Effect of extremely low frequency (ELF) magnetic field exposure on morphological and biophysical properties of human lymphoid cell line (Raji)

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Abstract

Human B lymphoid cells (Raji) were exposed for 72 h to a 50 Hz sinusoidal magnetic field at a density of 2 milliTesla (rms). The results of exposure showed a decrease in membrane fluidity as detected by Laurdan emission spectroscopy and DPH fluorescence polarization. Field exposure also resulted in a reorganization of cytoskeletal components. Scanning electron microscopy (SEM) revealed a loss of microvilli in the exposed cells. This change in plasma membrane morphology was accompanied by a different actin distribution, as detected by phalloidin fluorescence. We also present evidence that EMF exposure of Raji cells can interfere with protein phosphorylation. Our observations confirm the hypothesis that electric and magnetic fields may modify the plasma membrane structure and interfere with the initiation of the signal cascade pathways. © 1997 Published by Elsevier Science B.V.

Keywords: EMF; ELF; Fluidity; Membrane

1. Introduction

The possibility of negative effects on health from exposure to radio frequency (RF) or extremely low frequency (ELF) fields has stimulated the increase in recent years of publications on the biological effects of electric and magnetic fields [1–3]. Particularly debated is the question of the epidemiological evidence [4] of adverse effects of ELF fields generated by 50–60 Hz high voltage power transmission lines, video display terminals, electric blankets and other home appliances. Reported ranges of exposure are: magnetic flux density (B): $0.1–1000$ μT; and electric field intensity: $0.01–100$ V/cm (in air). This value of external field is reduced by about seven orders of magnitude to the $\mu V/cm$ range when air-capacity is coupled to conductive organisms or tissues. An electric field may also be set up in tissues and cells by a time-varying magnetic field according to Faraday's law. In humans, the peak values of this magnetically induced electrical field also fall in the $\mu V/cm$ range for ambient values of μT [5,6].

A strong objection to the acceptance of reported weak field effects is that their intensity is many orders of magnitude below the noise threshold [7] so that selective, cooperative or amplifying mechanisms, briefly reviewed in [8], must be postulated. The minimum value of applied field to which a cell would respond without any such mechanism has been calculated to be about 1 mV/cm for a large elongated cell and 20–40 mV/cm for a spherical cell [7,9]. On the other hand, it is known that some animals possess an extreme sensitivity to electric field (0.005 μV/cm for the shark) and to magnetic fields [10].

Much higher magnetic flux densities, pulsed or sinusoidal, in the 1–10 mT range (induced electric fields: 1–10 mV/cm) are produced by some industrial processes and by clinical (gradient fields in NMR imaging) and therapeutic procedures (healing of wounds and bone fractures). The efficacy of pulsed EMFs for the therapy of non-united bone fractures [11] is often cited as evidence that EMFs can influence physiological processes in organisms.

In general, a physiological framework for EMF effects has still to be established. This may be attempted by studying the mechanisms by which EMFs interact with cells under controlled laboratory conditions and by correlating in vivo evidence with in vitro data. Reviews of related studies on tissues and cells may be found in literature [12–14].

In their pioneering work Bawin and Adey in the middle 1970s [15] reported a 15% decrease of Ca^{2+} efflux in chick and cat cerebral tissue after a 20 min exposure to an electric field of 10 V/m in air (in the ambient range). Since then a great number of reports have been dedicated to the effect of EMF exposure on intracellular Ca^{2+} level with particular regard to cells of the immune system [16–18].

It is generally accepted that the plasma membrane surface is the site of interaction with EMFs and that some of the effects are dependent on Ca^{2+} regulation [1,14].

