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Coincident Nonlinear Changes in the Endocrine and Immune Systems due to Low-Frequency Magnetic Fields

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Electromagnetic field • Nonlinearity • Lymphoid phenotype • Chaos • Natural killer cells • Immunosurveillance

Abstract
Objective: The characteristic biological effects of low-frequency electromagnetic fields (EMFs) appear to be functional changes in the central nervous, endocrine and immune systems. For unapparent reasons, however, the results of similar studies have often differed markedly from one another. We recognized that it had generally been assumed, in the studies, that EMF effects would exhibit a dose-effect relationship, which is a basic property of linear systems. Prompted by recent developments in the theory on nonlinear systems, we hypothesized that there was a nonlinear relationship between EMFs and the effects they produced in the endocrine and immune systems. ***Methods:*** We developed a novel analytical method that could be used to distinguish between linear and nonlinear effects, and we employed it to examine the effect of EMFs on the endocrine and immune systems. ***Results:*** Mice exposed to 5 G, 60 Hz for 1-175 days in 7 independent experiments reliably exhibited changes in serum corticosterone and lymphoid phenotype when the data were analyzed while allowing that the field exposure and the resulting effects could be nonlinearly related. When the analysis was restricted to linear relationships, no effects due to the field were found. ***Conclusions:*** The results indicated that transduction of EMFs resulted in changes in both the endocrine and immune systems, and that the laws governing the changes in each system were not the type that govern conventional dose-effect relationships. Evidence based on mathematical modeling was found suggesting that the coincident changes could have been causally related.

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Introduction

Artificial electromagnetic fields (EMFs) having a wide frequency range and emanating from diverse sources are present everywhere in the environment, and their potential biological significance has received much attention [1, 2]. The fields are apparently not genotoxic, like ionizing radiation [3]. Instead, their primary biological effects appear to be functional responses in the central nervous, endocrine and immune systems [4]. This is problematical, however, because results of similar studies have often dif

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fered markedly from each other. For example, variability of results occurred in studies involving the effects of EMFs on intracellular calcium [5, 6], cellular transcription [7, 8], melatonin production [9, 10], lymphoid phenotype [11, 12] and the stress response [13, 14]. Explaining how EMFs might cause multisystem effects is a central problem in biology.

We proposed that the effects observed following the interaction of EMFs with living organisms were indirect consequences of field exposure [4]. According to this theory, following sensory transduction, afferent neuroelectric signals trigger efferent signals that activate the endocrine system, leading to changes in many different physiological parameters as a consequence of the ability of the body's sensory and effector systems to express and recognize each other's signals [15, 16]. The results of tests of various aspects of the theory have been presented [17–24]. Alternative theories have been proposed in which the biological changes caused by EMFs were considered to arise by means of a direct impact of the field on the affected cells or tissues [25–28].

Objections have been raised to all the theories, direct or indirect, primarily on the basis that the results of studies on the bioeffects of EMFs have been so variable, thereby suggesting to some that the reported effects were not actually caused by fields [1, 29–31]. This view is supported by the fact that not even one field effect has been shown to be readily reproducible in the hands of all interested investigators.

The biological effects of EMFs have almost always been analyzed under an assumption that a real effect would exhibit a dose-effect relationship, which is the characteristic behavior of a linear system (a system governed by a linear dynamical law). However, living organisms contain many complex control systems [1, 29–32], and it would therefore not be surprising if, for some agents, possibly including fields, effects could occur that were not linearly related to dose. This would imply some form of nonlinearity in the relationship between the agent and the response. For example, recent studies have unambiguously demonstrated the existence of a class of physical systems, called chaotic systems, that do not exhibit a dose-effect relationship because their activity is governed by a particular kind of nonlinear dynamical law [33, 34]. If the relation between EMFs and the bioeffects they produced were nonlinear, it might be possible to explain the variability in the reported studies.

We previously described a novel statistical procedure that was capable of revealing biological effects in animals exposed to EMFs without the need to resort to the assumption of linearity. We used the procedure to show that low-frequency magnetic fields consistently altered lymphoid phenotype via nonlinear processes [24]. The present study was undertaken to show that the same kind of changes occurred coincidentally in both the endocrine and immune systems, as would be predicted on the basis of our theory.

Methods

Experimental Design

In earlier studies on the bioeffects of EMFs, it was generally assumed that any real effect would be unidirectional and proportional to the field, and would occur more or less consistently in the variable chosen for measurement. We avoided these assumptions and allowed that a real effect could be either an increase or a decrease (depending on the animal), and that the particular variable affected by the field could be a priori undeterminable. A roulette wheel exemplifies the latter idea. An input (releasing the ball) always results in an output (ball in a slot), but the particular slot is not predictable [35].

It can be seen intuitively that the large-sample mean of a set of measurements of any particular variable in mice that responded unpredictably and bidirectionally to a field would be similar to the mean of the controls. In other words, if the input/output relation were as we hypothesized, a real effect of the EMF could not be observed by comparing means in large samples because oppositely directed changes would be averaged away. A single small sample might reveal the putative effect as a consequence of incomplete averaging, but statistical tests on small samples generally lack statistical power. To overcome the latter problem, we developed a novel statistical procedure that was suitable for inferring the kind of change that we hypothesized to occur.

