Normal and cancer-prone human cells respond differently to extremely low frequency magnetic fields

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Received 2 August 2000; revised 30 November 2000; accepted 4 December 2000

First published online 14 December 2000

Edited by Veli-Pekka Lehto

Abstract Human lymphoblastoid cells of normal origin and from genetic instability syndromes, i.e. Fanconi anemia (FA) group C and ataxia telangectasia, were continuously exposed to extremely low frequency magnetic field (ELF-MF). We report that ELF-MF, though not perturbing cell cycle progression, increases the rate of cell death in normal cell lines. In contrast, cell death is not affected in cells from genetic instability syndromes; this reflects a specific failure of the apoptotic response. Reintroduction of complementation group C in FA cells re-established the apoptotic response to ELF-MF. Thus, genes implicated in genetic instability syndromes are relevant in modulating the response of cells to ELF-MF. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Extremely low frequency electromagnetic field; Ataxia telangectasia; Fanconi anemia; Lymphoblastoid cell; Apoptosis; Cell cycle

1. Introduction

Human exposure to extremely low frequency electromagnetic field (ELF-EMF) has dramatically increased in the last decades in developed countries (reviewed in [1,2]). This has raised considerable interest in the biological activity of EMFs. A large number of epidemiological and biological studies, largely reviewed in [2–5], have been carried out with the dual aim of (i) establishing whether any correlation exists between EMFs and tumors and (ii) elucidating possible modes of action [2,6,7]. While there is a consensus that EMFs influence certain cellular processes, no definite conclusion has been drawn as yet on the potential health hazard associated with EMF, due to both the difficulty of defining exposure conditions and to the multiplicity of sources and adopted protocols (reviewed in [4,5]). Actually, the broad variations in exposure protocols in different laboratories make any direct comparison from independent studies extremely difficult to draw. Several studies have reported a co-stimulatory effect of ELF-EMF on lymphocyte proliferation, while inhibitory effects were recorded by other laboratories ([2,6] and references therein). Besides methodological differences among published studies, another possible explanation of the discrepancies may come from the observation that the response to EMF is highly dependent on the samples under study, and can vary with the age and/or state of health of donors, as well as the stimulation conditions in work with cultured cell lines. It is generally established that cells from old and diseased donors, as well as sub-optimally stimulated cells, are more sensitive to ELF-EMF than their 'healthy' counterparts ([8–10]; see also [11] for review).

Cells from human genetic instability syndromes are characterized by hypersensitivity to various genotoxic agents, defective repair and checkpoint controls and an increased incidence of cancers [12]. Examining the biological response of cells from such syndromes may help to pinpoint the pathways of genomic instability associated with the onset of neoplastic growth in the general population, and to identify environmental risks that may be particularly damaging to an individual carrying these disorders. Here we have studied the effects of ELF-EMF on human cells with different genetic background, i.e. lymphocytes from healthy donors and human lymphoblastoid cell lines of either normal origin or derived from two genetic instability syndromes, i.e. ataxia telangectasia (AT) and Fanconi anemia (FA). AT is a genetic disorder characterized by the recessive ATM mutation. The wild-type ATM gene encodes a protein kinase of the phosphatidylinositol 3-kinase superfamily, which is emerging as a versatile protein implicated in signalling pathways regulating cell cycle checkpoints, induction of DNA repair in response to ionizing radiations and apoptosis (reviewed in [13–15]). FA is also a recessive disorder, and is regarded as a DNA repair-defective syndrome associated with chromosomal instability, hypersensitivity to DNA damaging agents, and high predisposition to cancer particularly of myeloid origin (reviewed in [12,16,17]). A complex genetic determination underlies the syndrome: eight complementation groups are identified [18], four of which, termed A, C, F and G, have been cloned (reviewed in [17]; also see [19]).

Here we have examined a normal lymphoblastoid cell line (AHH1); two cell lines independently derived from homozygous patients for the ATM mutation; a FA cell line carrying a homozygous mutation in complementation group C (FANCC) [20]; and its corrected counterpart derived by stable transfection of the wild-type gene [21]. Experiments have

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Abbreviations: ELF-MF, extremely low frequency magnetic field; FA, Fanconi anemia; FANCC, Fanconi anemia complementation group C; AT, ataxia telangectasia; FACS, fluorescence-activated cell sorter

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PII: S0014-5793(00)02376-0

been carried out in two laboratories in Rome and Milan under identical extremely low frequency magnetic field (ELF-MF) exposure conditions. We report that (i) ELF-MFs do not affect the cell cycle in normal or genetic instability syndrome-derived cells; (ii) however, the spontaneous rate of cell death, revealed by both ultrastructural modifications and by the appearance of hypodiploid apoptotic cells in the cell population, significantly increases in healthy lymphocytes and in normal lymphoblastoid cells exposed to MF for over 72 h; (iii) in contrast, cells from the AT and FA syndromes fail to allow a significant apoptotic response to MF exposure; (iv) correction of the FA defect by transfection of the wild-type gene restores MF sensitivity.