The field of bioelectromagnetics as it emerges from the literature is characterised by various non-linearities (intensity, frequency and time windows) and peculiarities (cell type, age, treatment) so that extrapolations or duplications among laboratories can hardly be made [18]. However, for in vitro experiments, a choice of high levels of exposure, compared to ambient values, is more likely to overcome the noise threshold [7] and favour consistency and repeatability of experiments. A list of reports using 1–10 mT (and 1–10 mV/cm) may be found in [17]. A flux density of 22 mT at 60 Hz was used by Liburdy [19] to expose mitogen activated thymocytes placed in annular wells of different radii. With this method he was able to separate a directly magnetic from an electric effect providing evidence of an increased effect on Ca^{2+} influx from 1 to 1.7 mV/cm electric field.

EMF exposure at 2.5 mT (50 Hz) has been shown to modify the membrane surface as evidenced by microscopy [20], and to reduce the values of membrane electrical characteristics (conductivity and permittivity) in K562 leukemic cells [21] and in chick embryo myoblast cultures exposed to 1–10 mT, 50 Hz fields [22]. A maximum effect at 5 mT is reported in this work. An increase in membrane negative surface charge has also been described [23].

In the present work we provide evidence that 50 Hz magnetic field exposure of human lymphoid cells (Raji) results in modifications of cell morphology, membrane fluidity and cytoskeleton organisation. A point of experimental focus in EMF research is EMF effect on signal transduction pathways. In relation to this our results indicate that EMF appears to modify the protein kinase activities in the exposed cells.

2. Materials and methods

2.1. Cell cultures

Raji cells were grown in RPMI (Gibco Laboratories, Scotland) supplemented with 10% Fetal Calf Serum (Gibco Laboratories, Scotland) and antibiotics (110 IU/ml of penicillin and 100 μg/ml of streptomycin) at 37°C, 5% CO_2. Raji (obtained from ATCC) are EBV genome positive non producing human B-cell line [24].

2.2. Exposure solenoid

Cells grown to confluency were divided into two samples (2×10^5/ml cells in a total volume of 10 ml) and placed in 25 cc Corning flasks. One sample was exposed to a sinusoidal 50 Hz magnetic field at a flux density of 2 mT (rms) in a solenoid with a

water-jacketed, temperature regulated, container suspended at its center. Temperature regulation was 37 ± 0.5°C and 5% CO_2 was provided. The sham exposed sample was placed under the same conditions in a solenoid with no field.

The solenoid is placed with its axis vertical so that the magnetic flux is perpendicular to the base of the Corning flasks.

The solenoid has a diameter of 20 cm and an height of 40 cm. It is made of 600 turns of 2 mm dia. copper wire wound in three layers in continuous forward-backward-forward fashion. It is driven from the 50 Hz power mains through a variable autotransformer and generates a flux density of 2 mT (rms) for an applied voltage of 12 V (rms). Field density (B), measured with a calibrated Hall probe, is within -5% of center value inside the cylindrical exposure volume of 11 cm by 17 cm along the solenoid axis.

The measured geomagnetic ambient field is 32 μT (vertical component) and 16 μT (horizontal component). Stray ambient ac fields are below 0.1 μT.

An often overlooked fact related to solenoids is the presence of an almost homogeneous electric field oriented parallel to the magnetic field [25] unless adequate electric shielding is used [26]. An approximate estimate of this field may be obtained from the voltage across the extremes of the inner of the three layers and its length: respectively, 4 volts and 40 cm giving a value of field strength in air of 0.1 V/cm. The corresponding field induced by capacity coupling in the culture medium will be in the order of 0.01 μV/cm [27]. For comparison the value of electric field magnetically induced by the 2 mT field will go from 0 (at the centre) to a max. of about 80 μV/cm along the sides of the 4.8×5.2 cm base of the Corning flask [28].

2.3. Cell growth curves

Raji cells were exposed at 2 mT, 50 Hz in the solenoid region were the field gradient was within -5%. For each experiment cells were plated into 25 ml flasks (2.0×10^5/ml cells in a total volume of 10 ml). The flasks were kept in the exposure system continuously for 24, 48, 72 h. Cells were then counted and viability determined by Tripan Blue dye exclusion. The experiment has been repeated three times.

