



Cellular biology cellu

by Vijayalaxmi

Monday, June 21, 2004

Plenary Session 1

[Ultrashort electric pulses open a new gateway into cells.](#)

Schoenbach, K.H. USA.

Experimental studies in which human cells were exposed to ultra short high voltage electric pulses up to 30 MV/m amplitude and durations as short as 10 ns are reported to affect intracellular structures and result in 'electro-permeabilization' leading to a rise in intracellular free calcium levels and enhanced expression of genes. At increased electric fields, sub-microsecond pulses are shown to induce apoptosis in biological cells. This technology, using sub-microsecond pulses, has been applied to reduce/inhibit the growth of tumors and to increase the efficacy of gene therapy. Discussion: Dr. Schoenbach said that it is possible to electroporate intracellular organelles without 'porating' outer cell membranes. For example, application of 180 kV/cm pulses for 10 ns will open sub-cellular structures of oesinophils without damaging outer membranes.

Session 1

[Pulsed HV Research and applicaton](#)

Session-1.1. **Intracellular effects due to extremely large sub-microsecond electric field pulses: Theory and modeling.**

Weaver, J.C. USA.

Several groups have described experiments in which non-thermal electric field pulses of 10 to 300 ns durations and magnitudes of 1 to 150 kV/cm cause effects associated with sub-cellular structures. Elec-



gy and lar biophysics

tric field pulse-induced apoptosis is the most striking effect. The objectives of the studies are to create more realistic two-dimensional (2-D) models of mammalian cells containing not only the outer plasma membrane (PM), but also sub-cellular structures such as the nucleus, endoplasmic reticulum and several mitochondria. This provides micro-dosimetry at the cellular and sub-cellular level and allows the prediction of molecular and ionic transport within a cell model. A planar membrane patch ($3\ \mu\text{m} \times 3\ \mu\text{m}$) with a Smoluchowski equation-based model was used to investigate local electroporation behavior due to pulses of a wide range of durations and amplitudes. The general feature of these models is that supra-electroporation occurs extensively in the PM and less but significant electroporation occurs in membranes of the nucleus, endoplasmic reticulum and both the inner and outer mitochondrial membranes. The small residual pores have life-times of order seconds, which is a mechanism for translating sub-microsecond interactions to the physiological time scale of 0.1 millisecond to seconds. Translocation of membrane components is expected for both conventional (pulses with >100 microsecond durations and ~ 1 kV/cm magnitudes) and supra-electroporation, and the persistence of translocated phospholipids or proteins generates signals that can last for even longer times. Both molecular and ionic transport of residual pores and signaling by translocated membrane molecules may contribute to diverse and potentially specific intracellular effects that are caused by the exposure of cells and tissues to extremely large sub-microsecond electric field pulses.

Session-1.2. **Human gene expression in response to nanosecond pulsed electric fields.**

Tseng, C.C. USA.

The responses of human cells to nanosecond pulsed electric fields (nsPEF) depend on exposure conditions (field intensity, pulse duration, and number of pulses), and the effects range from alterations of gene expression (without immediate apoptosis) to necrosis. The present work focuses on gene expression using exposure conditions below the thresholds that can cause apoptosis and membrane electroporation. Human HL-60 cells were grown in RPMI + 10% FBS. Aliquot of $130\ \mu\text{l}$ cell suspension (10×10^6 cells/ml) were transferred to a 1 mm cuvette and exposed to 3 consecutive 60 nsPEF at 15kV/cm. Exposed cells from 5 cuvettes were pooled ($650\ \mu\text{l}$), diluted 10-fold with RPMI + 10% FBS, and incubated for 6 hours in a T-75 flask. Under this nsPEF exposure condition, there was no membrane electroporation as determined by ethidium homodimer uptake, no immediate apoptosis as indicated by the lack of caspase activation and the absence of annexin-V-FITC binding, and no cell death (approximately 100% survival). After nsPEF exposure and incubation, RNA was extracted and genome-wide gene expression was determined.

The procedure involved the preparation of total RNA \rightarrow first strand cDNA synthesis \rightarrow strand cDNA synthesis \rightarrow cDNA purification \rightarrow *in vitro* transcription \rightarrow biotin-labeling of cRNA \rightarrow cRNA purification \rightarrow fragmentation of cRNA \rightarrow hybridization of cRNA with probes on the microarray \rightarrow washing \rightarrow staining with fluorescent dyes \rightarrow scanning \rightarrow data analysis. From a total of 20489 genes analyzed, using the minimum

value of a 2-fold difference, 603 genes were found to be up-regulated or down-regulated. Of the affected genes, 1/3 were down-regulated and 2/3 were up-regulated. Many of the affected genes are involved in important biological activities such as signal transduction, cell adhesion, apoptosis, cellular defense, immunity, inflammatory and stress response, transcriptional control, DNA binding, ion transport, protein folding, phosphorylation and modification, and cell death. Altered gene expression was observed from both longer and shorter periods of incubation (2h, 12h, and 24h). These observations clearly indicated that the nsPEF exposure has a significant impact on gene expression, although the immediate cellular and sub-cellular changes leading to apoptosis are not apparent.

