Influence of a 50 Hz magnetic field and of all-trans-retinol on the proliferation of human cancer cell lines

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Abstract. *In vitro* exposure to power frequency magnetic fields (MF) has been reported to influence cell proliferation and differentiation. However, the nature of the response of different human cancer cell types to these fields has not been sufficiently characterized. The present work investigates the response of two proliferating human cell lines of neuroblastoma (NB69) and hepatocarcinoma (HepG2) to a 42 h, intermittent treatment with a weak, 100 μT, 50 Hz MF, alone or in combination with 0.5 μM all-trans-retinol (ROL), a retinoid currently applied in oncostatic therapies. In each experimental replicate the cell samples were submitted to one of the following treatment combinations: MF+/ROL+, MF+/ROL-, MF-/ROL+ or MF-/ROL-. The proliferative response was determined by cell counting (Trypan blue exclusion), BrdU incorporation and by spectrophotometric analysis of total protein and DNA content. The results show that when administered separately, the two treatments, MF and ROL, significantly enhanced cell proliferation in both cell lines. In NB69 simultaneous administration of MF and ROL induced an additive effect on cell proliferation, associated to increased DNA content. By contrast, in HepG2 the ROL-induced cell proliferation and increased protein content were partially blocked by simultaneous exposure to MF. Taken together, these data show that both agents, a weak MF and ROL at a low concentration, induce proliferative responses in the two assayed human cell lines. However, significant differences were observed between the responses of the two cellular species to the combined treatment with ROL and MF, indicating that the mechanisms underlying the cellular response to each of the two agents can mutually interact in a manner that is cell type-specific.

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Introduction

Residential exposure to extremely low frequency (ELF) magnetic fields (MF) has been suggested to be associated to increased risk for leukemia and brain tumors [see reviews by Mezei *et al* (1) and Kheifets *et al* (2)]. Also, epidemiological data on populations occupationaly exposed to ELF fields have been interpreted as indicative of increased incidence of those cancers and other malignancies [see reviews by Ahlbom *et al* (3), Park *et al* (4) and Kheifets *et al* (5)]. On the basis of the epidemiological evidence, particularly on leukemia, the International Agency for Research on Cancer (IARC) has classified weak ELF MF as a 'possible carcinogenic' agent (class 2B). One of the hypotheses proposed to explain the claimed carcinogenic action is that ELF MF could affect the recombination probability of radical pairs and therefore influence the free radical concentration in the biosystem (6). However, in the lack of adequate knowledge on the mechanism through which these fields might intervene in cancer processes, the international standards for non-ionizing radiation protection have considered the epidemiological evidence as non demonstrative. In fact, the current experimental evidence on *in vivo* responses to magnetic flux densities (B) below 500 μT, which corresponds to the reference level for protection of workers against potential harmful effects of short-term exposure to 50 Hz MF (7,8), has been repeatedly described as scarce and limited (9,10). Similarly, the results of *in vitro* studies investigating potential effects of weak MF on cell proliferation are considered inconsistent altogether [see Santini *et al* (11) for a review] due in part to the fact that a number of physical (magnetic flux density, exposure time, exposure cycle) and biological factors (cell type, cell genetics and/or cell physiology) seem to be critical to the cellular response (12,13).

In order to identify and characterize the cellular processes involved in the potential proliferative effects of ELF MF, a number of studies have investigated the *in vitro* response to those fields when administered in combination with different chemical species including antioxidants like melatonin or tamoxifen (14-19), or radical-inducing agents like menadione (20) and tumor promoters like DMBA (21).

The present study assesses the responsiveness of two human cancer cell lines, neuroblastoma NB69 and hepatocar-

cinoma HepG2, to a 100 μT, 50 Hz MF in combination with the retinoid all-trans-retinol (ROL), as regards to cell proliferation and total protein and DNA content. Retinoids are a group of natural and synthetic molecules that exhibit vitamin A-like biological activity. It has been reported that ROL and other retinoids exert protective effects such as scavenging of genotoxic oxygen species, modulation of signal transduction pathways, inhibition of cell transformation induced by physical and chemical agents, and facilitation of intercellular communication inhibited by genotoxic compounds (22-24). Taken together, these properties would be indicative of a potential protective role of retinoids on cancer diseases. This is consistent with the recognized cancer-preventive activity of ROL and other retinoids in animal models (25,26) and with epidemiological evidence suggesting cancer-protective effects of dietary intake or normal plasma levels of retinoids (27,28). However, this block of evidence and mechanistically based premises contrast with other data, including the results of recent clinical trials showing that vitamin A treatment could result in increased incidence of mortality in lung cancer patients [see review by Omenn (29)]. Such disparity in the epidemiological and experimental evidence reveals the need of better understand the phenomena involved in the cellular effects of retinoids and in their interactions with other chemical and physical agents.