The likelihood approach allows differences in means from replicate series of exposed and control groups to be combined to test an overall statistical hypothesis [36]. The log-likelihood ratio of the t statistic for a t test between an exposed and control group is:

$$l = 2N\,ln\left(1 + \frac{1}{2N-2}t^2\right),$$

where N is the number of animals in each group. The asymptotic distribution of l is χ^2 with 1 degree of freedom [37]. Using a Monte Carlo procedure, it can be shown that the distribution of l for $N \geq 5$ is approximately χ^2. For k pairs, the overall values of the test statistic, L, is

$$L = \sum_{1}^{k} l_i,$$

which also approximately follows the χ^2 distribution, with k degrees of freedom under the hypothesis of no treatment effect. Because L is sensitive to the *difference* between the exposed and control groups but not to the *direction* of the difference, L is suitable for testing a single overall hypothesis regarding the occurrence of true but not necessarily consistent EMF-induced effects in a series of k replicates. Of course, the same procedure can also detect consistent changes. Its novelty is its sensitivity to the kind of change not detected using standard methods. Therefore, the occurrence of changes due to exposure to 60-Hz magnetic fields was assessed on the basis of whether

$L > \chi^2_{k, 0.05}$, with $k = 3$ and N = 5. The null hypothesis was that the mean of the exposed mice in the first replicate was equal to the mean of the corresponding control, and the means in the second replicate were equal, and the means in the third replicate were equal. The alternative hypothesis was that one or more of the pairs of means differed for reasons that could not be attributed to chance (thus implying that transduction of the field must have occurred). Under these hypotheses, any kind of real change whatever justified rejection of the null hypothesis, irrespective of whether the data were consistent from replicate to replicate. We chose a group size of 5 because preliminary studies suggested it was large enough to characterize a population, but small enough to permit the large number of planned measurements on each animal. Controls included sham-exposed mice paired with each exposed group, and two experiments in which both groups were sham exposed.

In preliminary studies, we evaluated the use of the log-likelihood ratio for the F test for testing hypotheses involving variance (rather than hypotheses on means, as when the F test is used in ANOVA procedures). We found an unacceptably high number of false-positive results, and ultimately determined that they occurred as a result of the statistical properties of the variance. In particular, the false positives occurred more frequently than with the use of the log-likelihood ratio for the t test because the variance of sample variance is always greater than the variance of the sample mean. Consequently, differences in variance were not incorporated into our statistical design.

For the purpose of determining whether the kind of changes that occurred as a consequence of field exposure were consistent across the replicates, the data were also evaluated on the basis of whether $L > \chi^2_{1, 0.05}$, N = 15. The null hypothesis in this case was that the mean of all the mice exposed under the same conditions was equal to the mean of the corresponding controls. Thus, this method of analysis corresponded to the assumption that the field produced a consistent (average) change, which would suggest the applicability of a linear relationship between the field and the consequent changes in the measured variable. The use of the L statistic under this condition had a statistical power equivalent to a t test, and the results were identical to those found using a t test.

L for small samples (N = 5) is not precisely χ^2, but we showed that the error was immaterial (see Discussion below). The binomial theorem was used to assess whether the observed number of statistically significant L values in a particular experiment could reasonably be attributed to chance.

Approximately 9 experiments × 30 mice × 21 parameters = 5,670 individual measurements were made, of which about 15 differed by more than 5 standard deviations (SD) from their corresponding means. The outliers were included in the analysis because we had no objective basis to exclude them. Their inclusion had no effect on the interpretation of the data. Percentile comparison plots [36] (Statview, Abacus, Berkeley, Calif., USA) were made by normalizing each replicate by the mean of the control group and combining replicates. Data from different experiments were combined to yield distributions that were large enough to be analyzed. All mathematical calculations were made using Mathcad (Mathsoft, Cambridge, Mass., USA).

Exposure System

Magnetic fields were produced using an arrangement of four square coils [38], with construction details as specified elsewhere [39]. The outer and inner coils in each four-coil arrangement consisted, respectively, of 85 and 35 turns of 12-gauge magnet wire. Each coil was dipped in epoxy to minimize potential effects due to vibration and wrapped with grounded metal shielding to eliminate the possibility of electric-field effects. The shield was interrupted to prevent magnetic fields due to eddy currents. Each four-coil arrangement accommodated 4 cages on 2 shelves. Four sets of four coils arranged in an octapole configuration [39] constituted an exposure unit.

The units were designed using commercial software (MF3D, ERM Inc., Pittsburgh, Pa., USA) to produce homogeneous magnetic fields (± 5%) throughout the region occupied by the mice and a negligible fringing field. The predicted homogeneity and an absence of fringing field beyond 2 m from the unit were verified by direct measurements (Bartington MAG-03, GMW, Redwood City, Calif., USA).

Four identical units were built; two were used to produce magnetic fields, and the others were short-circuited and used to house the control mice. The exposure and control units were separated by more than 5 m in a room in the institutional animal care facility, which was chosen because of its low ambient 60-Hz magnetic fields and uniform geomagnetic field. The ambient 60-Hz field at the locations of the control units averaged 4 mG and was never higher than 7 mG.

The exposure units were energized by power supplies consisting of an isolation transformer, autotransformer and series capacitors, and were operated in series resonance at 60 Hz to eliminate powerline harmonics. Fourier analysis of the coil currents showed that the strongest harmonics were 50 dB below the fundamental. The power supplies were rack-mounted in solid copper boxes to minimize the magnetic fields created by eddy currents caused by the transformers.

The exposure room was continuously maintained under controlled temperature and humidity with an unvarying light/dark cycle (12/12 h). Room air was replaced 15 times/h with fresh air. A virtual instrument (Labview, National Instruments, Austin, Tex., USA) was created to continuously monitor and record room temperature, coil current, magnetic field and current harmonic content.

Animals

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, Minn., USA) were used in the study; they were 6 weeks old on arrival and were rested for a minimum of 2 weeks before use. The immediate environment of the mice was totally nonmetallic. The water bottle (glass sipper) was placed inside the cage to minimize differences in electrical potential between the mice and the water. Food pellets were placed in an accessible area of the bottle holder. The animal cages sat on plastic shelves whose indentations automatically centered the cages in the homogeneous portion of the magnetic field. The shelves were wall-mounted to prevent vibrational coupling between the coils and the cages. After commencing an experiment, the mice were exposed continuously, except for the time needed to service the cages (about 1 h/week). To avoid potential confounding effects due to switching transients, the magnetic field was never changed during the time the mice were exposed.

