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Radiofrequency-induced carcinogenesis: cellular calcium homeostasis changes as a triggering factor

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Abstract
The aim was to study the effects of radiofrequency (Rf) in a mice strain characterized by age-determined carcinogenesis of lymphatic tissues. Mice were treated with a 1 h/week Rf exposure for 4 months. A group submitted to sham exposure was used as control animals. The evolution of carcinogenesis was followed up to 18 months. The maximal life span of control mice was about 24 months. All dead animals were clinically and histologically examined to give an age-determined comparative quantification of the evolving carcinogenesis. A radiocalcium tracer method permitted the evaluation of Rf effects on transmembrane transport of extracellular calcium at 1 and 24 h after exposure. The determination of induced lipid peroxidation completed this second study. The findings show that Rf provoked an earlier general lymphocyte cell infiltration, formation of lymphoblastic ascites and extranodal tumours of different histological types, as well as an increased early mortality. The results suggest that in Rf-exposed mice, carcinogenesis may be induced earlier and with different pathological forms than in control animals. The modifications in cellular calcium homeostasis and the age-determined thymus involution appear to be important factors involved in this carcinogenesis process.

Keywords: *Carcinogenesis, radiofrequrency, cell calcium role.*

Introduction

Three decades ago, it was shown that microwave exposure produced an increase in the number of lymphoblasts in lymph node cells (Czerski 1975), while microwave radiation-induced acceleration of spontaneous and chemical carcinogen induced animal tumours was also reported (Szmigielski et al. 1982). On the other hand, a possible correlation between the incidence of leukaemia and occupational exposure to electric and magnetic fields was also reported (Milham 1982). More recent is the publication of an important statistical increase of brain tumour development among users of analogue cellular telephones (Hardell et al. 2003).

To understand better the biological modifications underlying carcinogenesis by radiofrequency (Rf), we have undertaken the present investigation.

Materials and methods

Studies were carried out on female Ico:OFI (I.O.P.S. Caw) mice, 4–5 weeks of age (Charles River Laboratories, IFA CREDO, France). This mouse model was developed in 1935 by Carworth Farms, and named CF1 (Carworth Farms strain 1). Since 1966, it was introduced in France by IFFA CREDO France (currently Charles River Laboratories, France) with the name OF1 (Oncine France 1). The life span of the mice is about 24 months, and the principal characteristic of the mice is to develop spontaneous lymphoid tumours in the course of ageing. According to IFFA CREDO France, after a 200-mouse study and at 18 months of age, the distribution of the developed tumours in the different organs an tissues, expressed as the percentage of the total tumour-bearing animals was as follows: in lympho-myeloid complex 33.2%, in lung 3.1%, in

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ovary 8.4%, in skin and muscle 2.2%, in salivate glands 0.4%, and in kidneys 0.4%.

The animal protocols used in our work were conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), and the care of the animals was supervised by the Veterinary Service of the University of Nancy.

Animals were treated on the same time schedule with 1 h of Rf exposure each week for 4 months. Control mice were subjected to a sham Rf exposure done under the same experimental conditions. The Rf exposure was carried out in a cylindrical polypropylene chamber in which the antenna of the cellular telephone (ALCATEL OT 501: 800 MHz) was placed in the centre. Mice were free to move a circular corridor 5 cm wide with a 2 cm separation from the antenna. The exterior walls of the chamber, excepting the top central area, 4 cm diameter that allowed the positioning of the telephone, were covered with 10 mg cm^{-2} aluminium foil. The temperature of the chamber was maintained within 23–25°C by means of cold air circulation.

During the whole experiment, animals showing health deterioration suggesting imminent death were sacrificed in order to avoid necrophagia. Dead mice were dissected for clinical and pathological examination of the principal organs or fluids involved in the carcinogenesis process: blood, ascites, spleen, liver, abdominal, thoracic and neck lymph nodes, lung, kidney, submaxillar glands and brain. Considering that the maximal life-time of these mice is about 24 months, to avoid the interference of the characteristic lymphatic pathology of ageing animals, the clinical follow-up was ended at 18 months of age. According to the breeder (IFFA CREDO, France), the mortality at 13 months of age (for $n = 300$ mice) is about 4%, and about 50% at 20 months of age. From the same source, only 1% of the mice present lymphomas and leukaemia at 13 months of age, and 6% at 18 months (for $n = 200$ mice).