2. Materials and methods

2.1. ELF-MF exposure apparatus

Experiments were carried out in laboratories in Rome and Milan using identical Helmholtz coils that generated a linearly polarized magnetic field. The magnetic field strength (H) was axial and was parallel to the coil axis. The magnetic field (50 Hz) was measured using a calibrated ELF-MF detector for frequencies below 100 Hz to ensure that the Helmholtz coil generated a uniform distribution accross the exposed surface. The apparatus frame was built in teflon with no glue, adhesive or degassing material and hence was totally chemically inert. The Helmholtz coil unit consists of two coils (35 turns each) made of 1 mm diameter enameled wire. The distance between the coils was set to obtain a peak magnetic induction of 60 μT at the center of the unit (e.g. where samples are placed during the experiment) and 58 μT at the radial position, corresponding to the outer edge of the dish. The 50 Hz signal was derived by a small transformer separated from the power supply circuitry. The signal obtained at the secondary winding was mostly free of higher harmonic distortion; furthermore, to ensure the highest spectral purity of the sinusoidal drive, the transformer output was passed through a two-section passive low-pass filter, which strongly attenuated all higher harmonics (mainly 100 and 150 Hz). FFT analysis of the signal indicated that less than 1% of the total spectral power was associated with higher harmonics. Line transients were eliminated by a two-section cascaded LC filter on the 220 V main. Spikes due to switching of neighboring equipment never appeared as noise in the coil current trace. The current-monitoring resistor included in the voltage-to-current converter is strictly non-inductive and does not act as a pickup even when the Helmholtz coils are placed in the vicinity of the power supply (a condition that was avoided in the present experiments). The wires do not warm up under standard conditions. The total wire resistance for a single coil is in the range of 0.3 Ω and the peak current is 0.25 A: thus, the Joule dissipation is negligible. Local heating-up of the wire can be ruled out as the skin effect at 50 Hz is also negligible. The magnetic field intensity in Rome is about 46 μT, the angle of declination 0.16° eastward and the inclination −60° downward with respect to the horizontal plan. The 50 Hz magnetic field was normal to the horizontal plan. It is assumed that the dc bias due to the terrestrial field does not significantly affect the experiments, as similar tests performed at different latitudes show similar results.

2.2. MF exposure

Cell cultures were split immediately before starting the experiment into two culture dishes (60×15 mm, Falcon) placed in the same humidified (84% H_2O) 5% CO_2 incubator at 37°C. The Helmholtz coil was placed in the upper left tray of the incubator with the culture dish to be MF-exposed, whereas the control culture dish was placed in the lower right tray where the background MF was lower than 0.01 μT. The temperature in the incubator was regularly monitored and was found to remain constant throughout the duration of the experiments.

2.3. Cell lines

Human immortalized lymphoblastoid cell lines include: AHH1, from a healthy donor; AT3189 and AT2782B, from two patients carrying homozygous mutations at the ATM locus; HSC536, from a patient carrying a homozygous mutation in the FANCC gene; and FACC11, resulting from the stable transfection of HSC536 cells with FANCC gene. Correction of the FA phenotype was previously demonstrated by assessing the acquired resistance to mitomycin C in the complemented but not in the parental HSC536 cell line [21]. Genetically characterized cell lines from genetic instability syndromes were a kind gift from F. Rosselli (CNRS Institut pour la Recherche sur le Cancer, Villejuif, France). Phytohaemagglutinin-stimulated lymphocyte cultures were set up from the peripheral blood of 10 healthy volunteers (seven females and three males, 32–49 years of age). 6 ml of blood were collected in heparinized tubes. A smear of each sample was stained with May Grunwald Giemsa: lymphocytes represented 20–38%, while monocytes ranged from 2 to 5%. Mononuclear cells were isolated using Histopaque-1077 (Sigma Diagnostics, Milan, Italy) according to the manufacturer's instructions. Peripheral lymphocyte cultures and lymphoblastoid AHH1 cells were routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA); syndrome-derived cell lines were cultured in the presence of 15% fetal bovine serum.