The results of the present study indicate that, when administered separately, a low dose of all-trans retinol and weak, power frequency MF can promote cell proliferation in two different human cell lines. However, in combination with the retinoid, the MF exposure can either enhance or counteract the ROL-induced proliferative effect, in a way that is cell species specific. These results provide new insight into the patterns of the cellular response to ROL and on its interactions with an MF stimulus that promotes proliferation in human cancer cells.

Materials and methods

Magnetic field exposure setup. Fifty-Hertz, sine wave magnetic fields at B=100 μT were generated inside pairs of coils in a Helmholtz configuration energized by a Newtronic wave generator, Model 200MSTPC (Madrid, Spain). The exposure set-up used in these experiments is based on that described by Blackman *et al* (30). Two identical exposure systems were used, each of them consisting of two 1000-turn, 20-cm-diameter coils of enameled copper wire, aligned coaxially 10 cm apart and oriented to produce vertically polarized magnetic fields. Cell culture dishes were placed in the uniform MF space within the coils for exposure or sham-exposure. Currents in the coils were adjusted and monitored using a multimeter (Hewlett Packart, model 974A, Loveland, CO) after the flux density was established with fluxgate magnetometers (Mag-03 Bartington, GMW Assoc, Witney, UK and EFA-3 BN 2245/90.20, Wandel and Goltermann, S.A, Eningen, Germany). The two identical pairs of coils were installed separately in the center of two identical, magnetically shielded (co-netic alloy) chambers (Amuneal Corp., Philadelphia, PA), each of them housed inside one of two identical incubators (Forma Scientific, Thermofisher, Waltham, MA, USA) with a 5% CO_2, 37˚C, 90% RH atmosphere (Fig. 1). The magnetic shielding allowed for significant reduction on the environmental DC (geomagnetic) and AC (power frequency) fields at the samples' locations (BDC <0.08 μT; BAC <0.1 μT). In each experimental run, only one of the two sets of coils was energized at random. The samples in the unenergized set were considered sham-exposed controls. Being the coils located inside shielding chambers installed in separate incubators, the magnetic environment of the control samples inside the unenergized coil was not influenced by fields emitted by the coil electrically stimulated for MF exposure.

Cell culture. Two different cell lines, the human hepatocarcinoma HepG2 and the human neuroblastoma NB69, were tested. The HepG2 cell line was obtained from ECACC (TDI S.A. Madrid, Spain) and cultured in Dulbecco's Minimum Essential Medium (D-MEM, BioWhittaker-Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated, fetal calf serum (FCS, Gibco-Invitrogen, Paisley, Scotland, UK), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin (Gibco-Invitrogen, Prat de Llobregat, Barcelona, Spain). The NB69 cell line was provided by Dr M.A. Mena (Hospital Ramón y Cajal, Madrid) and cultured in D-MEM supplemented with 15% heat inactivated FCS, 4 mM L-Glutamine, 100 U/ml penicillin-streptomycin and 0.25 μg/ml amphotericin B. Cells were grown in a humidified incubator with 5% CO_2 at 37˚C. Plasticware for cell culture was purchased from Nunc (LabClinics S.A., Barcelona, Spain). Cells were plated in 60 mm Ø Petri dishes at densities of $5x10^5$ cells/ml (HepG2) and $4.5x10^4$ cells/ml (NB69). When appropriate, the media were supplemented with ROL (Worthington Biochemical Corporation, LabClinics S.A. Barcelona, Spain) dissolved in absolute ethanol at a 1:1000 dilution. The vehicle, at the same dilution, was added to the corresponding control samples after proven not to affect significantly cell growth when compared to samples cultured with medium alone (pilot study, data not shown).

Test for selection of ROL concentration. HepG2 and NB69 cell samples were seeded and supplemented with ROL at concentrations of 0.1, 0.5, 2.0, or 5.0 μM. Samples with ROL vehicle were used as controls. The media were removed 72 h after plating and replaced with media supplemented with the corresponding ROL concentrations. Each concentration was quintuplicate tested (a total of 25 samples per experiment and cell type) and a total of 7 experimental replicates were carried out per cell type. At the end of day 5 of incubation the cells were collected in 1 ml of culture medium and used for viability analysis and cell counting. Each sample was double counted.