To assess Rf effects on cell membrane ionic calcium-transport, a group of 10 mice was intramuscularly injected with 10 μl isotonic calcium chloride (1.5 μCi ^{45}Ca), and 1 h thereafter the animals were exposed to Rf for 1 h. A control group (10 mice) received the same radioactive calcium chloride injection but it was subjected to a sham exposure. To calculate the radiocalcium specific activity of each tissue, as well as the tissue specific activity to blood specific activity ratio, immediately after Rf exposure mice were sacrificed, samples of blood, spleen, liver and brain collected and their radioactivity measured: the radiocalcium present is an indicator of the amount of extracellular calcium taken-up by the tissues (Figure 1).

In a second experiment, the transmembrane calcium transport was assessed in two groups of 12 mice intraperitoneally injected with 30 μmol calcium, given as isotonic calcium chloride (5 μCi ^{45}Ca). Thirty minutes after injection, one of the groups was exposed to Rf for 1 h, while the second group was subjected to a sham exposure. At the end of the exposure, six mice of each group were sacrificed. The remaining animals were killed after 24 h. Blood plasma, spleen and brain samples were measured for radiocalcium to calculate the 24:1 h radiocalcium uptake ratios in each group, and the radiocalcium uptake by tissues was compared with that of blood plasma. The lipid peroxidation induced after 24 h was determined in spleen and brain as thiobarbituric acid reactivity (Fodor & Marx 1988). The statistical significance of the differences between groups was determined by Student's *t*-test. A probability less than 5% ($p < 0.05$) was considered as significant.

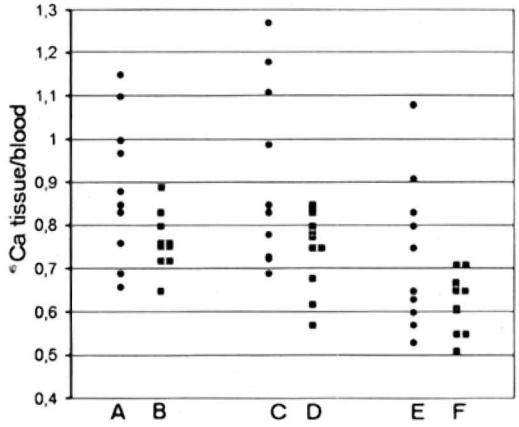

Figure 1. ^{45}Ca uptake as a specific activity in tissue to the specific activity in blood ratio by Rf-treated and control mice. (A) Rf-treated liver, (B) control liver, (C) Rf-treated spleen, (D) control spleen, (E) Rf-treated brain and (F) control brain.

Results

Immediately after Rf exposure, and compared with the control group, a radiocalcium uptake increase was noted in spleen, liver and brain of animals in this group (Figure 1). Increases in radiocalcium uptake after 24 h for the different organs, as well as the differences in induced lipid peroxidation are shown in Table I.

Table II summarizes the anatomic examination results of two age-determined groups: animals dead before 12 months of age, and those dead between 12 and 18 months of age. In the case of animals dead before 12 months of age, the most striking difference between Rf-treated and control groups was that Rf-treated animals showed a higher number of hypertrophied organs. It was the result of lymphocyte infiltration. Moreover, in three of the four dead mice,

ascites with lymphoid elements (in the range 14 450 – 26 700 cells mm^{-3}) were noted. An extranodal tumour (lung) was also observed in this group. In the second age group (12 – 18 months of age) the Rf-treated mice showed an increase of the characteristics also seen in the first age group. However, the same anatomic changes, although less in number and magnitude, were observed in controls. When sacrificed at 18 months of age (end of the follow-up), the number of control mice expressed as percentage of the initial animals (survival), was higher in the control (40%) than in the Rf-treated group (25%).