2.4. Actin labeling and confocal microscopy

Actin was labelled with FITC-Phalloidin after Bellomo [29] with the following modifications.

Exposed and non exposed cells were attached to polylysine (0.01% for 30 min at room temperature) treated glass cover slides. The cells were fixed in paraformaldehyde (2% 10 min), washed twice again with PBS, permeabilized with PBS containing BSA 1% and triton X-100 (0.2% for 5 min), washed again, incubated with FITC-Phalloidin (10 μg/ml) and finally washed with PBS and BSA. The fluorescence was monitored using a LEICA TCS 4D confocal scanning microscope, supplemented with Argon/Kripton laser and equipped with 40×1.00–0.5 and 100×1.3–0.6 oil immersion lenses. Three experiments were performed analyzing in total 600 cells. Images were recorded employing pseudocolor representation.

2.5. Laurdan labeling of cell membrane and laurdan emission spectroscopy

Laurdan labeling procedure was done according to Parasassi [30]. 1×10^6 cells were washed three times with PBS, then resuspended in 2.5 ml of PBS. After which 0.5 l of a 2.5 mM solution of Laurdan in DMSO were added to the cell suspension under mild stirring. Incubation was carried out in the dark for 20 min at room temperature. Cells were then pelleted and washed with PBS, resuspended in 2.5 ml of PBS, equilibrated for 5 min in the fluorescence spectrofluorometer at 20°C, then measured. Since Laurdan can diffuse from the plasma membrane to intracellular membranes, each set of cells (50 to 70 cells) were analyzed within 10 min. Laurdan emission fluorescence has been continuously recorded from 400 nm with a Perkin Elmer 650-40 fluorescence spectrophotometer using 360 nm as excitation wavelength. The experiment was repeated three times with the same results.

2.6. Fluorescence polarization measurements

1,6-Diphenyl-1,3,5-hexatriene (DPH) (Sigma) was used for monitoring the fluidity in the hydrocarbon core of the plasma membrane. Labeling of intact cells

Table 1
Quantitation of the autoradiograph in Fig. 6

Protein number	Phosphoroimager counts	
	Sham-exposed Raji cells	EMF exposed Raji cells
#1	4120 ± 60 [a]	1185 ± 12
#2	2411 ± 30	1584 ± 15
#3	1329 ± 12	975 ± 9
#4	1001 ± 10	625 ± 4
#5	6400 ± 80	3520 ± 36
#6	3408 ± 35	11319 ± 120
#7	7737 ± 91	8125 ± 95
#8	n.d.	3639 ± 30

Incorporation of 32P was measured in labeled protein by phosphoroimager analysis of the dried gels and expressed as phosphoroimager counts. Points represent the mean of three different set of experiments. Effect of EMF exposure on protein phosphorylation.
[a] Mean $\pm$ SD.

was performed by adding 2 μl of 1 mM DPH in tetrahydrofuran to 40 μg of protein/ml at room temperature. Then incubated in the dark at 4°C for 60 min. Fluorescence polarization was measured using a Perkin Elmer 650-40 spectrofluorometer using 360 and 450 nm as excitation and emission wavelengths, respectively. The degree of fluorescence polarization
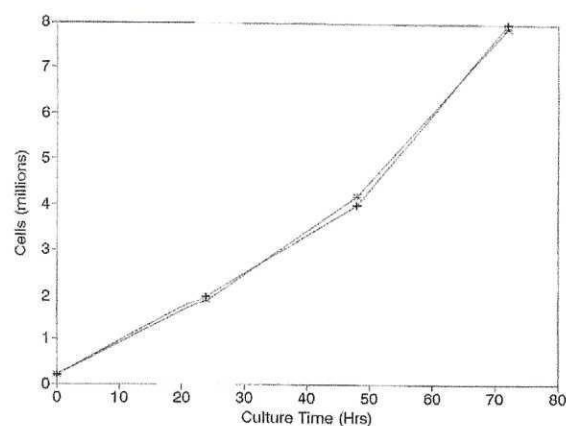
P, defined in the following equation, was then directly recorded.

$$P = (I_v - I_h)/(I_v + I_h)$$

where I_v and I_h are the emission intensities when the polarizers are oriented vertically and horizontally, respectively. The experiment was repeated three times with the same results.