Retinol and magnetic field treatments. In each experimental replicate 20 dishes with cells (10 with 0.0 μg/ml ROL and 10 with 0.5 μg/ml ROL) were incubated for 3 days inside unenergized Helmholtz coil sets. The media were renewed at the end of day 3 and the samples were distributed as follows: 5 dishes with 0.0 μg/ml ROL and 5 with 0.5 μg/ml ROL were placed inside one of the coil sets; the remaining ten dishes, 5 with 0.0 μg/ml ROL and 5 with 0.5 μg/ml ROL were located inside the second set of coils. The coil sets located inside the corresponding shielding chambers and incubators, were used alternatively, in a random sequence, for MF exposure or sham-exposure in consecutive experimental repeats. The MF-exposed samples were treated intermittently, in 3 h

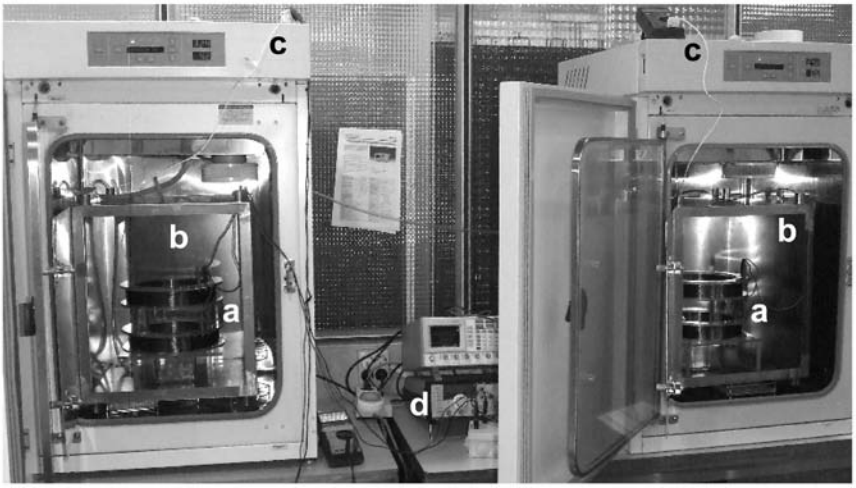

Figure 1. Two identical systems for magnetic field exposure consisting of two magnetically shielded chambers (a) with two Helmholtz coils (b) inside CO_2 incubators. (c) Temperature probe, (d) Magnetic field generator.

on/3 h off cycles, with 50 Hz MF at B =100 μT for a 42 h lapse. At the end of this period the MF-exposed and sham-exposed samples were analyzed for cell viability and cell growth. All experimental and analytical procedures were conducted in the blind for treatment condition. ANOVA followed by the Student's t-test were applied for statistical analysis. Differences $p<0.05$ were considered statistically significant.

Cell counting and espectrophotometric assays. After treated the cells were detached from the culture dishes and resuspended in 1 ml of media. Aliquots (50 μl) of each dish were prepared for hemocytometer quantification (double-counting) and the cell number was determined through Trypan blue exclusion assay. The remaining aliquots were used for quantitative estimation of total protein and DNA content by spectrophotometric methods: protein content was determined through application of Bradford's procedure (31), using albumin from bovine serum (Sigma, Steinheim, Germany) as a standard, and DNA quantification was performed according to Burton's method (32), with 2-deoxy-D-ribose (Sigma, Steinheim, Germany) as a standard.

5-bromo-2'-deoxyuridine labeling for identification of DNA-synthesizing cells. Samples were seeded on coverslips of 12-mm diameter placed on the bottom of 60-mm diameter Petri dishes. At the end of day 3 post-plating they were labeled with 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich, Madrid, Spain), at a 3-mM concentration, before being submitted to ROL and/or MF treatments. In each experimental run 2 coverslips placed inside 2 Petri dishes were used per experimental condition: Control; ROL; MF; ROL & MF. At day 4 post-plating, 21 h after BrdU application, the samples were fixed with 4% paraformaldehyde and permeabilized with 95/5 ethanol/acetic acid. Cells were incubated overnight with monoclonal primary antibody anti-BrdU (Dako, Denmark) at 4˚C. Anti-mouse Ig fluorescein-linked whole antibody (Amersham, Buckinghamshire, UK), the secondary antibody, was added to the cells and incubated at room temperature for 1 h. Preparations were counterstained and mounted in Hoechst-Non-Fade (Sigma, Steinheim, Germany) and studied through a Nikon Eclipse TE300 fluorescence microscope. Hoechst 33342 dye (Bisbenzimide, Sigma-Aldrich, Poole, UK) was added to the mounting medium for counterstaining of nuclei. Background controls without BrdU were included in the study. Twenty random microscope-fields per coverslip were evaluated. In each microscope-field the total nuclei (Hoechst 33342 fluorescent dye-positive) and the percent of BrdU positive cells were recorded and computer-assisted analyzed with AnalySIS 3.1 (Soft Imaging Systems GmbH, Münster, Germany). In each experimental run, a total of about 4500-5000 cells per experimental group were evaluated.