2.4. Electron microscopy

Control and exposed cells were resuspended in 2.5% glutaraldehyde in cacodylate buffer for 1 h at 4°C, washed in 0.1 M cacodylate buffer (pH 7.4), dehydrated end embedded in Epon-Araldite mixture (Fluka, Buchs, Switzerland). Thin sections were stained with toluidine blue, whereas ultra-thin sections were stained with uranyl acetate and lead citrate and examined under a Zeiss EM 109 electron-microscope (Carl Zeiss, Oberkochen, Germany) at 80 kW. Cell preparations were coded and independently analyzed by two 'blind' operators.

2.5. Fluorescence-activated cell sorter (FACS) analysis

To monitor cell cycle progression, cell cultures were exposed to 45 μM bromodeoxyuridine (BrdUrd) for the last 30 min before harvesting. Cells were fixed in 70% ethanol, washed in phosphate-buffered saline (PBS)/Tween 20 (0.5%) and incubated in 3 N HCl for 45 min; after partial DNA denaturation, cells were exposed to anti-BrdUrd antibody (IgG clone BU 20a, Dako, Glostrup, Denmark), to secondary fluorescein-conjugated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA) and subjected to biparametric analysis (WinMDI software) for simultaneous determination of BrdUrd incorporation and of the DNA content [22].

To detect apoptosis, two different flow-cytometry methods were employed: (1) determination of hypodiploid cells [23]: briefly, 10^6 cells were resuspended in PBS containing 0.1% Triton-X 100 and 100 U/ml RNase A (Sigma, St. Louis, MO, USA), stained with 20 μg/ml propidium iodide (Sigma, St. Louis, MO, USA) and analyzed using a FACStar plus (Becton-Dickinson) flow-cytometer (10 000 events per sample). The amplification scale was logarithmic for FSC-H and FL2-H parameters, and linear for SSC-H, FL2-A and FL2-W. The photomultiplier voltage was set so as to place the peak corresponding to 2C DNA content (G0/G1) at channel 200 in the FL2-A histogram. (2) Detection of dislocated phosphatidylserine residues on the outer cell membrane by annexin V–fluorescein isothiocyanate binding [24]. 10^6 cells were washed in PBS, resuspended in 0.1 ml binding buffer (10 mM HEPES, 140 mM NaCl, 2 mM CaCl, 5 mM KCl, 1 mM $MgCl_2$) containing 20 μl/ml annexin V–Fluos (Boehringer Mannheim) and incubated for 15 min at room temperature in the dark. Samples were then diluted by adding 0.4 ml binding buffer and analyzed by FACS using a linear amplification scale for FSC-H and a log scale for FL1-H.

3. Results and discussion

3.1. ELF-MFs do not affect cell cycle progression in lymphoblastoid cell lines

Human hematopoietic cells with different genetic backgrounds were exposed to continuous ELF-MF (50 Hz, 60 μT) and aliquots containing approximately 10^6 cells were harvested 0, 24, 48 and 72 h after exposure to be analyzed. Preliminary experiments using the trypan blue exclusion assay indicated that the basal viability of non-exposed cultures was comparable in all cell lines, ranging from 80% in AHH1 to 89% in HSC536 (mutant cell line in the FANCC complementation group) cultures. Lymphocyte cultures from

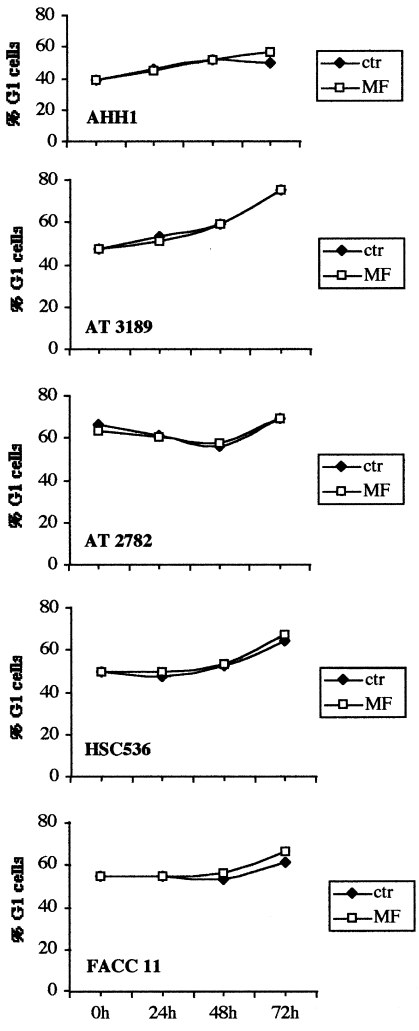

Fig. 1. Cell cycle progression in the presence or absence of MF. The graphs show the % of G1 phase cells in AHH1, AT (AT 3189 and AT 2782B), FA (HSC536) and FA cells transfected with complementation group C (FACC11) after the indicated times of incubation in the presence (MF) or absence (ctr) of MF. Three experiments were carried out with each cell line; representative panels are shown.