2.7. Scanning electron microscopy (SEM)

Exposed and sham exposed cells were washed in PBS, fixed with 2.5% glutheraldheide in 0.1 M Millonig's phosphate buffer for 1 h at 4°C and, after
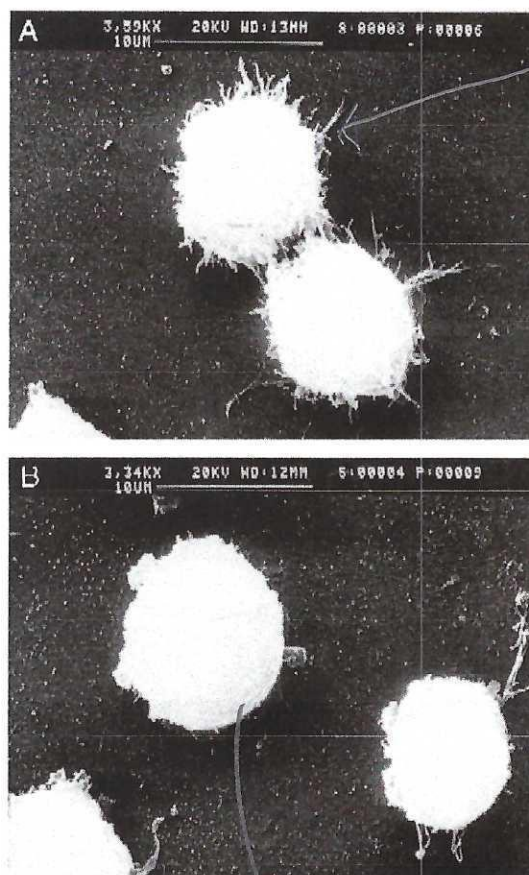

Fig. 2. Scanning electromicroscopy (SEM) of Raji cells: sham exposed (A) and exposed to a 2 mT 50 Hz magnetic field (B). Micrograph has been taken after 72 h at 37°C.

Fig. 1. Cell's growth. The reported curves represents cell viability of sham exposed (+) and 50 Hz 2 mT exposed (∗) Raji cells. For each curve cells were plated as described in Section 2. Growth curves represent the average of three different set of experiments.