Results

Cellular response to retinol as a function of the dose. The survival rates displayed by the control samples at day five post-plating were of about 98% in HepG2 and 85% in NB69. These values, which did not differ from those expected under normal growth conditions (33,34), were not changed significantly by any of the assayed ROL concentrations. However, after 5 days of treatment all ROL concentrations induced a general, statistically significant increase in the number of HepG2 cells with respect to the corresponding, vehicle-supplemented controls (Fig. 2A). In this cell line the dose of 0.5 μM, which is about 20% of the baseline concentration of retinol in human plasma (35), induced a robust and consistent increase (22.46% over controls, $p<0.001$). In NB69, the same dose of 0.5 μM was the only concentration inducing significant increases in the cell number average (15.12% over controls, $p<0.05$, in 7 experimental replicates; Fig. 2B), whereas at higher ROL concentrations, 2.0 and 5.0 μM, NB69 showed slight but significant reductions in the average cell count (2.7%, $p<0.05$ and 7.0% $p<0.05$ below controls, respectively). From these results, 0.5 μM was the dose chosen for comparatively studying the responses of the two cell lines to ROL and/or MF.

Proliferative response of HepG2 to a 42-h MF exposure in the presence or absence of ROL. As illustrated in Table I, both ROL

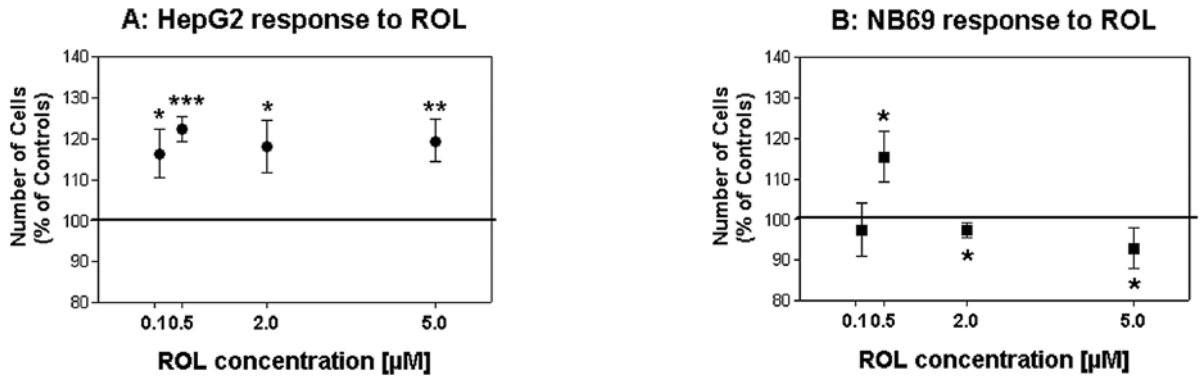

Figure 2. (A) HepG2 response to ROL and (B) NB69 response to ROL: Number of cells obtained at the end of day 5 of treatment to different concentrations of ROL. Data obtained from 7 independent replicates per cell line and normalized with respect to their respective controls. $^{*}p\leq0.05$; $^{**}p\leq0.01$; $^{***}p\leq0.001$, (ANOVA followed by unpaired Student's t-test).

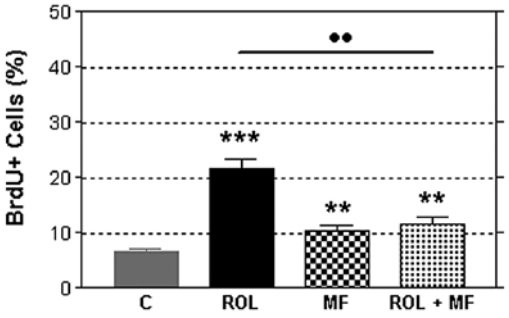

Figure 3. Percent of BrdU positive cells (HepG2) at the end of day 4 under the different conditions tested, controls (sham), ROL, MF, and the treatment combination of ROL plus MF, from 5 independent replicates, with 4 dishes per replicate. $^{**}p\leq0.01$; $^{***}p\leq0.001$, (ANOVA followed by unpaired Student's t-test).