all donors were also comparable, with a mean viability of 96 ± 4%. A first set of experiments was designed to assess cell cycle progression in the presence or absence of MF. If MF exposure evoked a damage checkpoint, then the cell cycle phase distribution may have been affected in exposed compared to control samples. Flow cytometric analysis of the genome size and of BrdUrd incorporation in replicating DNA enabled us to accurately determine the cell cycle phase distribution in each sample [22]. In Fig. 1, cell cycle progression is indicated by the percentage of G1 cells recorded at different times in the presence or absence of MF. Similar cell cycle profiles were recorded in MF-exposed and control cultures, regardless of normal, AT or FA background. All samples showed typical profiles of actively proliferating, asynchronous cultures during 72 h of exposure, roughly accounting for three cell division cycles.

3.2. Continuous exposure to ELF-MF increases the spontaneous rate of cell death in normal lymphocytes and lymphoblastoid cells, but not in FA or AT cell lines

After 72 h of exposure to ELF-MF, we noticed the fraction of G1 phase cells increased in all cell lines (Fig. 1); this was paralleled by a corresponding decrease in the number of S phase cells (data not shown). The G1 enrichment reflects a decline in the proliferating ability of the cultures, probably due to accumulation of cell metabolites and medium spoilage, which is typically accompanied by cell death in lines of hematopoietic origin. To further investigate this, cell death was directly examined by electron microscopy in all cell lines. Normal cells were first characterized in primary lymphocyte cultures from healthy individuals and in AHH1 cultures (Figs. 2A and 3A, respectively): prominent nucleoli and distinct cytoplasmic borders can be seen in a clear background without cell debris. MF-exposed samples showed submicroscopical changes ranging from reversible injuries (including minor plasma membrane alterations, mitochondrial changes, dilatation of the endoplasmic reticulum and nuclear alterations with disruption of granular and fibrillar elements), to truly necrotic and apoptotic morphologies. Apoptosis was characterized by the presence of highly condensed chromatin generally at the nuclear periphery, nuclear fragmentation and cell shrinkage (Figs. 2B,C and 3B–D). In Fig. 3B for example a late apoptotic cell can be seen, with interrupted plasma membrane, intracellular vacuoli and apoptotic nuclear fragments. Necrosis was characterized by overt discontinuities in plasma membranes, marked dilatation of mitochondria, intracytoplasmic myelin figures and osmophilic debris (Figs. 2D,E and 3E).

Since a controversial aspect in studies of MF effects on cellular systems is the reproducibility of MF-induced patterns, seven independent experiments were carried out with each cell line and coded. Two operators, both blinded to the code corresponding to each cell line and treatment, determined the number of aberrant figures in samples of 100 cells in each experiment. Table 1 summarizes the statistical analysis of these experiments. Morphologically aberrant cells and spontaneous cell death were recorded among both MF-exposed and control cultures after 72 h of incubation. However, a highly significant increase in the rate of cell death was measured in MF-exposed compared to control samples from normal lymphocytes and AHH1 cultures. No significant differences were

Table 1
Cell death (%) in cell lines exposed to LF-MF for 72 h as assessed by electron microscopy

	Lymphocytes		AHH1 cells		AT 3189 cells		HSC536 cells		FACC11 cells	
	control	MF	control	MF	control	MF	control	MF	control	MF
Mean	8.29	19.43	17.01	26.43	10.57	10.43	9.14	8.71	8.33	13.33
S.E.M.	1.83	4.68	2.88	3.46	6.27	4.49	1.81	2.49	2.56	1.49
P	0.0005***	0.0005***	0.0001***	0.0001***	0.9108	0.9108	0.7178	0.7178	0.0037**	0.0037**

Seven experiments were carried out; 100 cells from control and MF-exposed cultures were analyzed in each experiment. S.E.M.: standard error. *P*-values were calculated using the Student two-tailed *t*-test: **highly significant; ***extremely highly significant.