Discussion: Dr. Tseng mentioned that nsPEF exposures could help (a) in extending the current understanding of molecular pathways involved in signal transduction mechanisms, apoptosis, cell death, etc, and also in the identification of new molecular pathways.

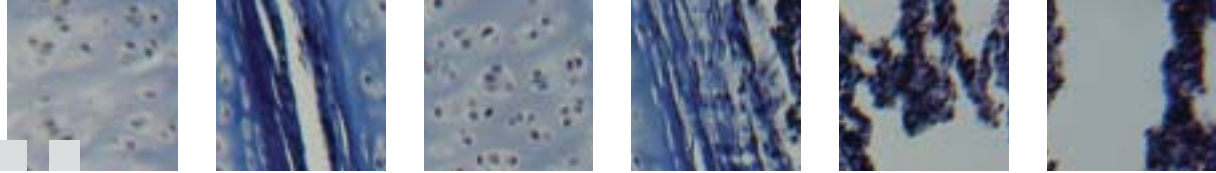
Session-1.3. **Dynamic effects of nanosecond pulsed electric fields on human cell signal transduction and function.**

Beebe, S.J. USA.

The objectives of the studies were to investigate the markers for apoptosis, intracellular calcium using fura-2 and fluorometry, green fluorescent protein expression by flow cytometry, and gene expression by microarray analysis in several cultured mammalian cells exposed to nsPEF with durations between 10, 60, 300 nanosec and electric fields between 4-300 kV/cm. In addition, mouse fibrosarcoma cells were exposed *in vivo* to nsPEF using needle electrodes and analyzed for apoptosis markers, tumor size and tumor weight. The results in HL-60 cells indicated that (a) when electric fields strength were high enough,

apoptosis signaling pathways were activated – this was determined by phosphatidylserine externalization, caspase activation, DNA fragmentation, and cytochrome c release into the cytoplasm, (b) when the nsPEF exposure was 60ns, 15kV/cm (which is below the threshold to induce apoptosis and classical plasma membrane electroporation) the molecular signaling was initiated by mobilization of calcium from the endoplasmic reticulum and the subsequent entry of calcium through the ‘porated’ channels in the plasma membrane, (c) in a total of 20,500 genes analyzed, using the minimum value of a 2-fold difference, the expression of 603 genes (~3%) was up-regulated (~67 %) or down-regulated (~33 %). The expression of ~60 genes were found to be affected by >4-fold. The affected genes were involved in signal transduction, ion transport, DNA binding, and transcription control, among others. In adherent HCT116 colon carcinoma cells, however, phosphatidylserine externalization and caspase activation were present but the cells did not die, suggesting that these two markers serve as non-apoptotic functions in these cells. Mouse fibrosarcoma tumors exposed *in vivo* to nsPEF exhibited DNA fragmentation and elevated caspase activity and were reduced in size and weight compared to contra-lateral sham-treated control tumors. These data demonstrated that nsPEF exposure profoundly modulate cell signaling mechanisms involving second messenger actions, ion transport, and transcription/translation that determine survival or death functions. Thus, nsPEF exposures can provide new tools in basic science research and/or in therapeutic clinics.

Discussion: Dr. Beebe mentioned that conditions of nsPEF exposure need to be modified to examine cell cycle effects, for example, killing of cells in S-phase may require higher pulses. Development of new ‘delivery systems’ are required to apply the nsPEF technology in therapeutic clinics.



Session-1.4. The rules of cell survival after exposure to high-intensity ultra-short electrical pulses. Pakhomov, A. USA.

The objectives of the investigations are to: (a) analyze the cytotoxic effect of different voltage (87, 125, and 174 kV/cm) and repetition rates of 10-ns electrical pulses (EP), (b) characterize the time dynamics and general mechanisms of cell death, and (c) develop quantitative criteria for assessing differential sensitivities of cells. Experiments were performed using cultured U937 (human histiocytic lymphoma) and Jurkat (human T-cell leukemia) cells which were grown in RPMI 1640 medium with 10 % fetal bovine serum. Aliquots of cells (0.2×10^6 cells/ml) were exposed to high-intensity 10 nsEP in gene transfer cuvettes. Pulse shape and amplitudes were monitored in digital oscilloscope using a custom-made high-voltage probe. Exposures were performed at 24-25°C, and the temperature of exposed samples did not exceed 37°C. Live and dead cell densities were determined, at intervals from 0.5 hr to 72 hr post-exposure, by counting cells in a hemocytometer using Trypan blue dye-exclusion method. The induction of apoptosis was evaluated by the specific cleavage of poly (ADP-ribose) polymerase (PARP) using Western blot, and by inter-nucleosomal DNA fragmentation using agarose gel electrophoresis. Exposure of U937 cells to brief pulses (e.g., 100 pulses at 100 kV/cm, or 200 pulses at 75 kV/cm) killed only few cells, if any. Exposure to longer pulses and/or at higher voltage caused both immediate (necrotic) and delayed (at least partially apoptotic) cell death. Live cell density reached its minimum at 8-16 hr after the exposure after which the surviving cells proliferated at a regular rate. Exposure Jurkat cells to nsEP showed the same immediate and delayed types of cell death, but the cells were far more sensitive to the same EP treatment than U937. Jurkat survivors failed to restore normal proliferation rate within 72 hr of post-exposure observation. Analysis of the cytotoxic

efficiency varied at different E-field values, numbers of pulses, and pulse repetition rates. Thus the data indicated that the indices of 'D₀' and 'n' values can be used for quantitative comparison of EP sensitivity of different cell lines. The respective values in our study were 108 J/g and 1.5 for Jurkat cells, and 266 J/g and 1.63 for U937. Cellular mechanisms responsible for differential sensitivity of these cell lines to nsEP are yet to be determined.