In 7 independent field experiments, mice were exposed to 5 G, 60 Hz for 1–175 days. The field strength was chosen because it is near the largest that would ordinarily be encountered in the environment. The frequency was chosen to be that of the North American electrical power system. To evaluate the reliability of the L procedure, 2 sham experiments were performed in which all mice received the control treatment. In each field experiment and each sham experiment,

Table 1. Effect on serum corticosterone in mice due to exposure to 5 G, 60 Hz

Day	Replicate	Corticosterone, ng/ml		*l*	*L*
		EMF	Control		
1	1	98.20±90.25	66.60±28.59	0.672	
	2	36.20±13.54	106.00±53.39	6.951	9.145*
	3	90.00±59.83	56.20±54.93	1.522	
5	1	71.00±23.36	91.80±70.67	0.477	
	2	70.50±43.79	62.20±22.26	0.176	0.653
	3	–	–	–	
21	1	36.20±8.41	68.20±39.07	3.372	
	2	42.00±21.11	48.50±27.20	0.208	3.580
	3	57.80±25.46	58.20±57.56	0.000	
49	1	94.60±27.98	43.40±19.60	8.770	
	2	51.00±20.53	41.80±21.80	0.573	14.710*
	3	28.80±14.67	69.00±34.78	5.367	
175	1	151.00±110.56	68.20±64.32	2.328	
	2	66.60±26.36	152.50±69.44	6.015	10.250*
	3	40.00±31.65	69.75±34.50	1.907	

Results are mean ± SD. A dash indicates that no data were obtained. * $p < 0.05$.

3 replicates were evaluated, each consisting of 5 exposed and 5 control mice. The 10 mice in a particular pair were sacrificed (cervical dislocation) on the same morning, and the minimum time between sacrifice of any 2 pairs was 1 week.

Immune Measurements

Flow Cytometry. Spleen and thymus cells were obtained by gently dispersing the organs between glass slides, and bone marrow cells were obtained by removing and flushing both femurs with phosphate-buffered saline (PBS). The cells were counted (Z1, Coulter, Hialeah, Fla., USA) and then resuspended at 10^7 cells/ml in staining buffer (PBS, 2% fetal bovine serum, 1 g/l sodium azide), and populations of interest were identified by two-color flow cytometric analysis using fluorescein isothiocyanate and phycoerythrin (Epics Profile II, Coulter). Cell populations were enumerated by staining with PK 136, for the NK1.1 antigen on natural killer (NK) cells of the B6 mouse, and GK1.5, 2.43, 2C11 and anti-Thy1.2 for the respective CD4, CD8, CD3 and CD90 antigens on T cells, and anti-IgM, anti-IgD and anti-CD45 for antigens on B cells. Antibodies were purified from hybridomas (ATCC, Rockville, Md., USA) or purchased (Pharmingen, San Diego, Calif., and Southern, Birmingham, Ala., USA). To prevent nonspecific binding, the cells were incubated with 50 µl of the appropriately diluted anti-Fc receptor antibody.

Assays. Cytotoxic T lymphocytes were generated in a one-way mixed lymphocyte culture by coculturing B6 spleen cells and gamma-irradiated A/J spleen stimulator for 5 days. Proliferation was quantified (after 3 days of incubation) using a commercial proliferation assay (CellTiter96 AQ, Promega, Madison, Wisc., USA). The result was expressed as the ratio of absorption units obtained from stimulated and unstimulated culture (stimulation index). The lytic activity of spleen NK cells was enhanced by culturing spleen cells for 18 h in medium containing IL-2 (800 units/ml) [40]. ^{51}Cr-labeled target cells were combined with various numbers of effector cells to give a range of effector-to-target (E:T) ratios (2:1, 3:1, 6:1, 13:1, 25:1, 50:1 and 100:1), which were each assayed in triplicate. The specific lysis was calculated as: specific lysis = (E – S)/(M – S), where E and S were, respectively, the counts per minute released in the presence of the effector cells and the presence of medium alone, and M was the maximum value (determined by lysing the target cells with acetic acid). For the determination of NK cell cytotoxicity, YAC-1 and P815 cells were used as positive and negative targets, respectively. YAC-1 and IL-4 cells were used as the respective positive and negative targets for the cytotoxic T lymphocytes generated in the mixed lymphocyte culture. For simplicity, the results are expressed in terms of a single predetermined E:T ratio for each assay.

Corticosterone Assay

Blood samples were obtained by exsanguination into the chest cavity. The blood was allowed to clot for 1 h and the serum was obtained by centrifugation. The corticosterone concentration in each sample was determined by radioimmunoassay (Diagnostic Products Corp., Los Angeles, Calif., USA) [41], using a standard curve.

Results

Serum corticosterone and 20 immune parameters were measured in 5 mice exposed to 5 G for 1 day and in 5 control mice, and the log-likelihood ratio statistic for the t test was calculated for each of the 21 comparisons between the two groups. The entire procedure was repeated twice, totaling 3 replicates, and 21 *L* values were computed by summing the corresponding constituent ratio statistics from each replicate (tables 1 and 2). We tested

Table 2. Immune parameters (mean ± SD) in mice exposed in 3 replicates to 5 G, 60 Hz, for 1 day