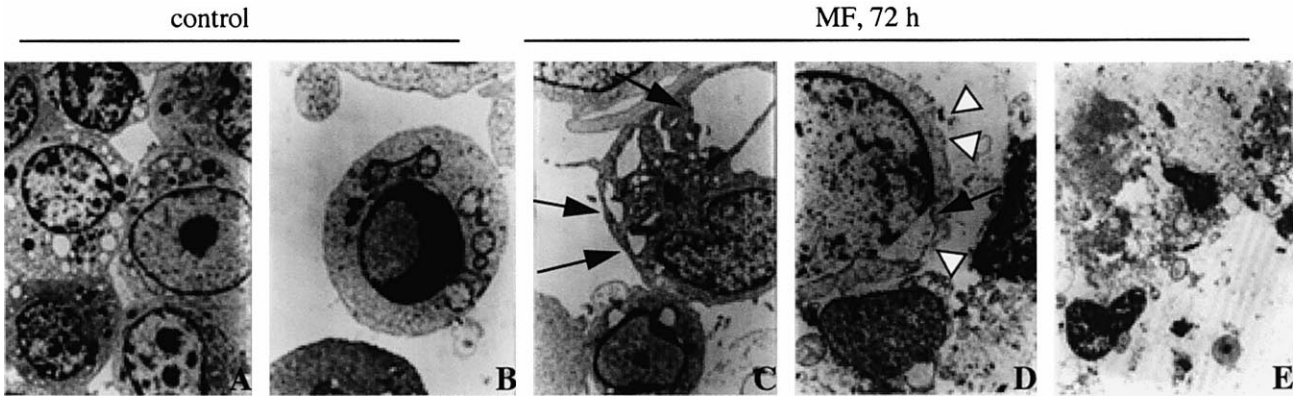

Fig. 2. Ultrastructural analysis of primary lymphocytes after 72 h of incubation in the absence (A, B) or presence (C–E) of MF. A: Well preserved morphology, with normal plasma membranes and mitochondria (original magnification 3200×). B: Apoptotic cell with a peripheral crescent-shaped mass of compact chromatin (original magnification 7000×). C: Lymphocytes with intracytoplasmic vacuoli (arrows) and dilatation of the perinuclear cisternae (original magnification 4500×). D: Lymphocytes with dilated perinuclear cisternae (arrows), interrupted cell membranes (arrowheads) and necrotic debris in the background (original magnification 7000×). E: Irreversible injury: necrosis and cell debris (original magnification 3200×).

recorded between MF-exposed and control cells from FA and AT syndromes. Correction of FA cells by transfection of the FANCC gene (FACC11 cell line) restored the MF-induced increase of cell death after 72 h.

3.3. *The rate of apoptosis increases in normal lymphocytes and lymphoblastoid cells exposed to ELF-MF, but not in FA or AT cells*

Both the AT [25,26] and FA [27,28] syndromes are generally defective in apoptosis in response to several stimuli, and this feature is regarded as a determinant of their cancer-proneness. Results in Table 1 suggest that the lack of MF-induced cell death may reflect a specific failure of the apoptotic response in AT and FA-derived cells. To investigate this question, apoptosis induction was determined in MF-exposed and control samples from all cell lines by biparametric FACS analysis: cellular granularity and DNA content were evaluated by measuring the 90° light scatter (SSC) parameter and the stoichiometric PI intercalation into DNA, respectively [23]. Apoptosis is revealed by the appearance of a population of highly condensed, granular cells [high SSC], whose DNA content is below 2C (see panels in Fig. 4A). AHH1 cultures underwent a low level of spontaneous apoptosis during the first 72 h of the experiment; after that time, the rate of spontaneous apoptosis began to increase. The increase was significantly greater in the presence of MF (Fig. 4B, left panel). In contrast, cells from genetic instability syndromes failed to undergo spontaneous apoptosis with time, and, furthermore, failed to express an increased apoptotic response to MF exposure: apoptotic rates remained very similar in MF-exposed and non-exposed samples from syndrome-derived cells after 72 (Fig. 5B) and 120 h (data not shown) of incubation. The different response between normal and cancer-prone cells did not reflect a difference in proliferation rates, since the average cell division time remained similar (24 h) in all lines as indicated by the data in Fig. 1. FACS analysis confirmed that corrected FA cells by the wild-type FANCC gene had a restored ability to induce increased levels of apoptosis in response to MF (Fig. 4B, right panel).