Discussion: Dr. Pakhomov mentioned that dose-response curves with nsEP exposure were similar to those reported for ionizing radiation exposures. This may suggest common interaction principles and/or cell killing mechanisms. The possibility of selective killing of certain cell types, with little impact on other cells, may have potential applications in biotechnology and medicine.

Session-1.5. Ultrawideband electromagnetic radiation (UWB EMR) exposures and activation of signal transduction pathways. Natarajan, M. USA.

Signal transduction is a fundamental process that the cells and organisms use to respond to external stimuli, and thus attempt to maintain normal function and homeostasis. Attempts were made to identify and understand the activation of signaling mechanisms initiated by UWB EMR exposure using cultured human monocytes. The cells were exposed intermittently to UWB pulses for a total of 90 minutes (30 minute on and 30 minute off, repeated 3-times). The pulse width was 0.8 ns, pulse rise time was between 258 to 273 ps, pulse E-field was 100 kV/m, and the pulse repetition rate was 250 pps. The temperature of the medium was maintained at 37°C in both sham- and UWB EMR-exposed flasks. Immediately after exposure the cells were transferred to 37°C and harvested after 10 min, 0.5 h, 4 h, 8 h, 24 h, and 48 h. Initiation of the NF-κB signaling pathway was ana-

lyzed by EMSA. The downstream effect of signaling pathway was examined in cells (which were transiently transfected either with NF- κ B.luciferase reporter vector or control vector) exposed as described above and harvested after 16 h. The differential expression of NF- κ B dependent genes was screened at 8 and 24 h post-exposure using NF- κ B super-array. The results indicated that the cells exposed to UWB EMR showed a marked increase in the NF- κ B DNA-binding activity compared to sham-exposed cells. The effect appears to be delayed until 24 hours post-exposure. Alterations in gene expression were not detected in cells exposed to UWB EMR.

Discussion: Dr. Natarajan mentioned that the response of the cells to UWB EMR exposure is only transient with undetectable effect on gene expression.

Session-1.6. **Tumor cell killing by 10 nsec, 80-300 kV/c electric pulses.**

Whitehead, T. USA. (This is presentation which was substituted for Roti Roti, J.L.)

The objective of the study was to determine whether 10 ns high intensity electric pulses (EP) can be used for therapeutic cell killing. Cultured HeLa cells (adherent and suspension cell) were exposed to 10 nsec, 80-300 kV/cm electric pulses, and the cell survival was determined using clonogenic assay. The data indicated a rapid induction of apoptosis as detected by Annexin V and flow cytometry. The cells in suspension appear to be slightly more resistant than adherent cells – suggesting that the high intensity EP have therapeutic role in clinics. Regarding the data to be presented by Roti Roti, Dr. Whitehead mentioned that exposure of cells to 95 GHz followed by 43°C treatment appear to affect DNA repair genes and DNA repair proteins.

Tuesday, June 22, 2004

Tutorial Session 1

[High-Throughput Screening techniques in EMF Research](#)

Tutorial Session-1.1. Proteomics and mass spectrometry: Tools for genome-era biology.

Grigffin, T. USA

The systematic and quantitative analysis of genes and proteins (gene products) in the Human Genome Project has revolutionized the research in modern biology. Mass spectrometry (MS) is one of the most powerful tools used for rapid identification, localization, quantification and determination of functional activities of proteins that are expressed in complex mixtures. This has led to the development of the field of PROTEOMICS. The core methodology has emerged from the ability to label proteins and peptides with stable-isotopes followed by automated mass spectrometric analysis. A wide-array of biological problems that cut across the fields of biochemistry, molecular and cellular biology, and clinical research can be addressed using MS analyses. The laboratory procedures involve extraction of proteins → digestion of proteins into peptides → multi-dimensional separation of peptides (gradient chromatography) → isolation of peptides → fragmentation of peptides (mass spectrophotometric analysis, 1000s of peptides in a matter of hours) → search in data base (tandom mass spectrum) → identification of proteins. Isotope-coded affinity tags (ICAT) are used to tag the proteins and peptides before multi-dimensional separation and analysis by cation exchange HPLC followed by quantitative proteomics. The other methodology discussed in detail included experimental design, sample preparation, application of MS-based proteomics for large-scale analysis of protein expression, characterization in macro-molecular complexes, interactions and analysis of sub-cellular protein localization, and future directions of research in EMF field. The data



from the protein modifications following exposure of cells to oxidative stress were presented.

Discussion: Dr. Griffin mentioned that a maximum of 2000 proteins can be identified using ICATs. The specific modifications that are examined in cells exposed to EMF were not answered.