three washes in the same buffer, seeded on polyly-sine-coated coverslips for 30 min, to allow adhesion to the glass surface. After washing, samples were post-fixed in 1% OsO_4 in Millonig's buffer, dehy-drated through a graded acetone series and critical point dried with CO_2 in a Balzers CPD 030 critical
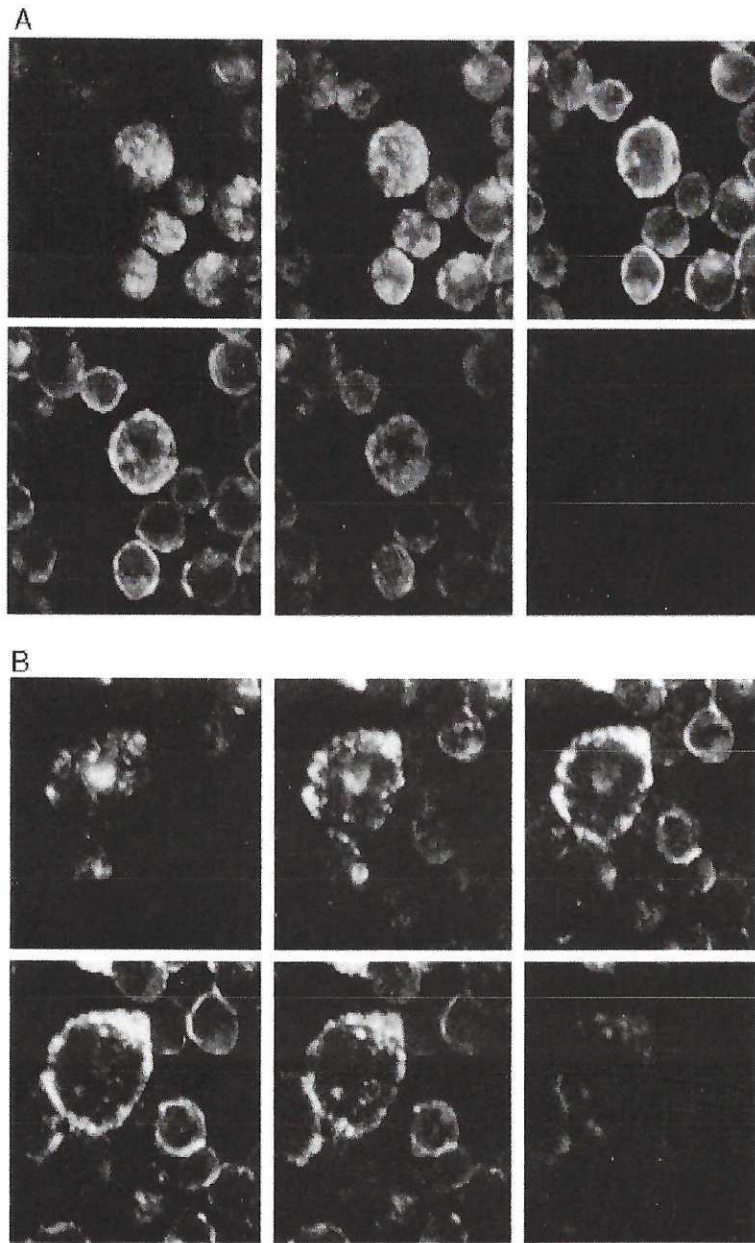

Fig. 3. Actin microfilament organization using confocal microscopy. Cells were exposed for 72 h at 37°C to the field, then the sham exposed (A) and exposed cells (B) were stained with FITC-phalloidin. Each micrograph represents a focus series from the top (upper left corner) to the bottom (lower right corner) of cells.

point drier. Specimens were coated with gold in a Balzers SCD 050 sputter instrument and observed on a Cambridge S240 scanning electron microscope.

2.8. In vitro phosphorylation

Labeling was carried out following the procedure of Eboli [31]. Raji cells (5×10^6), were rinsed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO$_3$, 2.3 mM CaCl$_2$, 5.6 mM glucose, 5 mM Hepes, pH 7.4) containing 1 mM MgCl$_2$. After washing cells were suspended in 100 μl Locke's solution containing 0.4 mM MgCl$_2$ and 0.3 mCi/ml [^{32}P] orthophosphate for 45 min at room temperature on a rocker. Cells were then briefly rinsed with (2×0.5 ml) Locke's solution and collected with (3×0.25 ml) chilled cell lysis buffer (1 mM MgCl$_2$, 0.5 mM EGTA, 0.5 mM dithiothreitol (DTT), 2% pyrophosphate, 5 mM Tris-Cl, pH 7.4). Ice-cold tri-chloroacetic acid (TCA) was quickly added to the lysates (20% w/v final) and after incubation on ice for 30 min, precipitates were collected by centrifugation. Pellets were resuspended in 50 μl of SDS sample buffer (9 M Urea, 0.14 M β-mercaptoethanol, 0.04 M DTT, 2% SDS, 0.075 M Tris-Cl, pH 8.0) and boiled for 5 min.