		Replicate	E	C	*l*	*L*
Cellularity, cells × 10^7						
Spleen		1	7.20±1.77	7.62±0.98	0.266	
		2	11.46±1.54	11.58±1.46	0.020	2.963
		3	9.90±1.45	8.40±1.57	2.677	
Thymus		1	2.10±0.66	2.46±1.09	0.484	
		2	5.88±1.57	6.40±1.43	0.367	0.871
		3	4.60±0.87	4.50±1.52	0.020	
Bone marrow		1	2.66±0.24	2.80±0.31	0.770	
		2	3.54±0.55	4.32±0.27	7.068	8.272*
		3	2.58±0.37	2.74±0.47	0.434	
Distribution, %						
Spleen	CD45	1	9.80±2.05	9.40±1.34	0.165	
		2	4.60±0.55	5.20±0.84	2.030	2.682
		3	3.60±0.55	3.80±0.45	0.487	
	IgM+	1	60.00±2.74	60.20±3.35	0.013	
		2	60.60±8.68	58.20±3.83	0.393	1.146
		3	61.20±2.28	60.00±2.55	0.740	
	IgM+IgD–	1	9.20±1.48	9.60±1.52	0.220	
		2	14.00±9.51	9.60±1.14	1.238	1.508
		3	9.60±0.89	9.80±2.05	0.050	
	IgM+IgD+	1	49.60±1.34	50.60±2.41	0.790	
		2	45.60±0.89	50.20±2.78	9.384	11.570*
		3	51.40±1.52	50.20±1.92	1.396	
	CD90+CD3+	1	23.80±1.79	23.00±2.45	0.426	
		2	18.60±6.23	19.20±1.10	0.056	8.506*
		3	11.60±0.89	10.00±0.71	8.024	
	NK1.1	1	2.80±0.45	3.40±0.55	3.714	
		2	3.60±0.55	3.40±0.55	0.408	6.353
		3	2.80±0.45	3.20±0.45	2.231	
Marrow	CD45	1	18.80±1.64	18.80±1.64	0	
		2	21.00±6.86	16.80±1.79	1.987	2.199
		3	16.60±1.34	17.20±2.95	0.212	
	IgM+	1	13.80±1.48	13.20±0.84	0.748	
		2	16.00±4.69	12.00±1.58	3.423	9.278*
		3	15.20±1.48	12.80±1.79	5.107	
	IgM+IgD–	1	8.00±1.00	8.00±1.41	0	
		2	12.80±6.98	6.80±0.84	3.754	9.348*
		3	9.40±2.07	7.00±0.71	5.594	
	IgM+IgD+	1	7.20±1.10	7.40±0.89	0.124	
		2	5.00±3.16	6.00±0.71	0.578	4.978
		3	8.40±1.14	6.80±1.30	4.276	
Thymus	CD90+CD3+	1	18.60±3.05	17.60±3.44	0.292	
		2	15.40±0.89	18.80±0.84	17.608	18.112*
		3	14.60±3.05	15.20±1.10	0.212	
	CD4+CD8–	1	17.80±3.19	18.60±2.88	0.214	
		2	10.80±1.48	11.00±4.53	0.011	1.041
		3	11.20±1.64	12.00±1.41	0.816	
	CD4–CD8+	1	3.20±0.84	3.20±1.30	0	
		2	1.60±0.55	2.00±0.00	2.877	3.229
		3	3.00±0.71	2.80±0.45	0.352	
	CD4+CD8+	1	74.60±3.72	73.80±3.70	0.144	
		2	84.80±1.64	83.60±4.34	0.410	1.417
		3	81.40±2.07	80.20±2.39	0.863	

➤

Table 2 (continued)

	Replicate	E	C	l	L
Function					
SI, Ratio of cell numbers	1	6.15±1.14	6.90±0.75	1.722	
	2	13.30±3.37	13.00±2.54	0.032	1.893
	3	1.48±0.16	1.44±0.20	0.139	
CTLa, %	1	70.80±2.28	69.40±5.94	0.298	
	2	67.80±19.18	63.60±26.29	0.104	0.402
	3	–	–	0	
NKa, %	1	41.40±13.72	38.20±7.40	0.260	
	2	22.20±1.92	26.50±3.13	1.592	2.742
	3	26.20±3.10	29.20±4.82	0.890	

A dash indicates that no data were obtained. * $p < 0.05$.

E = Exposed; C = control; SI = stimulation index. CTLa = cytotoxic T lymphocyte assay (E:T, 13:1); NKa = NK cytotoxic assay (E:T, 25:1); l = log-likelihood ratio statistic for the t test for a particular replicate; L = sum of the statistics from each replicate.

Table 3 Immune parameters (mean ± SD) in mice exposed in 3 replicates to 5 G, 60 Hz, for 5 days

		Replicate	E	C	l	L
Cellularity, cells $\times 10^7$						
Spleen		1	10.28±1.48	10.22±1.19	0.006	
		2	10.92±0.74	9.08±1.60	5.180	5.187
		3	12.90±2.30	12.86±1.28	0.001	
Thymus		1	4.92±1.87	3.88±0.64	1.600	
		2	6.28±1.68	7.44±2.76	0.775	3.150
		3	8.32±4.76	6.52±1.57	0.775	
Bone marrow		1	3.62±0.13	3.94±0.46	2.455	
		2	2.06±0.34	1.96±0.09	0.484	2.939
		3	2.06±0.05	2.06±0.14	0	
Distribution, %						
Spleen	CD45	1	6.00±0.71	6.00±1.00	0	
		2	3.40±0.55	3.20±0.45	0.487	4.201
		3	2.80±0.45	3.40±0.55	3.714	
	IgM+	1	63.46±0.98	58.44±1.40	18.536	
		2	64.00±0.49	63.62±0.49	1.732	21.200*
		3	64.22±2.66	63.04±1.36	0.932	
	IgM+IgD–	1	9.20±2.17	8.40±1.14	0.645	
		2	8.80±1.10	11.00±1.00	8.651	12.173*
		3	11.80±0.45	11.00±1.00	2.877	
	IgM+IgD+	1	48.40±3.21	52.80±1.79	6.400	
		2	54.00±1.87	51.60±1.95	4.008	12.438*
		3	52.60±0.89	53.80±1.79	2.030	
	CD90+CD3+	1	19.40±1.82	17.00±2.45	3.273	
		2	18.80±3.56	15.20±1.30	4.462	
		3	18.80±1.48	19.60±1.14	1.082	8.817*
	NK1.1	1	3.30±0.62	3.10±0.56	0.352	
		2	3.10±0.21	2.68±0.61	2.345	3.330
		3	3.42±0.24	3.26±0.43	0.633	

Table 3 (continued)