Tutorial Session-1.2. **Analysis of gene expression in EMF research.**

Maercker, C. Germany

An approach to analyze whole genome is essential since genes not only act together but also interact with each other. Methods for whole-genome analyses using micro-arrays (high-throughput, HTPT) have been established about six years ago. Since then, these methods have evolved into promising standard procedures and are applied in many laboratories all over the world. One advantage of the HTPT methods is that by the use of a single assay, the networks of all genes becomes visible at the same time, in the whole genome of a given cell – without pre-selection. This is important because, for example, heat shock genes can have many different functions and such functions are hard to be discriminated by single gene assays using RT-PCR method. The basic procedure in HTPT involves the extraction of total RNA → preparation of cDNA (reverse transcription) → biotin labeling of cRNA (*in vitro* transcription) → fragmentation of biotin labeled cRNA → control and test samples are labeled with 2 different fluorescent dyes that gives red and green colors → hybridization with gene chips → wash and stain → scanning and quantitation. Red color spots → up-regulated genes; green color spots → down-regulated genes; yellow color spots → genes that are expressed equally in control and test samples. A total of up to 30,000 genes can be analysed using a single gene array on a microscope slide. One of the aims of REFLEX project funded by the European Union was to determine if gene expression tech-

nology fits into requirements for EMF research. Experiments were conducted using primary fibroblasts (ES1 cells) exposed for 24 hours to intermittent 50 Hz EMF (5 min ON and 10 min OFF). Statistical analysis of the data showed a significant down-regulation of genes involved in NF- κ B and Ras/MAPK pathway. Similar observations were made when the cells were exposed to 1800 MHz (GSM) at 20 W/kg SAR for 1 hour. Furthermore, there was an increased expression of lectins and indications for alterations in calcium signaling pathways following exposure of the cells to EMF/RF. The results from other experiments showed increasing ribosome biogenesis in HL-60 leukemia cells after EMF/RF exposure which was accompanied by over-expression of several heat shock proteins. Therefore, heat shock genes might be involved, but the EMF/RF response was obviously different from the heat shock response. Gene expression analysis of endothelial cells treated with EMF/RF revealed altered regulation of cytoskeletal genes, and these results could be confirmed by protein data. The gene array technology has its limitations. The expression of genes depends on cell type, developmental stage, environmental influences, genetic background, etc. Therefore, repetition of the experiments to get statistically significant data for all genes is essential. Also RNA stability and post-translational modifications like phosphorylation of proteins can not be investigated on the level of transcriptomics. Pilot experiments using antibody chips are necessary to confirm the observations. These antibody chip arrays are able to specifically bind to the different cellular proteins and are therefore a versatile tool for gene expression and phosphorylation studies. Altogether, the gene array technologies open a more detailed view of the genome/proteome of cell and are therefore promising steps towards functional genome analysis of cells exposed to EMF/RF. **Discussion:** Dr. Maercker mentioned that the reproducibility of gene expression in micro-arrays was AP-

PROXIMATELY 80% for up-regulated genes. Ageing may have an effect on the expression and interaction of genes.

Tutorial Session-1.3. Protein microarray technology – Principles and applications in proteomics.

Stoll, D. GERMANY.

Methods which can be used for the determination of changes in time-dependent or stimulus-dependent expression of proteins are valuable tools to investigate the biological effects at molecular level. Protein micro-arrays are now available to screen entire genome for proteins that interact/act as substrates for protein-modifying enzymes and/or targets of immune responses. These micro-arrays are useful tools for proteome analyses and supplement other technologies like 2D-PAGE (2-dimensional polyacrylamide gel electrophoresis), liquid chromatography or MS methods. The laboratory procedures involves (a) flow cytometry using beads of different colors (and no image analysis necessary) to analyze up to 1000s of samples/day and (b) planar surface micro-array assay (image analysis) which is highly sensitive to detect up to 3000 fluorescence spots and can analyze up to 20,000 samples/day. Theoretical advantages, expectations and limitations of miniaturized multiplexed ligand binding assays were also discussed together with their huge potential for proteomic research and diagnostic purposes. Discussion: Dr. Stoll mentioned that the numbers of protein spots that can be detected are generally ~1,500; however, the numbers of protein that can be analyzed depend on the cross-reactivity of antibodies. The spot size should not be too small (~80-150 microns) and should be able to capture at least one molecule.

Tutorial Session-1.4. 2-DE/MS proteomics studies – some practical aspects of analysis.

Nylund, R. FINALAND.