To further confirm these results, the annexin V binding

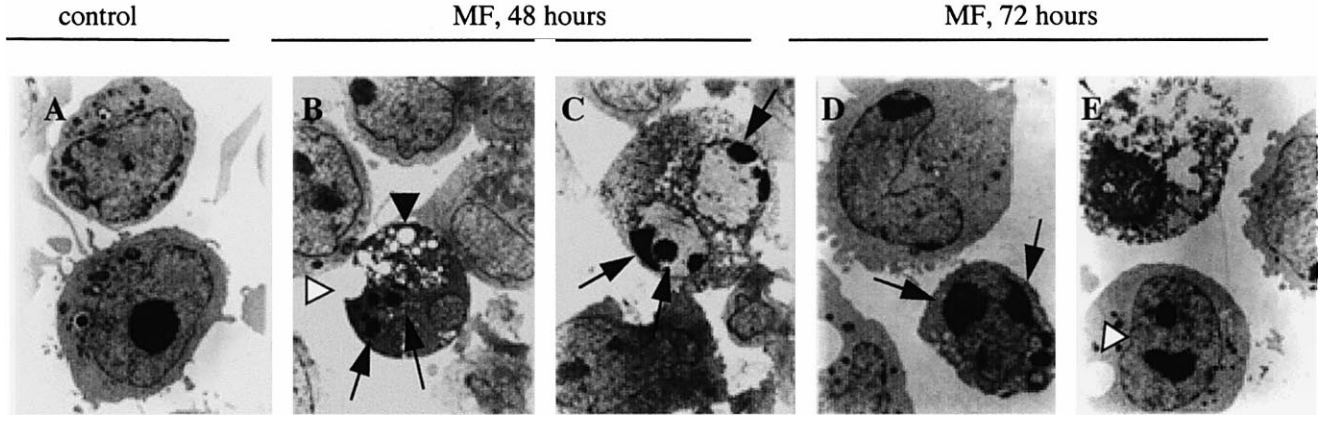

Fig. 3. Ultrastructural analysis of lymphoblastoid cells after incubation in the absence (A) or presence (B–E) of MF. A: AHH1 cells with well preserved morphology, normal membranes and mitochondria. Several pseudopods and nucleoli are visible (original magnification 3200×). B: AHH1 late apoptotic cell with interrupted plasma membrane (open arrowhead), intracellular vacuoli (plain arrowhead) and nuclear fragments with uniformly dense chromatin (arrows); no recognizable mitochondria (original magnification 3200×). C: FACC11 apoptotic cell with nuclear fragments with uniformly dense chromatin (arrows), interrupted cell membranes and intracellular vacuoli (original magnification 3200×). D: AHH1 apoptotic cell with condensed, fragmented chromatin (arrows) (original magnification 4500×). E: ACC11 necrotic cell (top), neighbored by a cell (below) with evident cytoplasmic vacuoli (arrowhead) and dilated endoplasmic reticulum (original magnification 3200×).