		Replicate	E	C	l	L
Marrow	CD45	1	19.00±3.94	18.20±1.64	0.217	
		2	15.40±1.82	18.20±1.64	5.970	10.649*
		3	18.40±1.52	16.60±1.14	4.462	
	IgM+	1	13.20±1.48	12.60±1.34	0.547	
		2	13.40±2.07	13.60±1.82	0.033	3.945
		3	15.60±1.82	13.20±2.39	3.365	
	IgM+IgD–	1	8.80±3.11	7.80±0.45	0.613	
		2	6.80±1.10	10.20±1.79	9.714	18.919*
		3	9.20±0.45	7.80±0.84	8.592	
	IgM+IgD+	1	6.60±1.95	6.60±0.89	0	
		2	7.60±2.51	6.40±0.89	1.193	1.358
		3	6.40±0.55	6.20±1.10	0.165	
Thymus	CD90+CD3+	1	13.80±3.90	14.80±1.10	0.374	
		2	14.80±3.70	16.60±1.95	1.095	2.928
		3	13.40±2.41	16.80±6.34	1.459	
	CD4+CD8–	1	13.20±1.30	10.20±1.30	9.762	
		2	9.00±1.00	9.60±1.52	0.660	10.454*
		3	9.00±1.22	8.80±2.49	0.032	
	CD4–CD8+	1	1.54±0.52	1.90±1.09	0.541	
		2	2.90±1.66	2.00±0.46	1.582	2.415
		3	2.12±0.57	2.28±0.46	0.292	
	CD4+CD8+	1	80.60±2.79	78.20±11.30	0.262	
		2	85.80±1.30	84.40±1.52	2.671	3.166
		3	85.60±1.52	84.60±4.93	0.233	
Function						
SI, Ratio of cell numbers		1	5.17±1.75	4.71±1.12	0.291	
		2	2.55±0.43	2.54±0.28	0	0.414
		3	1.67±0.29	1.61±0.35	0.123	
CTLa, %		1	65.00±6.96	61.60±8.41	0.588	
		2	52.60±9.26	69.00±13.56	4.844	10.922*
		3	75.60±4.56	70.00±2.45	5.490	
NKa, %		1	29.00±6.24	33.40±7.27	1.238	
		2	33.40±17.40	23.20±6.50	1.727	4.552
		3	35.40±6.23	39.20±3.70	1.587	

* $p < 0.05$. E = Exposed; C = control; SI = stimulation index; CTLa = cytotoxic T lymphocyte assay (E:T, 13:1); NKa = NK cell cytotoxic assay (E:T, 25:1); l = log-likelihood ratio statistic for the t test for a particular replicate; L = sum of the statistics from each replicate.

hypotheses concerning the occurrence of field-induced change in a particular parameter by assessing whether L exceeded the critical value ($L > 7.83$, $p < 0.05$) of the χ^2 distribution with 3 degrees of freedom. The distribution of corticosterone levels was significantly affected by the field (table 1), and there were 6 significant differences among the 20 immune parameters (table 2).

Following 5 days of exposure, there were statistically significant differences in 8 immune parameters (table 3), but corticosterone was not affected (table 1). The experiment was repeated 5 additional times, corresponding to exposures of 10, 21, 49, 105 and 175 days, except that corticosterone was not measured following field exposure for 10 or 105 days. After 10 days, there were 9 significant differences among the immune parameters, and thereafter the number of immune system changes varied from 2 to 4, depending on the exposure duration (detailed data not shown, but see table 4). An effect of field exposure on corticosterone was found after 49 and 175 days, but not after 21 days (table 1).

The statistically significant differences observed in the immune system in all experiments are listed in table 4,

Table 4. L values for statistically significant comparisons involving immune parameters in mice exposed to 60-Hz magnetic fields or control conditions

		Exposure period, days						
		1	5	10	21	49	105	175
Cellularity	Spleen	–	–	–	–	–	10.4	10.5
	Thymus	–	–	12.5	–	–	–	–
	Bone marrow	8.3	–	–	–	–	7.8	–
Distribution	Spleen							
	CD45	–	–	13.3	–	–	–	–
	IgM+	–	21.2	11.0	–	–	9.4	–
	IgM+IgD–	–	12.2	15.1	–	–	15.2	–
	IgM+IgD+	11.6	12.4	11.4	10.1	7.8	–	–
	CD90+CD3+	8.5	8.8	16.2[1]	12.9	–	–	–
	NK1.1	–	–	18.9	10.7	–	–	12.1
	Marrow							
	CD45	–	10.6	–	–	–	–	–
	IgM+	9.3	–	–	–	–	–	–
	IgM+IgD–	9.4	18.9	9.8	–	–	–	–
	IgM+IgD+	–	–	–	–	–	–	–
	Thymus							
	CD90+CD3+	18.1	–	–	–	9.2	–	–
	CD4+CD8–	–	10.4	–	–	–	–	–
	CD4–CD8+	–	–	–	–	–	–	–
	CD4+CD8+	–	–	–	–	–	–	–
Function	SI	–	–	–	–	–	–	–
	CTLa	–	10.9	–	–	–	–	–
	NKa	–	–	14.0	–	–	–	25.7

The mice were studied in a series of 3 replicates, each consisting of 5 exposed and 5 control mice. The L value for all pair-wise comparisons for which $L\,\chi^2_{3,\,0.05}$ (L ≥ 7.8) is listed. SI = Stimulation index; CTLa = cytotoxic T lymphocyte assay (E:T, 13:1); NKa = NK cell cytotoxic assay (E:T, 25:1).

[1] Evaluated using only 2 replicates; L was adjusted to the equivalent 3-replicate value.

and the cumulative frequency of significant results as a function of L is shown in figure 1. Also shown in figure 1 are the results of two sham experiments in which the field was not applied to either of the two groups [24].