Over the past years HTPT screening techniques have become widely used in the several different research fields. Screening for the expression of proteins is usually referred as proteomics. There are various ways to screen protein expression. One of the most common techniques is a combination of the two-dimensional gel electrophoresis (2D-E) (to determine the molecular weight by isoelectric focusing) followed by mass spectrometric analysis (MS). Repeatability is crucial for 2D-E approach: several repeats are needed to be combined and compared against each others to obtain reliable results. The sample preparation is a key component for successful 2D-E separation of proteins and the method varies depending on the nature of the sample. The samples are usually lysed by homogenization using a detergent/glass beads/sonication/freezing-thawing. A protease inhibitor needs to be included to prevent the occurrence of proteolysis. The proteins are then precipitated with trichloroacetic acid, denatured and subjected to ultracentrifugation. A proper percentage SDS or PAGE gel (10-12.5%, depending on the size of the proteins under investigation) need to be used. The loading capacity of the samples in the gels need to be adjusted (100-500 microgm total protein) for optimal resolution. The amount of protein appears to influence the method and the reliability of the results. Commercially available strips (7-24 cm) or tubes can be used for isoelectric focusing, The protein spots should be cut, digested using trypsin, visualized (autoradiography where radioactive materials need to be used, silver staining which is non-radioactive but has poor linearity, fluorescent dyes which exhibit good linearity and rapid or coomassie blue staining which is easy to use but low sensitivity) for peptide-finger-printing using MS analysis. Typically staining method causes some



limitations either in the detection sensitivity or in the linearity of the detection. A gel-based scanner with densitometer and a computer to store images should be available.

Discussion: Dr. Nylund mentioned that one of the most common problems in MS studies are contaminants which are in the sample. Some non-natural protein modifications caused by staining may also hinder the analysis. The optimization of the technology takes time - from some weeks to months. However, an optimized system is efficient to screen several proteins at the same time and will provide a good overview of the general proteome appearance, in which unknown effects can be efficiently studied.

Tutorial Session-1.5. **Use of high-throughput screening techniques in EMF research.**

Leszczynski, D. FINLAND.

New EMF/RF frequencies and modulations are continuously introduced into the society with the development of new telecommunication systems. In spite of the years of research there is still uncertainty whether low-energy EMF/RF can induce biological effects and whether such effects pose any adverse health hazard in humans. The results from the most commonly studied 'obvious' effects of EMF/RF exposures on carcinogenesis, blood-brain-barrier, development, hypersensitive responses effects are not reproduced in independent investigations. The biological effects thus far reported are uncertain since the biophysical mechanisms behind their occurrence are unknown. The discussion is now to determine whether EMF exposure can induce some weak effects (without causing any disease) that are detrimental to the quality of life – sleep disorders, headaches etc. There is a need for a relatively simple and rapid screening test(s) that would determine whether new EMF frequency or modulation will induce unpredicted/unexpected biological effects. The use of the HTPT for proteomics

and transcriptomics has been proposed as a useful approach to determine if EMF exposure has any potential biological targets at cellular level. The HTPT have several advantages as well as many limitations and uncertainties. However, when cautiously used, HTPT offers a unique opportunity to rapidly determine which genes and proteins respond to EMF exposure. The data thus obtained from HTPT could help not only in finding biological effects of EMF but also in understanding the biophysical mechanism behind the biological effects. Research efforts and resources can then be focused on these known targets of EMF radiation.

Discussion: Dr. Leszczynski said – if we consider using HTPT, the potential candidate genes/proteins need to be identified. There should not be too many or too few of such genes/proteins for testing. Consideration must also be given to establish a network of laboratories to perform these standardized tests.

Wednesday, June 23, 2004

Tutorial Session 8

Biophysical and biological dosimetry. I

Session-8.1. **Genotoxicity in mice exposed to millimeter waves.**

Vijayalaxmi. USA

A multitude of beneficial uses of millimeter waves (MMW) in medicine have been described in the literature. MMW technologies are also increasingly used in traffic and military radar systems, wireless communication devices, etc. It was considered relevant to investigate the genotoxic potential of MMW exposure in experimental animals since such damage is very often linked to carcinogenesis. The objective of the investigation was to determine whether or not: (a) MMW wave exposure induces genotoxicity, and (b) MMW exposure will modify the genotoxic effects of cyclophosphamide (CP), a chemotherapeutic drug used in the treatment of various malignancies. MMW

radiation was produced using a Russian-made YAV-1 generator and transmitted at 42.2 ± 0.2 GHz (7.1 mm). The peak SAR and peak incident power density were determined as 622.0 ± 100.0 W/kg and 31.0 ± 5.0 mW/cm², respectively. The experiment was conducted over a period of four days. A total of 48 male BALB/C mice were randomized to 6 groups of 8 animals each: (1) untreated controls, (2) exposed to MMW for 30 min/day, for 3 consecutive days, (3) sham-exposed as in group 2, (4) injected with CP (i.p. 15 mg/kg body weight) on the second day of the experiment, (5) 30 min of MMW exposure on the first day, injected with CP (15 mg/kg body weight) immediately after 30 min of MMW exposure on the second day, and 30 min of MMW exposure on the 3rd day, and (6) sham-exposures and CP treatment as in group 5. All mice were sacrificed at 24 hours following the last treatment. Peripheral blood and bone marrow smears were examined to determine the extent of genotoxicity which was assessed from incidence of micronuclei (MN) in polychromatic erythrocytes (PCEs). The average frequencies of MN/2000 PCEs were 6.0 ± 1.6 in untreated mice, 5.1 ± 1.5 in MMWexposed and 5.1 ± 1.3 in sham-exposed mice. In the bone marrow, the values were 9.1 ± 1.1 in untreated mice, 9.3 ± 1.6 in MMWexposed and 9.1 ± 1.6 in sham-exposed mice. In contrast, mice which were injected with CP exhibited a significant increase in MN/2000 PCEs, 14.6 ± 2.7 in peripheral blood and 21.3 ± 3.9 in bone marrow cells ($p < 0.0001$). However, the values were not significantly different in mice which were additionally exposed to MMW (14.3 ± 2.8 in peripheral blood and 23.5 ± 2.3 in bone marrow) or sham-exposed mice (15.4 ± 3.0 in peripheral blood and 22.1 ± 2.5 in bone marrow cells). Thus, there was no indication that MMW exposure was capable of inducing genotoxicity in both peripheral blood and bone marrow cells. Also, MMW exposure did not influence cyclophosphamide-induced MN in both tissues. Discussion: Dr. Vijayalaxmi men-