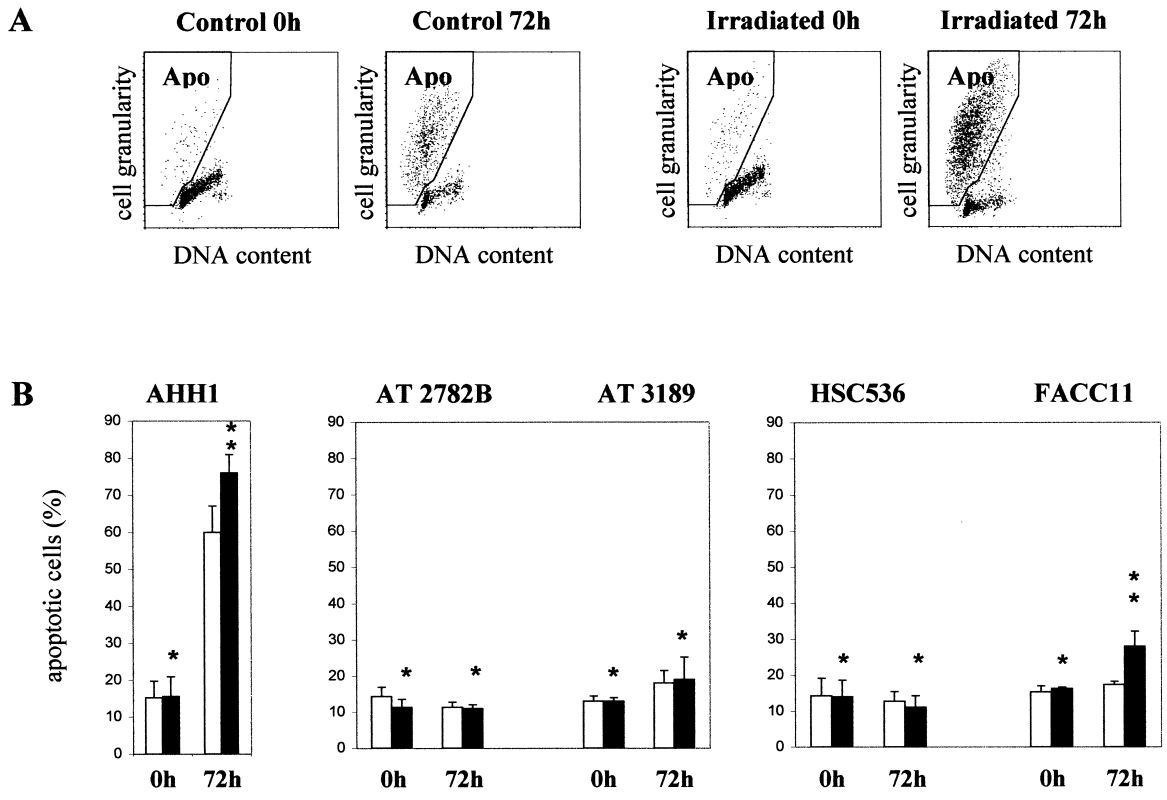

Fig. 4. Apoptotic response of lymphoblastoid cells in the presence or absence of MF. A: Examplifying FACS profiles of the apoptotic cell population in AHH1 cultures. B: Apoptosis frequency in AHH1 and syndrome-derived cell lines (AT 2782B, AT 3189, FA HSC536 and FACC11 transfected with complementation group C). Three experiments were carried out; histograms represent mean values (empty histograms, control;: black histograms, MF-exposed samples); bars represent standard errors. *P*-values between exposed and non-exposed samples after 72 h of incubation were calculated using the Student two-tailed *t*-test; $*P > 0.1$, $**P < 0.05$.

assay was employed to independently assess the apoptotic response in normal AHH1, AT 3189, FA HSC536 and FA-corrected FACC11 cell lines. Annexin V specifically binds to phosphatidyl-serine residues which become externalized on the outer cell membrane during the early phases of apoptosis [24]. Fig. 5 shows the flow-cytometry detection of apoptotic cells by fluorescein-labeled annexin V after 72 h of exposure to ELF-MF. ELF-MF exposure significantly increased the proportion of annexin V-positive cells in the normal AHH1 cell line but did not affect the rate of apoptosis of either the AT 3189 or the FA HSC536 cell lines. Correction of the FA defect in the FACC11 cell line fully restored the apoptotic response to ELF-MF. Thus, measuring early apoptotic events by annexin V gave fully consistent results with those obtained by assessing the distribution of hypodiploid cells typical of advanced apoptosis.

In summary, the present results show that a 50 Hz, 60 μT MF, though not affecting progression through the cell division cycle, stimulates the spontaneous rate of cell death in human healthy lymphocytes and normal lymphoblastoid cells. Previous studies suggested that MF exposure increases apoptosis in rodent cells [29,30]. A novel aspect emerging from our study is the defective apoptotic response to MF exposure of cell lines derived from FA and AT syndromes, both of which are characterized as repair-defective and cancer-prone. Cells from human genetic instability syndromes have not been examined before in response to MF. Our results are generally consistent with the notion that the origin and state of health of cells are highly influential in the response to MF [11]. In addition, our experiments suggest that both the ATM kinase and the FA C gene product can influence the response to MF exposure: two independent cell lines lacking ATM, and a cell line carrying a homozygous mutation in the FANCC gene, proved deficient in MF-induced apoptosis. Reintroduction of the FANCC gene in defective cells restored the response to MF exposure. A large body of work has recently focussed on the role of ATM in checkpoint controls (reviewed in [13–15]): ATM is implicated in both p53-dependent and p53-independent apoptotic pathways [25,26] and a cytoplasmic fraction of the ATM protein kinase pool is thought to play specific roles in signal transduction [13–15]. The molecular role of FANCC gene product is less clear, yet a general role in preventing apoptosis is accepted [28,31], which can also be exerted in a p53-independent manner [32,33]. The possibility that ELF-MFs act on DNA is controversial [6,7,34,35]; therefore, the failure of AT and FA cells to undergo apoptosis in response to MF exposure is not necessarily related to the deregulated apoptotic response to genotoxic agents in these syndromes, but may reflect a more general impairment in cellular signalling. MFs are thought to influence the intracellular calcium flux in cells of the immune system [30,36–38], which is in turn a key factor in modulation of apoptosis. Further work will be required to elucidate the molecular steps implicated in the signalling pathways underlying the apoptotic response to MF. From the data reported here, we believe that an indication is beginning to emerge concerning the genetic