Table I. Treatment-induced changes in the number of cells, total protein and DNA content.

	ROL+/MF-	ROL-/MF+	ROL+/MF+
HEPG2			
No. of cells	126.56±3.05^{c}	111.91±2.17^{c}	117.33±5.54^{a}
Protein	114.81±2.51^{c}	109.22±2.80^{b}	107.58±2.19^{a}
DNA	117.63±3.57^{c}	106.89±4.01	113.57 ± 4.28^{b}
NB69			
No. of cells	109.68±3.32^{a}	115.08±1.63^{c}	120.90±2.08^{c}
Protein	116.01±5.67^{a}	101.51±5.68	116.25±4.70^{b}
DNA	111.79±9.73	113.82±8.89	139.06±9.07^{b}

Cell number (x10^4) and total amount of protein (mg/dish) and DNA (mg/dish) at the end of day 5 of treatment with 0.5 μM ROL and/or 50 Hz, 100 μT MF. Standardized data (%) over the respective controls, treated with vehicle and/or sham-exposed to MF. Six experimental replicates (5 dishes per replicate) per cell line were conducted. Each figure in the table represents the mean ± SEM of 30 dishes. ANOVA followed by the Student's t-test; $^{a}p\leq0.05$; $^{b}p<0.01$; $^{c}p<0.001$.

alone and MF alone significantly increased the mean number of hepatocarcinoma cells when compared to the respective controls, either treated with vehicle (26.56±3.05% p<0.001) or sham-exposed to MF (11.91±2.17% p<0.001). When in combination with MF, ROL also increased significantly the cell number (17.33±5.54% over the corresponding control group, sham-exposed plus vehicle-treated). However, this response seems to be weaker than that elicited by ROL alone; which indicates that MF exposure might interfere or modulate the proliferative response induced by ROL on HepG2. This hypothesis would receive support from the data illustrated in Fig. 3, which show that the enhancement of BrdU incorporation by HepG2 cells is significantly higher (p<0.001) after treatment with ROL alone (21.52±1.83% over controls) than when ROL is applied in combination with MF (11.56±1.22% over the corresponding controls). As for cell viability, no significant differences were found between the values obtained under treatment with ROL alone (98.50±1.25%), MF alone (98.94±1.33%) or ROL + MF (97.24±1.82%) when compared to the average control figures (98.60±1.56%).

Protein and DNA content in HepG2 after 42-h MF exposure in the presence or absence of ROL. As summarized in Table I, the three assayed treatments, ROL alone, MF alone and ROL + MF induced in HepG2 significant increases in protein content when compared to their corresponding controls (14.81±2.51%, p<0.001; 9.22±2.80%, p<0.01 and 7.58±2.19%, p<0.05, respectively). However, the response to the combined treatment with ROL + MF seems to be weaker than that elicited by ROL alone. As for DNA content, it was increased significantly by ROL, both when administered alone (17.63±3.57%; p<0.001) and when in combination with MF exposure (13.57±4.28%; p<0.01), whereas MF alone did not change significantly the DNA content (6.89±4.01% over controls). Taken together, these results reinforce the indications that MF could interfere or modulate the response to ROL in the hepatocarcinoma cells.

Proliferative response of NB69 to a 42-h MF exposure in the presence or absence of ROL. As shown in Table I, when administered separately, ROL and MF induced significant increases in the number of NB69 cells with respect to the corresponding controls (9.68±3.32%, p<0.05 and 15.08±1.63%, p<0.001,

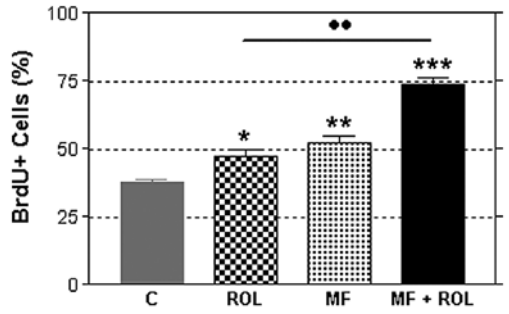

Figure 4. Percent of BrdU positive cells (NB69) at the end of day 4 under the different conditions tested, controls (sham), ROL, MF, and the treatment combination of ROL plus MF, from three independent replicates, with four dishes per replicate. $^{*}p\leq0.05$; $^{**}p\leq0.01$; $^{***}p\leq0.001$ (ANOVA followed by unpaired Student's t-test).