2.9. Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [32]. Sample volumes of 20 μl corresponding to 1×10^6 cells were loaded. Two-dimensional gel electrophoresis was carried out according to Eboli [31]. Equal volumes of samples (11 μl/well) were loaded. The first dimension, non equilibrium isoelectric focusing gel, (1.8×75 mm) containing a 3% total carrier ampholytes mixture composed of 75% pH 3.5–9.5, 25% pH 5.0–8.0, 9 M Urea, 0.5% Nonidet P-40, 1.6% 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulphonate (Chaps) and 5% acrylamide were run for 1500 Vh; the second dimension was carried out on 6–14% SDS polyacrylamide gradient gel. Incorporation of ^{32}P-label was revealed by auto-radiography and, in two dimensional gels, quantitated with a Phosphorimages Densitometer (Molecular Dynamics) (Table 1).

The experiment has been repeated three times.

3. Results

3.1. Growth curves

Standard growth curves were obtained for a quantitative evaluation of the effect of 50 Hz 2 mT EMF on Raji cells. The increase in cell number as a function of time for up to 72 h for the exposed and sham exposed cells is illustrated in Fig. 1. No significant differences between exposed and sham exposed Raji cells could be detected.

3.2. SEM analysis

Electron microscopy was performed on three different experiments on both sham exposed cells and cells exposed for 72 h to a 2 mT fields. Sham-exposed Raji cells appeared with a round shape showing a plasma membrane fully covered by microvilli. (Fig. 2, panel A). By comparison the EMF exposed cells showed morphological alterations of the plasma membrane such as a complete loss of microvilli (Fig. 2, panel B) and in some case a more elliptical shape (data not shown). The number of cells with these injuries represent 82% of the total. No broken microvilli could be detected in the frame.

3.3. Actin distribution

The distribution of actin filaments through the whole cell volume in sham exposed and exposed Raji
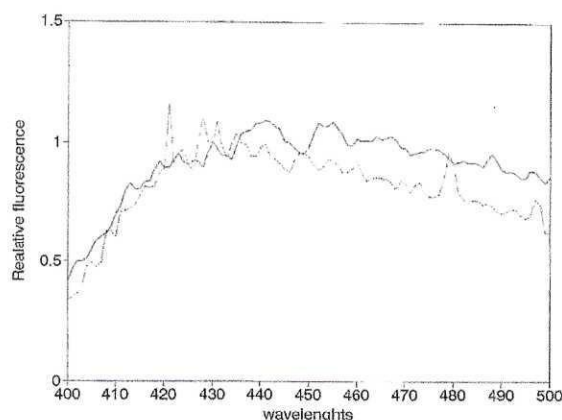
Fig. 4. Laurdan emission spectra of sham exposed (—) and 2 mT exposed Raji cells (· · ·). Spectra were recorded using 360 nm as excitation wavelength.
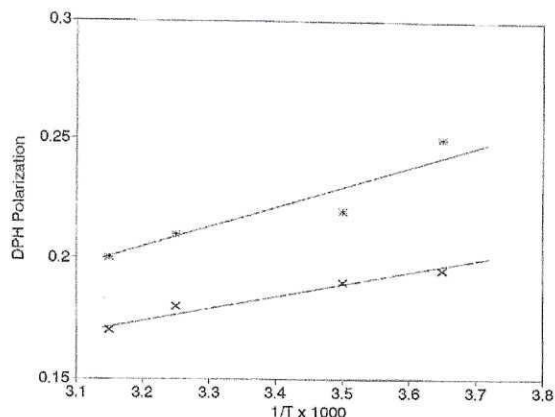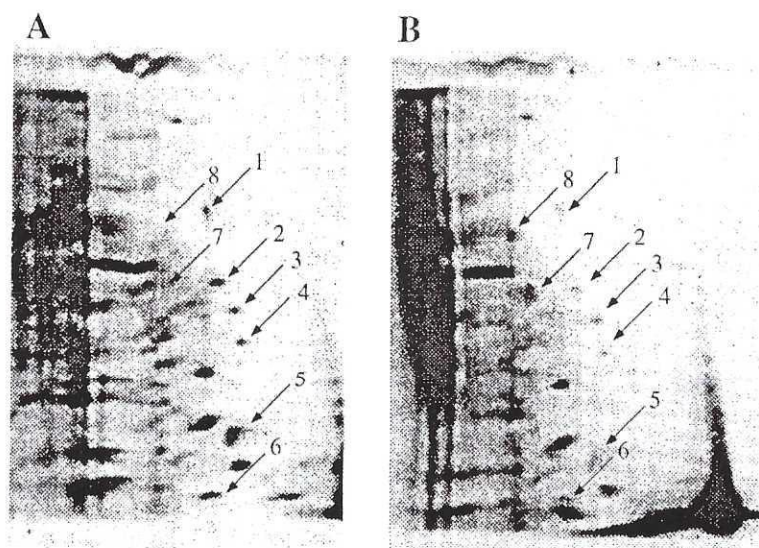
Fig. 5. DPH fluorescence polarization of sham-exposed ($\times$) and 2-mT exposed Raji cells ($*$). Fluorescence polarization were recorded using 360 and 450 nm as excitation and emission wavelengths respectively. Depicted is the Arrrenius plot of DPH polarization between 4°C and 40°C. Data point represent the average of three separate experiments with duplicate readings at each temperature.