The occurrence of consistent change among similarly exposed mice was evaluating by ignoring the replicate structure and computing L directly from the 15 exposed and 15 control mice, and then assessing whether $L > \chi^2_{1,\,0.05}$. The procedure produced no significant differences in any instances where effects were noted initially (tables 1 and 4).

To assess whether the effect of the field on corticosterone in mice exposed for 49 and for 175 days (table 1) occurred primarily in a subgroup of animals, percentile plots were made for the combined group and for the corresponding combined control group. We found that the measurements in the upper tail of the distribution were selectively increased followingfield exposure (fig. 2a). A similar difference was not found when the data from the short-term experiments were combined (fig. 2b).

We next considered whether field-induced changes in specific immune parameters were also distribution dependent. Two clear cases could be examined. Sometimes a parameter was affected by long-term but not short-term exposure, as, for example, spleen cellularity (table 4). When the animals were split into two groups with the dividing line determined by the presence or absence of an effect on spleen cellularity, we again found that the field had a selective impact on mice in the upper part of the distribution in the sense that the high values of spleen cellularity were higher than the corresponding percentiles of the controls (fig. 3). In other cases, the effect of the field occurred only in association with relatively short-term exposure, for example marrow IgM+IgD–. Here again,

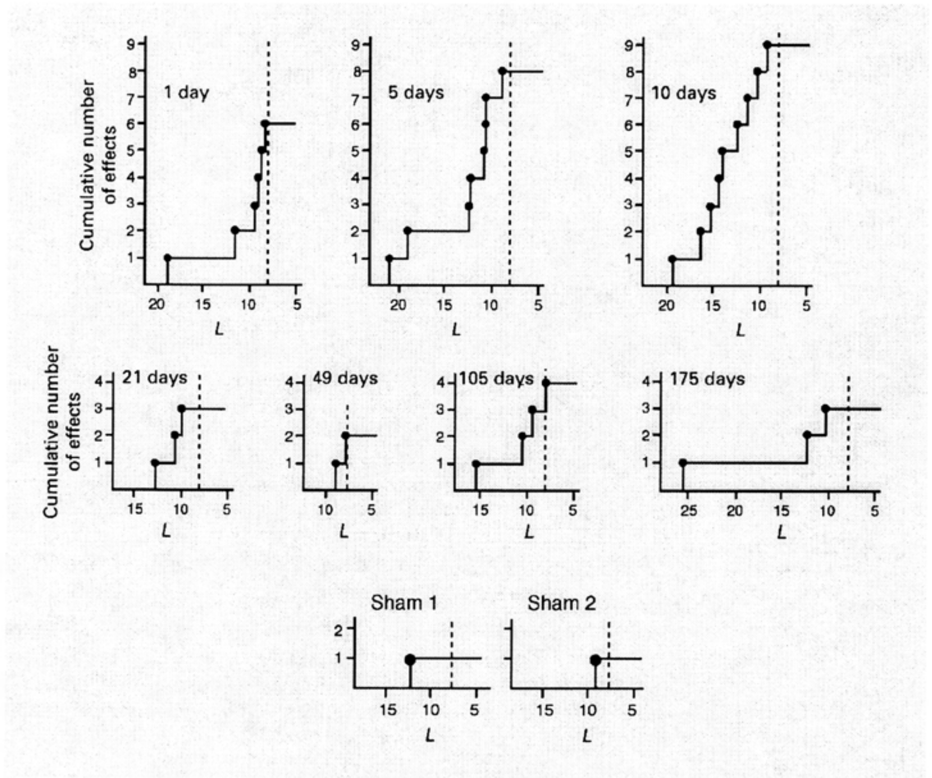

Fig. 1. Cumulative frequency of immune parameters (out of 20) that were significantly affected by EMF exposure for the duration indicated, as a function of the magnitude of the test statistic. Regions beyond the dotted line indicate $p < 0.05$ ($L > 7.8$). The results from the sham studies were described previously [24].

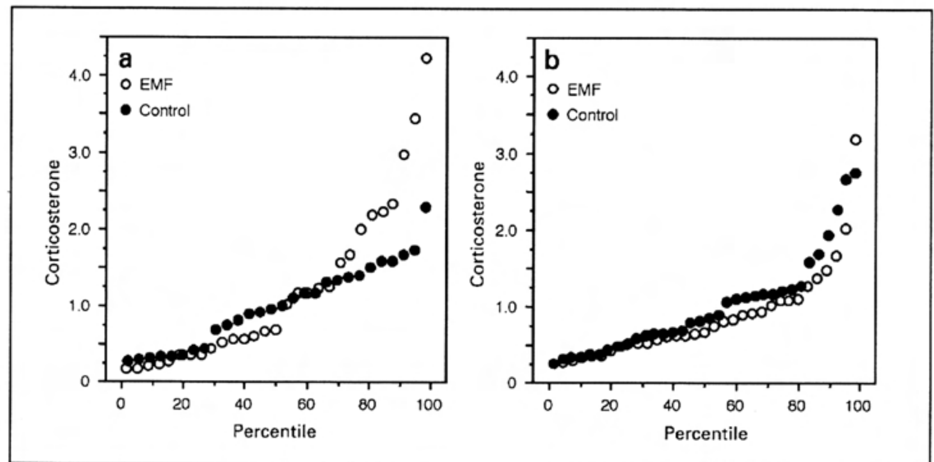

Fig. 2. Percentile plots of corticoid measurements in mice exposed to 5 G, 60 Hz. **a** Combined results for mice exposed for 49 and 175 days. **b** Combined results for mice exposed for 1, 5 and 21 days.