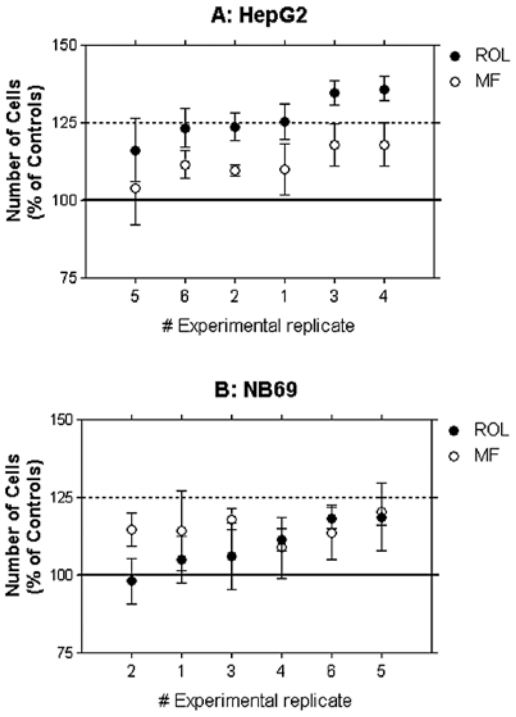

Figure 5. (A) HepG2 growth responses and (B) NB69 growth responses (estimated as number of cells obtained at the end of day 5 by Trypan) to ROL alone and to MF alone represented in crescent order of ROL-effect for the 6 experiments performed. In the figure the replicates are numbered following the sequential order in which they were carried out, being #1 the first experimental run conducted and #6 the last replicate. Each measure in the figure represents the mean ± SD of 5 dishes. Data normalized vs. the respective controls (sham). In HepG2 cells a significant linear correlation was revealed (r^2=0.973; $p\leq0.001$).

respectively). The combined treatment with ROL + MF also increased significantly the cell number with respect to controls (20.90±2.08%, p<0.001). This effect was significantly stronger than that induced by ROL alone (p<0.05), which is indicative that MF could potentiate the response of NB69 cells to the chemical treatment. The results on BrdU incorporation (Fig. 4) showed that the increment in the proportion of BrdU+ cells induced by the combined treatment (73.90% over controls) was significantly higher (p<0.001) than that induced by ROL alone (47.35% over the corresponding controls). These data reinforce those on total cell count, indicating that both treatments in combination could elicit a synergistic, proliferative response in NB69 cells. Concerning cell viability, no significant differences were observed at any of the assayed conditions: ROL (89.98±1.55%), MF (90.40±1.36%) or ROL + MF (85.94±1.81%) when compared to the average viability rate in controls (89.73±1.92%).

Protein and DNA content in NB69 after 42-h MF exposure in the presence or absence of ROL. Data in Table I show that ROL, alone or in combination with MF, significantly increased the protein content in NB69 cells with respect to controls (16.01%±5.67%, p<0.05 and 16.25%±4.70%, p<0.01, respectively), whereas MF alone did not change the cellular protein content (1.51±5.68% over controls). On the other hand, samples treated separately with ROL or with MF showed modest, non-significant statistically increases in DNA content (11.79±9.73% and 13.82±8.89% over controls, respectively). However, when in combination ROL + MF acted in a synergistic or additive manner, inducing significant increase in DNA content (39.06±9.07% over controls, p<0.01). This effect on DNA content after 42 h of combined treatment is consistent with the additive response of increased cell number, registered in the same samples (Table I) and is coherent with the effect observed on BrdU+ cells at day 4, after 21 h of treatment with ROL + MF (Fig. 4).

Comparative growth responses to ROL and MF in both cell lines. Fig. 5A compares the cell growth responses of HepG2 samples to ROL and to MF treatments along 6 individual experimental replicates. In the figure, the data are displayed in an ascending sequence, from lowest to highest strength of the response to ROL. The replicates are numbered following the sequential order in which they were carried out, being #1 the first experimental run conducted and #6 the last replicate. As can be observed, the strength of the cell growth response to MF in the individual replicates shows a pattern similar to that for ROL. In fact, the patterns of both responses are linearly correlated (r^2=0.957; p<0.001), indicating that the more sensitive an individual sample was to ROL, the stronger its response to MF. It is therefore paradoxical that when in combination with ROL, the MF exposure seems to interfere with the different aspects of the cellular response to ROL, including those concerning the protein and DNA contents.

Fig. 5B compares the cell growth responses of NB69 samples to ROL and to MF. As in Fig. 5A, the results are ordered in an ascending sequence of ROL strength effect in 6 experimental replicates. In contrast to that observed for the HepG2 line, in NB69 the pattern of response to ROL did not seem to correlate with that of the MF-induced effect, indicating that in this neuroblastoma line, the regulation of cell growth response to ROL differs from that of the response to MF. This is consistent with the additive effect observed on cell growth after combined treatment with ROL plus MF (Table I).