tioned that MMW are extensively used in clinical medicine in Russia and in Eastern European countries. Patients are exposed to MMW on forearm or forehead to alleviate severe pain in the stomach or to treat gastrointestinal disorders. The hypothesis which has been proposed for such beneficial effects is that MMW exposure induces cytokines which are distributed systemically in the body and those cytokines exert their influence to relieve pain in remote organs. Similar hypothesis was used in the current study by exposing the mice to MMW in the nose area only and the beneficial effect (reduction in the incidence of CP-induced MN?), if any, was investigated in distance areas, i.e., in bone marrow and peripheral blood cells. The data did not indicate any beneficial effect of MMW exposure on CP-induced MN. But, MMW exposure *per se* was not genotoxic and did not induce MN.

Thursday, June 24, 2004

Tutorial Session 3

[Research related to the effect of radiofrequency radiation on micronucleus formation](#)

This tutorial session is organized by the Center for Devices and Radiological Health (CDRH) of the Food and Drugs Administration (FDA). A couple of years ago, the FDA signed a cooperative research and development agreement (CRADA) with the Cellular Telecommunication and Internet Association (CTIA) to address the public's concerns and to answer the questions related to the safety of using wireless phones. Under the terms of CRADA, the FDA had funded 3 research laboratories to investigate the effects of exposure to radiofrequency radiation (RFR) emitted from wireless communication devices on micronucleus (MN) formation. Discussing their research findings were Dr. Bernd Gortitz, Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany, Dr. Raymond Tice, Integrated laboratory



systems, Research Triangle park, NC, USA, and Dr. Maria Rosaria Scarfi, Interuniversity center on interaction between electromagnetic fields and biosystems, Naples, Italy. Three invited panel members – Dr. James McGregor (FDA), Dr. Greg Lotz (NIOSH) and Dr. Russel Owen (EPA) participated in the discussions.

Tutorial Session-3.1. Effects of 1-week and 6-weeks radiofrequency (RF) exposure on micronucleus formation in B6C3F1 mice.

Gorlitz, B. Germany.

The aim of the study was to investigate a potential induction of MN in restrained mice exposed (whole body) to RFR for 2 hours/day for 1 week or 6 weeks. The SARs used for 1 week exposure studies were 0, 0.44, 1.33 and 4.0 W/kg. The SARs for 6 weeks exposure investigations were 0.0, 0.33, 1.0 and 3.0 W/kg. A total of 80 mice (10 males and 10 females) in each 902 MHz and 1747 MHz RFR groups and their respective sham-exposure groups were used in the experiments. At the end of the RFR exposures, all mice were sacrificed by an overdose of carbon-dioxide. Microscope slides were prepared from bone marrow (1 week exposure), peripheral blood (6 weeks exposure), and skin keratinocytes from tail root and cultured lymphocytes in the spleen (1 and 6 weeks exposures). In each mouse, 1000 spleen lymphocytes and 2000 cells in bone marrow, peripheral blood and keratinocytes were examined to determine the incidence of MN in coded slides. The results were decoded after the completion of the analyses. The data indicated that there were no significant differences between RFR-exposed and sham-exposed mice in their mean body weights, percent polychromatic erythrocytes and the incidence of MN. Thus under the experimental conditions used RFR exposure had no effect on MN formation in mice.

Discussion: Dr. Gorlitz mentioned the following. (1) The exposures were designed to simulate short-term

and long-term human usage. (2) Cage controls were not included. (3) Mice injected with cyclophosphamide were included as positive controls. However, there was no positive response in skin keratinocytes. (4) Pair-wise comparisons in MN indices were made between RFR-exposed and sham-exposed mice and the error rates (alpha-level) were controlled for multiple comparisons. (5) Because of insufficient numbers of cells available for analysis, only 1000 cells were examined in some cases.

Tutorial Session-3.2. Effect of exposures to radiofrequency radiation (837 MHz RF) emitted by a TDMA cellular telephone on the frequency of micronuclei in cultured human blood lymphocytes.

Tice, R. USA.