The histological examination of tissues from Rf-treated mice showed the following findings: in spleen, a marked peliosis and infiltration by atypical, immature and blastic lymphocytes with some of plasmocytoid type. In liver, sinusoid dilatation and significant infiltration of blastic lymphocytes, which sometimes showed a moruloid form. In kidney, atypical lymphocyte foci showing pleomorphisms and large nuclei. In thymus, a massive lymphoblastic infiltration affecting peripheral tissues (lymph nodes and adipose deposits) and some showed lymphocytes of plasmocytoid aspect (B-cells). In lymph nodes (abdominals, mediastinals, and axillars), as well as in parotid and submaxillar glands, an infiltration by atypical and lymphoblastic elements showed moruloid form at perivascular areas. A lung showed a malignant adenomatous tumour without lymphoid infiltration. An ovary presented a massive lymphoblastic infiltration. A meningeal lymphocytic infiltration without penetration of the cerebral parenchyma was seen in the brain, and some of these cells showed an oeosinophil cytoplasm. In general, these histological manifestations were less impressive in aggressiveness and proliferation in

Table I. *In vivo* ^{45}Ca uptake and lipid peroxidation in mice exposed to Rf, and to a sham exposure (controls). (a) ^{45}Ca uptake immediately after Rf exposure for controls value = 100, (b) ^{45}Ca uptake 24 h to 1 h ratios for a 1 h value = 100, and (c) Rf-induced lipid peroxidation at 24 h for controls value = 100.

(a)		
Spleen	112 ($p<0.025$)	
Liver	111 ($p<0.05$)	
Brain	112 ($p<0.05$)	
(b)		
	Rf exposed	Controls
Spleen	14 ($p<0.05$)	13 ($p<0.05$)
Brain	67 ($p<0.005$)	47 ($p<0.005$)
Blood plasma	12 ($p<0.025$)	7 ($p<0.05$)
Brain-to-blood plasma ratios		
7 ($p<0.05$)	6 ($p<0.005$)	
(c)		
Spleen	77 ($p<0.05$)	
Brain	43 ($p<0.0005$)	

Table II. Values corresponding to Rf-treated mice and controls for the two age-determined groups.

	Rf treated	Controls
Up to 12 months of age		
Dead mice	4	2
Spleen weight (g)	0.54*(0.31 – 0.72)**	0.26 (0.10 – 0.41)
Liver weight (g)	1.55 (0.75 – 1.94)	1.7 (1.63 – 1.77)
Kidney weight (g)	0.37 (0.35 – 0.41)	0.54 (0.50 – 0.58)
Hypertrophy	S(4), L(2), K(1), AL(4), O(1), T(1), P(2)	S(1), K(2)
Extranodal tumours	Lu(1)	–
Blood lymphocytes	3000 – 62 800 mm^{-3}	3500 – 8600 mm^{-3}
Ascites	(3) 0.5 – 2.0 ml	–
Ascites lymphocytes	14 400 – 26 700 mm^{-3}	–
Survival rate (%)	80	90
From 12 to 18 months of age		
Spleen weight (g)	1.10 (0.18 – 7.6)	0.89 (0.13 – 5.89)
Liver weight (g)	3.17 (1.59 – 12.3)	2.54 (1.42 – 5.31)
Kidney weight (g)	0.63 (0.46 – 1.07)	0.51 (0.29 – 0.60)
Hypertrophy	S(11), L(9), K(9), AL(7), O(2), T(2), P(1)	S(6), L(5), K(2), AL(6)
Extranodal tumours	(4) 0.6 – 12 g	(1) 2.4 g
Blood lymphocytes	2000 – 45 000 mm^{-3}	5000 – 300 000 mm^{-3}
Ascites	(2) 1 – 2 ml	–
Ascites lymphocytes	14 500 – 40 500 mm^{-3}	–
Initial number	20	20
Dead before 18 months	15	8
Survival rate (%)	25	50

S, spleen; L, liver; K, kidney; AL, abdominal lymph nodes; O, ovary; P, parotid and submaxillary glands; Lu, lung; T, thymus.
Values in parentheses are the number of affected animals.
*Mean weight; **range of weights (g).

tissues of control animals. Especially remarkable was the scarcity of the meningeal lymphocytic infiltration in brain. The different histological types of extra-nodular tumours observed in Rf-treated and control mice are shown in Table III.