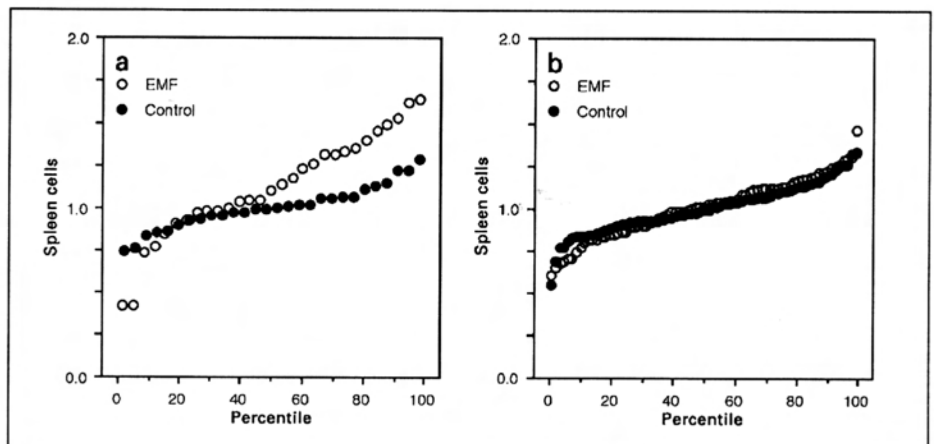

Fig. 3. Percentile plots of the number of spleen cells in mice exposed to 5 G, 60 Hz. **a** Combined results for mice exposed for 105 and 175 days. **b** Combined results for mice exposed for 1, 5, 10, 21 and 49 days.

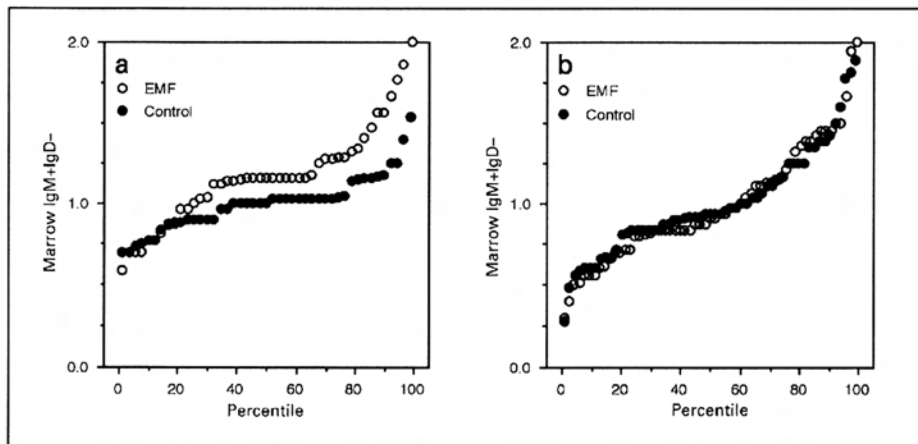

Fig. 4. Percentile plots of the percentage of marrow IgM+IgD– cells in mice exposed to 5 G, 60 Hz. **a** Combined results for mice exposed for 1, 5 and 10 days. **b** Combined results for mice exposed for 21, 49, 105 and 175 days. Points greater than 2 are shown as 2.

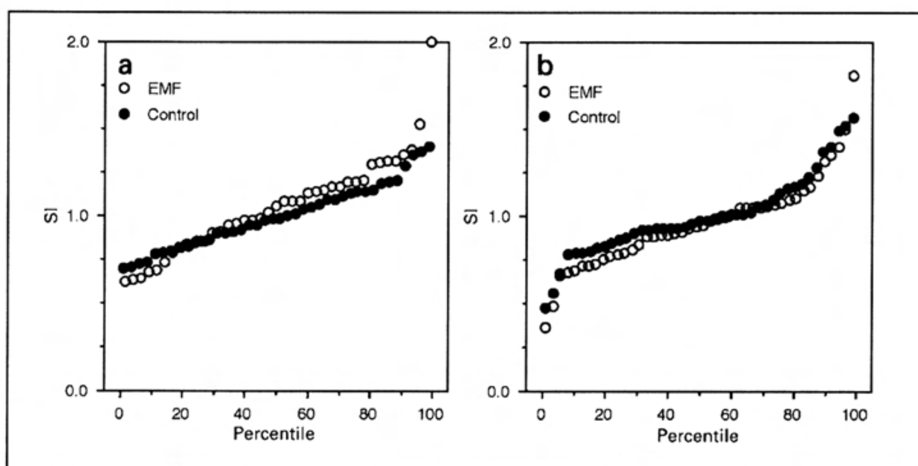

Fig. 5. Percentile plots of the stimulation index (SI) in mice exposed to 5 G, 60 Hz. **a** Combined results for mice exposed for 1, 5, 10 and 21 days. **b** Combined results for mice exposed for 49, 105 and 175 days. The point greater than 2 is shown as 2.

the high values were higher than the controls only in the mice in which the parameter was affected by the field (fig. 4). As a control procedure, we performed a similar analysis using the stimulation index, which was not affected by field exposure in any experiment. The distributions of measurements were identical in the exposed and control groups for both short-term and long-term exposure (fig. 5).

Discussion

In 20 measurements of the immune system after 49 days of exposure, we found two significant effects (fig. 1), which occurred at p values of 0.031 and 0.006. There was only a 5.4% chance that both could have occurred due to chance. In the other 6 field experiments, the number of significant differences and their associated p values were such that a possible role for chance in explaining the results could be firmly rejected. In the endocrine system, only a single endpoint was measured, but the results showed clearly that the corticosterone levels were altered as a consequence of long-term exposure (table 1).

It might be argued that the significant differences observed in the various experiments were somehow due to the use of the χ^2 distribution, because L for small samples (N = 5) is not precisely χ^2. However, no more than the expected number of false-positive results were found in each of 2 sham experiments (fig. 1, table 4), suggesting that the use of χ^2 cannot account for the observed significant differences. Moreover, it can be shown that small-sample p is overestimated when $|t| < 2.05$ (ranging from 20% for t = 0.1 to 1% for t = 2.0); otherwise, it is underestimated [42]. Application of the appropriate correction factor to each l in the study did not alter the results. Thus, it was appropriate to use the χ^2 distribution to evaluate the data, and thereby avoid the laborious computation of numerous randomization distributions.