The results from previous investigations (Tice et al, Bioelectromagnetics, 23, 113-126, 2002) indicated a significant increase in MN in human blood lymphocytes, from a single donor, exposed for 24 hours, in culture tubes, to RFR signals (at ≥ 5.0 W/kg SAR) in transverse electromagnetic cells (TEM). There were 3 primary objectives for the current research project. (1) Comparison of MN obtained in human blood lymphocytes exposed for 24 hours to 837 MHz (SARs of 2.5, 5.0 and 10.0 W/kg) in TEM cells and in a radial transmission line system (RTL). Blood samples obtained from 3 males and 3 female donors were exposed either in petridishes in TEM cells or in culture flasks in RTL facility. This is to assess the influence of exposure conditions (petridishes versus culture flasks) and donor-to-donor variability. (2) Evaluation of MN in human blood lymphocytes, exposed in petridishes for 24 hours at 35°C, 39°C and 42°C (prior to cell culture) in TEM cells, without RFR exposure. (3) Evaluation of MN in human blood lymphocytes, exposed in petridishes for 24 hours at 39°C and 42°C (prior to cell culture) in TEM cells, with RFR exposure at SARs of 5 and 10 W/kg. The results indicated the

following. (1) Exposure of cells (from 3 males and 3 females) to 837 MHz RFR in TEM cells or in RTL did not increase MN at all SARs tested (except in one female donor at 10 W/kg SAR). There were no significant differences between the cells exposed to RFR in petridishes and in culture flasks. Also, there were no significant differences between the donors. (2) Exposure of cells (1 male and 1 female donor), in petridishes, for 24 hours to different temperatures, 35°C, 39°C and 42°C, in TEM cells, without RFR exposure, resulted in a sporadic but inconsistent pattern of increase in MN. (3) Exposure of cells (1 male and 1 female donor), in petridishes, for 24 hours to 39°C and 42°C in TEM cells, with 837 MHz RFR exposure (SAR of 10 W/kg), did not induce MN. Thus the data did not show increased MN in human blood lymphocytes exposed to 837 MHz RFR, in TEM cells or in RTL system, under different exposure conditions. These data are in contrast with those observed in previous studies (Tice et al. 2002). The major difference was in the use of petridishes in the current investigations while culture tubes were used in previous investigations. Also, the absence of an increase in MN in cells exposed to 39°C and 42°C with and without RFR signals suggested that increased temperatures are not likely the cause for increased MN observed in previous studies (Tice et al 2002).

Discussion: Dr. Tice said the following: Positive controls were not included in the study. Water was circulated around samples to maintain the temperature within the range: engineers were adjusting the fluctuating temperatures throughout the exposure period. Presentation of the data on the basis of individual donors is preferred since in some donors, there was a definite increase in MN in cells exposed to RFR: the significant difference between RFR-exposed and sham-exposed cells in such donors would NOT have been identified if the data from all donors were pooled. Dr. Tice maintained that the data from his previous study

(Tice et al 2002) was valid. Based on the current data, temperature increases could NOT explain the increases in MN observed in previous studies. Dr. Tice suggested that “regulators” must make a careful examination of the differences in RFR exposures carried out in culture tubes (increase in MN) and petridishes (no such increase in MN). Dr. Tice did not give detailed answers to the questions related to: (a) the dosimetry - whether the SAR was based on homogeneous distribution of blood sample and if so, the influence of the blood being settled at the bottom of culture tubes, petridishes and culture flasks? (b) The data presented as fold-differences between RFR-exposed and sham-exposed cells is difficult to understand since it is not possible to know whether a particular sham-exposed value dropped 50% from the level of other sham-exposed cells or whether the RFR caused a 50% increase in MN compared to all sham-exposures.

Tutorial Session-3.3. **Induction of micronuclei in human blood lymphocytes after in vitro exposure to mobile telecommunication radio frequency signal.**

Scarfi, M.R. Italy.

Collaborative investigations were conducted in two separate laboratories, in Rome and in Naples. There were 2 objectives. (1) Comparison of MN obtained in human peripheral blood lymphocytes (10 donors – 5 donors tested in each laboratory) exposed for 24 hours to 900 MHz (GSM signal) at peak SARs of 0, 1, 5, 10 W/kg: the temperature was maintained at 37°C during exposure. (2) Evaluation of MN in human blood lymphocytes (4 donors - 2 donors tested in each laboratory) exposed for 24 hours to conventional heating (35°-42°C) with simultaneous exposure to 900 MHz at peak SARs of 1, 5 W/kg. Sham-exposed and positive controls (0.33 microg/ml mitomycin C, MMC) were included in all experiments. A written consent was obtained from each of 10 blood donors who