Discussion

Following public concerns about potential health risks linked to the exposure to the 50/60 Hz MF emitted by electric power lines and electrically powered apparatuses, numerous

experimental studies have been conducted examining the possibility that ELF fields can induce cancer in man [see Kheifets *et al* (2) for a review]. However, to this day, there is still no general agreement on the exact biological effects of power frequency MF, on the physical mechanisms that may be behind these effects or on the extent to which these effects may be harmful to humans. Under these circumstances there is general consensus that *in vitro* experimental studies are critical to better understand the potential cellular and molecular mechanisms responsible for cancer induction by ELF fields. Interactions between ELF-MF and endogenous or pharmacological agents are phenomena that challenge contemporary models and mechanisms and can modulate the risk of adverse effects from exposures to MF. The present work describes cellular interaction between a weak, 50 Hz MF and a low dose of ROL, a vitamin A related compound, in two human cancer cell lines.

A vast bulk of evidence has provided support to the view that vitamin A and carotenoids play protective roles in tumor promotion and progression (36-39). However, the full range of the biological effects of vitamin A (ROL) and its derivatives, the retinoids remains incomplete. In fact, recent studies have provided evidence that retinol could enhance carcinogenesis, especially at doses that exceed the normal dietary intake or in conditions of enhanced oxidative stress (40-43). This evidence would be supported by the results of a number of experimental studies in different biological systems, *in vivo* and *in vitro*, showing that pro-vitamin A, β-carotene, retinol and retinoids have pro-oxidant properties, which might lead to cell oxidative damage and carcinogenesis (44-48). Thus, in the present scenario of apparently contradictory evidence, further research is needed in order to elucidate the mechanism(s) by which retinoids may influence human carcinogenesis.

Our data on the cellular response to ROL are coherent with previous studies reporting that retinol increases the activity of ornithine decarboxilase, a key enzyme in growth processes, and induces cell cycle progression in cultured rat Sertoli cells (49). Also using Sertoli cells, other studies have reported that 7-10 μM ROL induces mitogenic signaling, accompanied by increased DNA synthesis and focus formation, mediated by superoxide anion generation. This effect is reversed by antioxidant treatment (50,51). By contrast, lower ROL concentrations 1-5 μM, which are considered to be within the physiological range for Sertoli cells (52) increased neither reactive species production nor proliferation. On the other hand, in human promyelocytic leukemia cells HL-60 concentrations of 2-5 μM have been reported to induce oxidative DNA damage (53). In the present study a dose as low as 0.5 μM ROL, is enough to induce a proliferative response in human cancer cells. Whether or not this proliferative response involves mechanisms related to oxidative stress remains to be elucidated. As for the differences observed in the ROL dose-response patterns displayed by HepG2 and NB69, they support previously reported indications that ROL present different active properties in biological systems (51). The biphasic pattern of proliferarive/antiproliferative response of NB69 as a function of the ROL concentration, has also been described in different cell types treated with retinoids (54,55) or with other agents that exhibit antitumor activity (56). Taken together, this block of data would highlight the importance of maintaining ROL levels within the physiological range in normal cells, since slight variations in concentration may trigger significant changes in the cellular behaviour. The data also suggest that, in general, cancer cells are more sensitive to ROL and can display behavioural changes at ROL levels below the normal physiological range.

After exposure to the MF alone, the increased growth effect in NB69 was similar to that observed in the HepG2. However, in this hepatocarcinoma cell line the MF also induced a significant increase in protein content that was not observed in NB69. In HepG2 a significant correlation was observed in the sensitivity of the different cell samples to the proliferative effects of ROL and MF when administered alone. Such a correlation was not observed in NB69, indicating that in this cell type the sensitivity of the different samples to ROL was not related to their sensitivity to the MF. Moreover, in NB69 the combined treatment with ROL plus MF induced additive or synergic effects of increased cell number and DNA synthesis, as indicated by the incorporation of BrdU into DNA. By contrast, in HepG2 the ROL-induced cell proliferation and increased protein content were partially blocked by a simultaneous exposure to MF. Taken together, these results indicate that although in both cell lines the MF affects proliferation to a similar extent, in the presence of ROL, the MF action on these cell types seems to be different, and probably is highly dependent on their specific characteristics of the retinol-regulation.