bution of actin filaments within the cells. Exposure for 72 h to the 2 mT field causes a rearranging of actin microfilaments in 78% of the total cells, with depolymerization and marginalization from the center towards the cell membrane (Fig. 3B). This difference in actin filament distribution for sham exposed and field exposed cells was obtained each time the experiment was repeated (three times).

3.4. Membrane fluidity

We utilized the sensitivity of two fluorescent membrane probes, DPH and Laurdan, to study ELF magnetic field effect on plasma membrane fluidity. DPH estimate of membrane fluidity is based on fluorescence depolarization measurements [33], while Laurdan is known to be sensitive to the polarity of the environment, displaying a large red shift of the emission in polar solvent compared to non polar solvents [34]. Laurdan emission from exposed Raji cells (72 h) produced a spectra with a peak (430 nm) blue shifted from that obtained by sham exposed cells (450 nm) (Fig. 4). This situation is typical of the probe in a more rigid membrane (gel phospholipids phase). This experiment was repeated up to three times, showing the same blue shift in the emission spectra of the field exposed cells.

cells is illustrated in Fig. 3 which represent a focus series: the first frame on the upper left corner shows the top and the lower right corner shows the bottom of cells. Confocal microscope analysis of sham exposed cells (Fig. 3A) revealed an homogenous distri-

Fig. 6. Effect of EMF on total protein phosphorylation. Sham exposed cells (A) and 2 mT exposed cells (B). Cells homogenate were separated by charge in the first dimension and in SDS-PAGE in the second dimension. Autoradiograph of the gel shows labelled spots (arrow) corresponding to protein with a variation in ^{32}P incorporation.

To investigate in further detail whether Raji cells exposure to EMF could generate differences in cell membrane fluidity, as suggested by Laurdan emission spectroscopy, we analyzed membrane fluidity by steady-state DPH fluorescence polarization. Polarization values for exposed cells (72 h) were higher than those from control cells. This indicates that the membranes of field exposed Raji cells are significantly less fluid than those of sham exposed cells (Fig. 5).

3.5. Effect of EMF exposure on protein phosphorylation

A typical autoradiograph from Raji cells lysate (control cells) of the electrophoretic analysis of the phosphorylated species resolved in two dimensional gels is given in Fig. 6A. Cell membrane exposure to EMF resulted in a different modulation in the incorporation of ^{32}P in phosphoprotein (Fig. 6B): some of these proteins are more phosphorylated (see spots number 6, 7 and 8) when the field was present, while in others the incorporation of ^{32}P seems to be inhibited (spots number 1, 2, 3, 4, and 5). The experiment was repeated three times with no variability in the electrophoretic pattern.