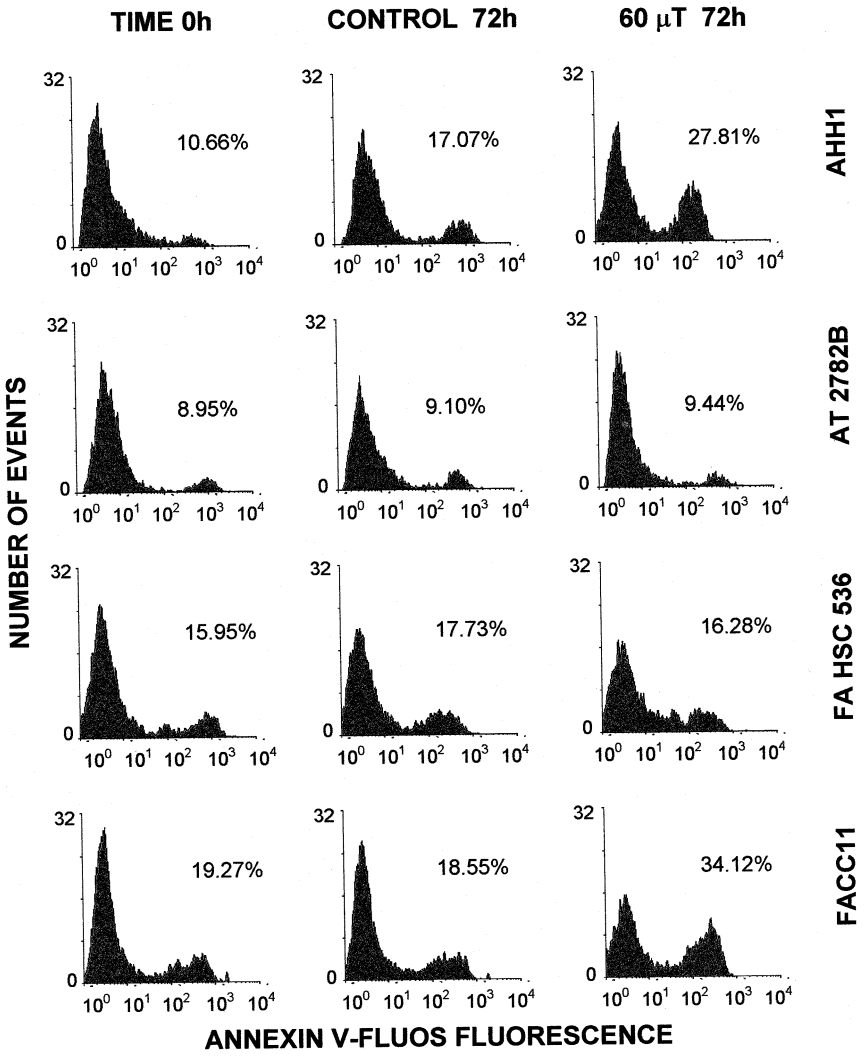

Fig. 5. FACS analysis of early apoptotic events in lymphoblastoid cell populations in the presence or absence of MF as detected by the annexin V binding test. Two independent experiments were carried out and representative panels are shown. The low fluorescence peak (left peak in each histogram) corresponds to annexin V-negative viable cells. Annexin V binding to apoptotic cell membranes is revealed by the appearance of a second high fluorescence peak. The percentage of positive cells in the whole population is indicated.

background required for expression of the MF-sensitive phenotype.

Acknowledgements: We are grateful to Filippo Rosselli (CNRS, Villejuif, France) for providing syndrome-derived cell lines. We also thank Norma Ridi and Flavio Fontana for providing the Helmholtz coil devices and MF measurements; Patrizia Bernardelli for providing laboratory space and support to M.C. and M.C.B.; and Filippo D'Ottavio for excellent assistance throughout this work. This study was supported by the Italian National Institute of Health (Istituto Superiore di Sanità, Project 'Biological effects of low frequency electromagnetic fields'), the CNR and the 'Emilio Bernardelli' Foundation.

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