After exposure to MF alone the spectrophotometric analysis showed in the two cell lines modest, non significant statistically, increases on total DNA content, associated to the described significant increases in cell number. However, when the MF effects on DNA synthesis were analyzed through BrdU incorporation in the DNA molecule of dividing cells, statistically significant increases were obtained in the two cell lines. This indicates that the MF growth-promoting action could be exerted only on the proliferative fraction of the exposed samples.

A number of studies have reported before that ELF electromagnetic fields can elicit proliferative responses in various cell types (57-61), whereas others have described no field effects or even antiproliferative responses in different biosystems (62-65). These results are not necessarily contradictory. As a matter of fact, the *in vitro* response to ELF fields has been shown to be modulated by a variety of physical and/or biological parameters that could potentiate or inhibit the cellular sensitivity to MF. These parameters include the field strength, the wave form, the exposure cycle and duration, the genetic characteristics of the cells (66), the presence of pharmacological drugs or endogenous hormones like melatonin (19,67) or the redox status of the system (68). Pirozzoli *et al* (69) reported a significant increase (10%; $p \leq 0.05$) in proliferation of the human neuroblastoma cell line LAN-5, after 7 days of continuous exposure to 50 Hz, 1 mT MF. In our experiments a much shorter (2 days) intermittent exposure to a weaker (0.1 mT) MF induced a similar increase in proliferation (15%, $p \leq 0.001$) in other neuroblastoma line. Two parameters could be responsible for the high responsiveness of our system when compared to that of Pirozzoli and co-workers. The NB69 cell type could be more MF sensitive than the LAN-5 line and/or, under the applied experimental conditions, a short, intermittent MF stimulus might be more effective at inducing proliferative effects in neuroblastoma cells than a longer but continuous exposure. The application of intermittent fields, not

continuous, showed an impact on the induction of DNA strand breaks in human diploid fibroblasts after a 24 h exposure to 50 Hz, 1.0 mT MF (70).

It has been proposed that ELF MF may affect biological systems by increasing free radical activity in cells (71-73). Also, it has been reported that ELF MF could potentiate the DNA damage induced by H_2O_2 (74) or influence the radical pairs generation during the oxidative or enzymatic processes of DNA repair (75). Herein reported results show that a 42-h exposure to MF potentiates the ROL-induced stimulation of DNA replication and cell growth in NB69 cells. This could reveal the existence of common intracellular oxidative stress mechanisms by which MF and ROL stimulate proliferation in human cancer cells. Also, previous results reported by our group (76) indicate that the cell growth promoting effects of a 10-μT power frequency MF on the HepG2 line can be prevented by melatonin, a powerful free radical scavenger (77,78). This would provide additional support to the hypothesis that the herein reported MF effects could be mediated by free radicals. In any case, more research is needed to test these hypotheses and to elucidate the basic mechanisms involved in the cellular response to ELF fields.

In summary, the present results show that two human cancer cell lines, HepG2 and NB69, present different cell growth patterns when treated with increasing doses of ROL. However, both lines show similar proliferative responses when submitted to a ROL concentration as low as 0.5 μM. This effect is consistent with the bulk of epidemiological and experimental evidence suggesting that ROL and other retinoids can modulate the proliferating rate in tumors in a way that is dose-dependent and cancer-type specific. When in combination with a 100 μT MF, 0.5 μM ROL elicited different responses in the assayed cell lines. An additive, proliferative effect was obtained in NB69, which might indicate that in this cell type, the mechanisms triggered by these two agents are different. In contrast to this, in HepG2 samples, submitted to the combined treatment, the MF and ROL induced opposite, mutually compensatory effects on cell proliferation. This is indicative that those agents are able to influence common growth signaling pathways but in an antagonistic manner. As for the effects of MF alone, both cell types showed similar, significant proliferative responses after a 42-h interval of intermittent exposure, indicating that the treatment could stimulate the same pathways of response in these two human cancer cell lines. Such proliferative responses were elicited by a weak magnetic flux density, equivalent to 20% of the reference level recommended by ICNIRP for the protection against the short-term deleterious effects of occupational exposures to 50 Hz MF. This *in vitro* effect in human cancer cell systems cannot be envisioned as suggestive that exposure to the herein applied MF parameters is carcinogenic to humans. However these data provide new information on the conditions under which cells are sensitive to low, ELF MF, on the patterns of the cell response and on the pathways involved in such interactions. This information adds to the bulk of experimental and epidemiological data aimed to better understand the mechanisms underlying the bioeffects of low frequency fields. Such understanding is crucial to establish efficacious strategies for non-ionizing radiation protection of the public and workers, as well as to the potential development of new, MF-based applications.

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