of TPA can cause differentiation at 12 hours. Following exposure to 1 G and 2 G EM fields, it takes 24-48 hours to see the significant induction. The effect of inducing HL-60 cell differentiation by EM field is equivalent to that of 250-500 pg/ml TPA at 24 hours. In evaluating the specificity of the HL-60 differentiation, cells treated with phorbol and 4α-PDD do not show functional and morphological changes, while PDB can induce increase of phagocytic cells under higher concentrations. The difference of HL-60 cells responses to various intensities and time exposure in this study support a "window effect".

**Tumor promotion and signal transduction**

One of the proposed EM field-cell interaction mechanisms is that some unknown event(s) at the cell surface is translated by coupling to the cell's signaling pathways. There are other studies that showed EM field exposure could affect cell membrane receptor tyrosine kinase activity which could also lead to activation of PKC (Uckun *et al.*, 1995). Exposure of B-lineage lymphoid cells to an elf-EM filed 60 Hz, 1 G magnetic field led to the activation of the protein tyrosine kinases lyn and syk, and downstream activation of PKC. The PKC activity increased later than the tyrosine kinase activity and could be abolished by pretreatment with protein tyrosine kinase inhibitors. Lyn and syk are cell surface membrane associated tyrosine kinases. Dibirdik *et al.* (1998) demonstrated that in chicken lymphoma B-cells, lyn kinase is essential for phospholipase C-γ2 activation and inositol 1,4,5-triphosphate turnover is stimulated by exposure

to 60 Hz, 1 G EM field. These experiments are consistent with the proposed mechanism of EM field-cell interaction initiated at cell surface.

Byus *et al.* (1984) reported a transient increase in the activity of PKC in lymphocytes after about 60 minutes of exposure to a 450 MHz, EM field. Further, Monti *et al.* (1991) reported an enhanced activity of PKC in HL-60 cells following exposure to a triangular magnetic field pulse (80 G peak, 9-msec rise and fall followed by a 2-msec pause) for periods ranging from 10-20 minutes. The increased activity of PKC remained constant for at least 60 minutes. The variant responses of PKC activity suggest the early involvement of a transductive pathway which may play a contributive role in terms of long period consequences of cellular responses and that further elaborating the role of PKC to EM field exposure needs further study.

Early gene expression in HL-60 cell exposed to EM fields observed in previous work done in our laboratory implied that the PKC could play a critical role in mediating weak EM field signalings. Pathways hypothesized as important to interaction between EMF and the cell were inhibited in some of these determinations, with the result that the effects of EMF were negated. The choice of inhibitors was based on what is currently proposed as possible transduction pathways involved in EMF-cell interactions. One of the most consistent findings in EMF research invokes a direct role for changes in calcium flux (Wallaczek and Liburdy, 1990). The correlation of calcium with EMF activity supported the premise that signal transduction processes involving PKC could also be a pathway putatively induced by EMF exposure. Chelerythrine, an inhibitor of PKC, was used to test the latter proposal. Verapamil was used as an inhibitor of active L-type calcium channels. Consistent with proposed mechanisms, *fos* transcripts levels were not affected by EMF exposure in the presence of either chelerythrine or verapamil, suggesting that previous

proposals on the relationship of EMF exposure to cell metabolism were valid. The present study demonstrated that TPA, as an HL-60 cell differentiation inducer and mice skin cancer tumor promoter, can initiate early steps consistent with proliferative induction before cells precede to differentiation and PKC depletion. In the present study, significant PKC translocation is observed as the initiating event in phorbol ester induction. However, a differential response of PKC to 60 mG and 1 G EM fields exposure occurs and at most time points the increased PKC translocation is not statistically significant. This cellular reaction to EM field also shows a difference from TPA. Making a clear conclusion of PKC involvement in EM field induced cell differentiation in this study is difficult at this point. However, it is plausible that other alternative signaling pathways may involve in EM field induced cell differentiation because at an early phase of cellular response, EM fields may affect other components of the cell membrane receptor, where cross-talk signaling occurs.

TPA induced transformation is a good model to directly study tumor promotion and its underlying mechanism. It is not clear whether activation or depletion of PKC is important for the tumor-promoting effects of phorbol esters since it has been known that long term treatment of TPA deplets all forms of PKC. Down-regulation of protein kinase C δ by treatment with TPA can transform cells that over-express the non-receptor class tyrosine kinase c-src (Lu *et al.*, 1997). The down-regulation of PKC-δ by TPA as related to transformation is due to the loss of function of PKC-δ in tumor-suppressing effect, while PKC-δ is tyrosine-phosphorylated in cells. In a cell division model, it is suggested that PKC-δ affects cell cycle progression through a $G_1$/S cell cycle checkpoint (Zang *et al.*, 1997, Lu *et al.*, 1997, Armand *et al.*, 1999). Both "competence" and "progression" factors are needed to leave the resting stage of the cell cycle ($G_0$) and transverse $G_1$

into S phase. Over-expression of c-Src or the epidermal growth factor (EGF) receptor is postulated to act as a competence factor which either prevents entrance into the resting ($G_0$) state or facilitates exit from $G_0$. Inhibition of PKC δ is postulated to be a progression factor. Therefore, in tumor promotion model, inhibition or depletion of the tumor-suppressing PKC δ allows cells cycle progression and , therefore, the amplification of the initiated (c-Src- or EGF receptor-over expressing) cells. Other members of gene family, such as p53, $p21^{cip}$, p27, $p16^{ink}$ family, and retinoblastoma protein are called a tumor suppression pathway (Sherr, 1996). A defect in this tumor-suppressing pathway, which blocks cell cycle progression in late $G_1$, is essential for all human tumors. In the present study, TPA down-regulated total PKCα. In contrast with expectation, EM field in long term exposure did not down-regulate total PKC α.

In other words, TPA treatment causes significant PKC translocation while EM field exposure results in inconclusive data. It suggests that either up or down-regulation of PKC α is involved in TPA induced cell differentiation. Either more sensitive tests for PKC α translocation should be employed or there is the possibility of the existence of alternative signaling pathways which are affected by EM field. Some studies have pointed out that membrane tyrosine kinase receptor is involved in EM field signaling pathways. It is not clear how this relates to the tumor promoter TPA induced cell differentiation. It also needs to elucidate the difference between TPA and EM fields induced cell differentiation.

## Conclusions

The purpose of this study was to examine the hypothesis that cancer-related changes could occur in cells exposed to elf-EM fields. The strategy is to study the outcome of elf-EM exposure and cell differentiation in HL-60 cells and to study the mechanisms for EM field-cell interactions.

The present data demonstrate that a specific and sensitive method is, in particular, necessary to measuring the end-point effects of EM fields. A cell model which characterizes transformation, proliferation and differentiation with a known mechanism and one which is closely related to a tumor promotion model is required. The study of HL-60 cell differentiation supports the notion that EM field exposure plays as a factor or cofactor during long term cellular responses to external stimuli. The study of PKC translocation resulted in ambiguity about the similarity between EM field-cell interaction and TPA.

The present study supports the hypothesis that EM fields exposure cause biological effects in cells. Broadening the study of the effects of EM fields to other cell types is necessary. In the present system, it is also necessary to further identify the role of other isoforms of PKC and other signaling proteins in the consequences of EM field exposure.

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