Discussion

Rf exposure provokes modifications of the calcium transmembrane transport which changes the intracellular calcium ion homeostasis. In the present work, the immediate increase of ^{45}Ca-ion influx observed in spleen, liver and brain was higher in Rf-treated mice than in controls. The after 24-h higher ^{45}Ca-ion uptake by brain in comparison with that corresponding to after 1 h indicates an impairment of the buffering systems controlling the cellular calcium ion homeostasis

Nanomolar calcium ion influx is known to induce lymphocyte proliferation (Lichtman et al. 1983), and in the case of Rf exposure, it is possible that the equilibrium existing between ions, polyanionic macromolecules and glycoproteins of the cell surface can be disrupted by variations of the surrounding ionic concentrations by the rhythmic modulation of the Rf energy (Bawin et al. 1975). Consequently, an extracellular calcium ion influx by passive diffusion could provoke pulses of calcium ion-signal which induce lymphocyte proliferation. On the other hand, a chain of events: preneoplasia, neoplasia and eventual cellular death by over-calcification (necrosis) may follow an important change in intracellular calcium ion concentration modification (Anghileri 1995).

The absence of increased lipid peroxidation in tissues of Rf-treated mice corroborates the independence of the intracellular calcium homeostasis changes from the oxidative stress (Anghileri 1995). With respect to the thermal effects of the Rf exposure, the low-energy radiation, the experimental conditions of the current study (such as free movement in a range from 2 to 6 cm from the receiver antenna) and temperature-controlled exposure chamber, assure a minimal interference of these effects. In relation to this, an athermal hypothesis on the biological effects of electromagnetic radiation has been stated based on experimental evidence (Tyler 1975).

The importance of cellular calcium ion homeostasis in carcinogenesis is incontestable. The dependence of neoplastic transformation on great changes in the responsiveness to calcium ion-signal driving cell cycles and triggering cell differentiation is well reflected by the shedding of cyclic-adenosine monophosphate-dependent control during neoplastic transformation of lymphocytes. This is a consequence of the reduction of the dependence on calcium ion-signal resulting from an increased calcium ion influx. It decreases its intracellular concentration by eliciting the saturation of calcium buffering structures (Whitfield 1990).

Thymic factor activity is a relevant factor in tumour induction. The thymus is very important in the development of cellular immune functions. Mitotic agents lead a resting T-lymphocyte to enter the cell cycle by a previous increase in cytoplasmic calcium ions. In mice, the ageing process leads to an accumulation of memory lymphocytes bearing PGP-1, a 95-KD glycoprotein found in many cells and tissues. It fails to proliferate, to secrete lymphokines or to differentiate into mature effectors (Miller 1990). On the other hand, the stimulation of T-lymphocytes via the T-cell antigen receptor/CD3 complex activating a number of intracellular signal-transduction pathways is notably dependent on the calcium ion signal (Guse 1998). In mammals, ageing leads to a decline in the ability of T-cells to proliferate in response to antigens and mitogens (Walters & Claman 1975, Antel et al. 1980). Accumulated experimental evidence is consistent with the notion that calcium ion transport is an important site of age-dependent failure in the T-cell system (Kennes et al. 1981). In humans, the involution of the thymus contributes to the decrease of immunological vigour with advanced age. In mice, involution of immunological reactivity is developed at an earlier age in short-lived as opposed to longer-lived strains (Hori et al. 1973). This involution

Table III. Histological characteristics of the extra nodular tumours in mice treated with RF exposure.

Rf exposed	Controls
Non-lymphoid tumours	
Limb: carcinosarcoma (11.5 g – 14 months)*	Limb: fibro/leiomyosarcoma(5.1 g – 18 months)*
Limb: subcutaneous carcinosarcoma (0.6 g – 18 months)	
Abdominal wall: fibro/leiomyosarcoma (5.1 g – 18 months)	
Lymphoid tumours	
Limb: subcutaneous atypical lymphocytes with infiltration of fat and muscular tissues (1.8 g – 14 months)	

*Tumour weight – age.

appears to be related to the current findings with Rf on the onset of tumours at about the half of the maximal life span of the mice. Coincidentally, at about the half of their life-spans, in both humans and mice thymus-dependent immunity is reduced to approximately one-fifth of the newborn activity (Miller 1990), and immunological involution is an important factor that influences survival in mammals (Lewis et al. 1978).

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