It might also be argued that, in the case of the immune parameters, the large number of statistically significant

differences was due to correlated data. However, in our experimental design, assessment of the existence of a deterministic effect of field exposure on the immune system depended only on the number of significant differences. The presence or absence of correlations was not a relevant factor in assessing the existence of an effect.

When the endocrine and immune data were analyzed on the basis of a linear model (*L* computed directly from the 15 exposed and 15 control mice, based on the χ^2 distribution with 1 degree of freedom), no significant differences were found in any instance where effects were noted initially (table 1, fig. 1). The pivotal role of nonlinearity in revealing the effect of EMFs on the endocrine and immune systems indicated that the field-induced changes were actually nonlinear.

The percentile plots suggested that the effect of the field on both the endocrine and immune systems was concentrated on a subgroup of the exposed group, namely the animals whose measurements fell at the upper end of the distribution of normal measurements (fig. 2–4). This result could help to explain why previous studies on the bioeffects of EMFs have been inconclusive, because the use of linear methods to analyze data such as those depicted in figures 2–4 can be hopelessly insensitive.

Some limitations of this study can be seen by considering a particular experiment, for example the 175-day-exposure experiment (tables 1 and 4). Our conclusion that the field altered the endocrine and immune systems applies only to group behavior. We cannot exclude the possibility that the two types of changes occurred in different subgroups of the exposed mice. Moreover, we cannot resolve the questions of whether and how the two changes were causally related. The results were consistent with our hypothesis that endocrine changes alter lymphoid phenotype, but they cannot prove that was the case. Both limitations could be overcome by suitable modifications of our methods.

A salient feature of our results is their suggestion that the endocrine and immune systems can interact to produce reciprocal changes that are not predictable in magnitude or direction. It is possible to mimic this phenomenon mathematically, thereby showing how it might occur. For this purpose, we used the Lorenz system of three nonlinear equations which we arbitrarily chose to represent corticosterone, IgM+ and NK cell cytotoxicity. We assumed that a specific change occurred in the corticosterone level (the 'input') in an animal at t = 0 as a consequence of EMF transduction, and then calculated the mean of the time series for each of the three parameters over 5 s, beginning 5 s after the input; the mean can be thought of as the value

a

Animal No.	Input (%)	Δ Cort. (%)	Δ IgM+ (%)	Δ NK (%)
1	+1	-19	-20	+22
2	-1	-29	+10	-12
3	+1.5	+12	-15	-14
4	-1.5	+20	+14	-12

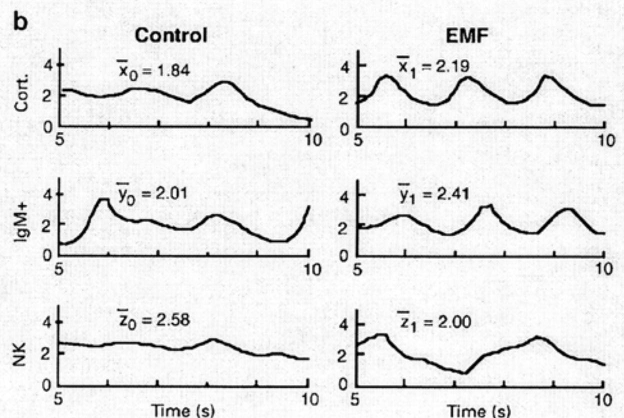

Fig. 6. Nonlinear interaction of the endocrine and immune systems, mimicked using the Lorenz equations [46]. **a** It was assumed that each animal transduced an EMF that resulted in a discrete change in corticosterone at t = 0 of the amount shown, and the resulting average percentage change in each of the three parameters that occurred 5–10 s later was calculated. The initial conditions (t = 0) for animal 1 for control and EMF conditions, respectively, were: $x_0 = y_0 = z_0 = 1$ and $x_1 = 1.01$, $y_1 = z_1 = 1$. The initial conditions for animals 2–4 were identical, except x_1 was 0.99, 1.015 and 0.985, respectively. The system was tuned to operate in the chaotic mode ($\alpha = 16$, $\mu = 45.92$ and $\beta = 4$) and solved using a fourth-order Runge-Kutta method with a time step of 0.0125 s. **b** Time series and means for each parameter in animal 1 before and after application of the EMF at t = 0. Cort. = Corticosterone; NK = NK cell cytotoxicity.

that would have been observed in a measurement. The inputs chosen were ±1% and ±1.5% of the baseline at t = 0. Because the equations were known, it was possible to also calculate the means that would have been measured in the absence of EMF transduction, and therefore the change in each parameter occasioned by transduction could be ascertained. The results (fig. 6a) showed that corticosterone changes of ±1–1.5% led to apparently random but nevertheless completely deterministic and nontrivial changes in all three parameters. This example shows that (1) the results of interactions may not be predictable in nonlinear systems and (2) even small initial changes can lead to appreciable changes in the other parameters. To preserve causality, it is necessary only that the result would have been otherwise but for the presence of the field.

A basic property of the endocrine and immune systems is the consistency and graded nature of the responses that occur when stimuli are repeated or intensified. Why are the effects of 60-Hz magnetic fields so different? It is reasonable to view the processes responsible for the faithful correspondence between stimuli and responses as resulting from evolution by natural selection, leading progressively to physiological systems that conferred a selective advantage because they were more reliable. Conversely, in the absence of natural selection there is no process by which the phenomenon of consistency in response to a stimulus can come about. Power-frequency fields were negligible throughout the period of evolution of life on earth and became a prominent part of the environment only within the last century. They could not have served as an agent of evolutionary change, and consequently, a physical mechanism capable of producing a predictable response to fields did not develop.

Finally, the question of the possible biophysical mechanism responsible for the interaction of power-frequency EMFs with biological receptors has been considered by others [43–45] but was not addressed here. The further question of the biological significance of the type of change reported here was similarly not addressed in our experimental design.

Acknowledgments

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