4. Discussion

It is unlikely that 50 Hz EMF is able to transfer a significant amount of energy to cells. Recently, however, concern has emerged about the possibility that EMF exposure from residentially proximate power lines, household electrical wiring and appliances may contribute to elevate the risk of some types of cancer and in particular the risk of childhood acute lymphoblastic leukemia [35]. The molecular mechanism by which EMF exposure could induce cell proliferation and development of malignancy has not been deciphered. An important work of Liburdy and colleagues [36] demonstrated an interaction between EMF and the cell's signal transduction cascade. They found an increase in the susceptibility of mitogen activated lymphocytes in the calcium uptake and in c-MYC m-RNA transcript when the cells were exposed to 60 Hz EMF. In our study, we investigated the effects of exposure at 2 mT, 50 Hz magnetic field on physical and biochemical properties of the Raji lymphoblas-

toid cell line. Raji cells are latently infected by the Epstein Barr Virus (EBV) genome. Furthermore, when the latent EBV genome is expressed and the first viral antigens are produced, they can provide a useful tool in the search for the potential neoplastic effect of EMFs, because it is known that EBV replication can be associated with appearance of malignancies [37].

SEM analysis of exposed cells clearly indicates a morphological effect of the field on the organization of the plasma membrane. Compared to sham exposed cells, magnetic field exposure resulted in a loss of microvilli. These data are in accord with previous results of Paradisi and coworkers [20] that demonstrated absence of microvilli after exposure to a 50 Hz field of human erytroleukemia K562 cells. Microvilli are known to be very important in lymphocyte migration and traffic into sites of inflammation and are mainly composed by cytoskeletal components such as actin filaments. As a consequence of cell EMF exposure, we could also monitor by confocal microscopy a rearrangement in actin filament distribution within the cytoplasmic department. Since actin polymerization is one of the mechanisms regulating microvilli formation in the mature lymphocytes it is possible to argue that the loss of microvilli following EMF exposure could be related to actin depolymerization.

We also attempted to quantify membrane viscosity in the absence or presence of the EMFs using two fluorophores, Laurdan and DPH. Such probes monitor membrane fluidity through their sensitivity to the microenvironment. Laurdan is susceptible to changes in fluidity through changes in its emission spectra, while DPH monitors membrane fluidity through its sensitivity to the polarized light. Both probes showed a change in their spectral properties after 50 Hz EMF exposure: the Laurdan emission spectra was shifted towards the blue, this spectral region is typical of Laurdan in a rigid membrane; while the increase in DPH polarization suggested greater hindrance of the probe mobility in the 50 Hz exposed Raji cell's plasma membrane.

These last results are consistent with changes both in morphology and in actin filament distribution, since changes in the interaction between cytoskeletal component and membrane proteins can results in a different phospholipid organization. Our finding of

actin rearrangement induced by EMF exposure, following a previous report on the relation between activation of protein kinase C (PKC) and signal cascade pathways [37] prompted us to perform protein phosphorylation analysis of 50 Hz EMF exposed Raji cells. The bidimensional electrophoresis analysis of the total phosphorylated proteins from the exposed cells showed an altered pattern of protein phosphorylation: some proteins were more phosphorylated while in some we could detect an inhibition in phosphorylation. It is known that cytoskeleton rearrangement during leukocyte activation is accompanied by the rapid, PKC-dependent, phosphorylation of Myristoilated, alanine rich C-kinase substrate (MARKS). This substrate has been proposed to regulate actin-membrane interaction, as well as actin structure in the membrane [38]. MARKS can be found in focal contacts were actin filaments attach to the substrate-adherent plasma membrane, in association with vinculin, talin and PKC. In the light of the above findings it is possible to speculate that the loss in microvilli and the decrease in actin filament can be originated by an impaired phosphorylation of substrates needed for actin polymerization.

This data shows that exposure culture lymphoid cells to a 2 mT, 50 Hz magnetic field produces a modification of membrane and cytoskeletal organization, together with an alteration of protein kinases activity, without affecting cell proliferation and confirms the hypothesis that 50 Hz EMF can affect plasma membrane, influencing the signal transduction pathways.

Acknowledgements

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