were between 20-30 years of age. Information regarding each donor's current working environment, smoking habits, alcohol consumption, exposure to chemicals, recent history of viral infection, vaccination, diagnostic X-rays, was also collected. The experimental protocol involved exposures of duplicate aliquots of 3 ml of diluted whole blood (1ml whole blood + 2ml medium) to RFR followed by stimulation of cells with phytohemagglutinin and incubation at $37\pm 1^\circ\text{C}$. Cytochalasin B ($6\ \mu\text{g}/\text{ml}$) was added to all cell cultures at 44 h of incubation. All cells were harvested after a total culture period of 72 hours, treated with hypotonic solution, fixed and stained with Giemsa. Coded slides were exchanged between both laboratories which allowed independent scoring of the same slides. The scoring criteria used were that in each culture, the incidence of binucleate cells (BCI) should be 30-60% and the spontaneous MN frequencies should not exceed 12 in 1000 BCI. A wire patch cell system was used to expose the cells (in 35 mm petridishes) to 900 MHz. Numerical and experimental dosimetry was used to determine the distribution of SAR levels in petridishes. A four channels system has been set up to allow simultaneous exposures at four different power levels. The power levels and temperatures during experimentation was recorded and stored in a data base in the computer. The data thus far collected in both laboratories indicated no significant differences between RFR-exposed and sham-exposed cells in the incidence of BCI (40-48%), lymphocyte proliferation index (>1.5 and <2.0), and the frequency of MN ($<5/1000$ BCI). Positive control cells treated with MMC exhibited decreased BCI ($<40\%$), decreased lymphocyte proliferation index (<1.5) and increased incidence of MN (25-30/1000 BCI). Thus there was no evidence of genotoxicity in human blood lymphocytes exposed to 900 MHz RFR at SARs upto 10 W/kg. The experiments on the objective #2, i.e., the influence of temperature, are in progress.

Discussion: Dr. Scarfi said that data collected in Rome and in Naples did not confirm the observations reported by Tice et al., in 2002. Attention was also drawn to the peer-reviewed publications by several independent investigators who (used adequate temperature controls during RFR exposures) failed to confirm the data reported by Tice et al., in 2002.

At the end, several attendees at the session were left with the impression that Dr. Scarfi's investigations were appropriately conducted, and the data collection as well as their presentation was excellent.

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Glossary

Electropermeabilization	disruption of the cell membrane induced by an electric field pulse of high intensity and short duration
Apoptosis	Apoptosis is defined as programmed cell death controlled by the biological cell itself, in contrast to necrosis.
Organelles	In biology, an organelle ('small organ') is a subcellular functional structure encompassed by a membrane.
Electroporate	method to create holes in biological membranes, e.g. to insert DNA into living cells
Eosinophil	type of white blood cell from the group of phagocytes (engulfing cells) which respond to allergens and parasites and ingest them
Endoplasmic reticulum	the endoplasmic reticulum (endoplasmic = 'within the cytosol'; reticulum = 'small network', in short ER) is an important cell organelle found in all eukaryotic cells; among its functions are protein synthesis and signal transduction
Translocation	In genetics, translocation generally means the migration of chromosome or chromatid fragments within a given chromosome set. Here simply the transport of membrane components.
Necrosis	In biology and medicine, necrosis is a condition where an area of tissue dies. The rest of the organism stays alive, only part of it is destroyed.
Aliquot	[Lat. aliquot = some, several] part of the sample (e.g. in microbiology), if the whole sample cannot or shall not be examined
Caspase	caspases are a family of protein-processing enzymes; most important enzymes in apoptosis
Protein expression	the production of a protein according to the information stored in a gene
'second messenger'	links within the signal system of hydrophilic hormones and neurotransmitters; the activation of a 'second messenger' allows the enhancement or differentiation of the signal
S-phase	stage of cell division; S stands for synthesis, the genetic information in the cell is doubled
Agarose gel electrophoresis	gel electrophoresis is an analytical method used in chemistry and molecular biology to separate different types of molecules; (see below: electrophoresis), a combination of molecules that are to be separated are exposed to an electric field and migrate through a gel, in this case an agarose gel in a ion buffer solution
Electrophoresis	the migration of electrically charged particles through a material serving as a carrier within an electric field

Adherent cells	cells that are attached to surfaces
Proteomics	Study of the proteome, i.e. all proteins expressed in a cell or a living organism
Ribosome	ribosomes are protein-RNA complexes occurring in large numbers in the cytoplasm of each cell; their purpose is to produce the protein from the sequence information of the mRNA; this process is called protein biosynthesis or translation
Biogenesis	(Greek) the origin of living beings; it comprises the individual development of organisms (ontogenesis), as well as the developmental history of the species (phylogenesis); here either the synthesis of ribosomes themselves, or synthesizing activity of ribosomes
Gene array technology (DNA microarrays)	method for the analysis of gene expression, i.e. for the determination of gene activity of a cell, tissue or organism. Numerous, densely packed samples of known small, one-strand DNA fragments ('oligonucleotides') are mounted on a glass slide ('DNA chip') at exactly determined spots in a grid of test fields ('array'; 'spotting'). When linking the DNA fragments previously marked with a fluorescent dye to the chip, the chip DNA and the test DNA bind and form more or less measurable fluorescent double-strand fragments ('hybridization'), dependent on DNA base-pair matches. The degree of hybridization of known DNA fragments gives information on gene expression in the unknown sample. This method is increasingly used also to determine protein volumes.
Micronucleus	In cell division, the nucleus membrane is dissolved during mitosis. The reconstruction of the nucleus membrane occurs towards the end of mitosis. However, if chromosomes are not close together, chromosome groups or even single chromosomes can be enclosed by an own nucleus membrane. Similar processes are observed also after mutagen exposure. As these subnuclei most often contain only single chromosomes or chromosomal fragments, they are called micronuclei (MN).

For other questions regarding terminology please go to www.femu.de, www.wikipedia.org or www.google.com (type in "define:", then the search word).