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The effect of successful low-dose immunotherapy ascertained by provocation neutralization on lymphocytic calcium ion influx following electric field exposure

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Abstract:
Background: Low-dose immunotherapy affects baseline levels of intracellular calcium. However, the effect of background electric fields is yet to be ascertained. The aim of this study was to test the following hypotheses: desensitization by low-dose immunotherapy is associated with reduced calcium ion influx during electric field exposure; the effect of low-dose immunotherapy on intracellular calcium ion concentration does not depend on electric field exposure; and the intracellular calcium ion concentration is amplified by electric field exposure.
Methods: The experimental design was balanced and orthogonal. Intracellular lymphocytic calcium ion concentrations were assayed in 47 patients, following incubation with picogram amounts of 12 test allergens, using a cell-permeable calcium-sensing ratiometric fluorescent dye and fluorescence spectroscopy, both at baseline and following successful provocation neutralization treatment with low-dose immunotherapy. Duplicates were also exposed to an electric field which replicated the frequency spectrum measured in a non-Faraday shielded room.
Results: A significant or trend-level main effect was found for low-dose immunotherapy for: benzoate; formaldehyde; metabisulfite; natural gas; nitrosamines; organophosphates; salicylate; azo-dyes and precursors; nickel; and petrol (gasoline) exhaust. Significant or trend-level main effects for electric field exposure were observed for: formaldehyde; mercury (inorganic); natural gas; nickel; nitrosamines; petrol exhaust; salicylate; benzoate; and metabisulfite. There was no evidence of a statistical interaction between these two factors. Electric field exposure was associated with a higher intracellular calcium ion concentration.
Conclusion: There was support for all three hypotheses. The results suggest that patients may experience increased sensitivity to allergens as a result of exposure to everyday electric fields.

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Introduction

We have recently shown that low-dose immunotherapy, which is a form of allergen-specific immunotherapy in which low doses of the antigen are administered intradermally or sublingually, affects baseline levels of intracellular calcium in lymphocytes, supporting the premise that allergens affect cell signaling in immune cells and provocation neutralization immunotherapy helps to promote more normal immune cell signaling [1]. However, in practice, patients who suffer from chemical sensitivities are also subjected to background electric fields, for example the fluctuating electromagnetic field produced by mains electricity, which has the same frequency as that of the electricity supply (50 Hz in the United Kingdom). In turn, such fluctuating fields may cause changes in living tissues, with endogenous ions being the current carriers [2, 3]. In particular, owing to its structure as a phospholipid bilayer, the lymphocyte membrane acts as a capacitor, with capacitance C, such that

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$$C\frac{dV}{dt} = I_{ch} + I_e,$$

where the derivative on the left-hand side refers to the rate of change in electrical potential, while, on the right-hand side, I_{ch} refers to the current flow through ion channels and I_e is the external current, in this case induced by an external fluctuating field [4]. For each open channel, the following generalized form of Ohm's law applies:

$$\mathbf{I} = \sigma\mathbf{E},$$

where the vectors **I** and **E** refer to the current density and electric field, respectively, and σ is the conductivity (that is, the reciprocal of the resistivity) [3]. The current flow through calcium ion channels may be considered to take the form

$$-\sum (g_{Ca^{2+}})(N_{Ca^{2+}})(f_{Ca^{2+}})\left(V - V_{Ca^{2+}}\right),$$

where $g_{Ca^{2+}}$ is the conductance of open calcium ion channels, $N_{Ca^{2+}}$ is the total number of calcium ion channels, $f_{Ca^{2+}}$ is the fraction of the calcium ion channels which are open, and $V_{Ca^{2+}}$ refers to the reversal potential [4].

If the state of lymphocyte calcium ion channels is modeled as being binary (either open or closed), with $f^{eq}_{Ca^{2+}}$ representing the corresponding activation curve (equilibrium probability as a function of $V - V_{1/2}$), then the dynamic state of these ion channels is given by

$$\frac{df_{Ca^{2+}}}{dt} = -\frac{1}{\tau_{Ca^{2+}}(V)}\left(f_{Ca^{2+}} - f^{eq}_{Ca^{2+}}\right),$$

where $\tau_{Ca^{2+}}(V)$ represents the time constant for relaxation to equilibrium [4].

While low-dose immunotherapy has been demonstrated to be effective in the treatment of food, chemical, and inhalant-particulate sensitivities [5–8], its underlying molecular mechanism of desensitization is not yet established [1]. Our recent previous study supported the possibility that this mechanism might involve reduced lymphocytic calcium ion influx but did not take into account the influence of ambient electric field exposure, which by the above argument might be expected to amplify this effect in sensitive patients [1]. There is not, however, any reason to expect that the effect of low-dose immunotherapy depends on electric field exposure.

We therefore formulated the following three hypotheses: (i) desensitization by low-dose immunotherapy is associated with reduced influx of calcium ions into lymphocytes in the presence of electric field exposure; (ii) the effect of low-dose immunotherapy on intracellular calcium ion concentration does not depend on electric field exposure (a null hypothesis); and (iii) both before low-dose immunotherapy, and after low-dose immunotherapy, the intracellular calcium ion concentration is amplified by electric field exposure.

The aim of this study was to test these hypotheses by measuring intracellular lymphocytic calcium ion concentrations, following incubation with picogram amounts of the test allergens, of patients before and after desensitization by low-dose immunotherapy to a range of pollutants and foods, both with and without exposure to an electric field. The experimental design was balanced and orthogonal.

Materials and methods

Subjects

The study consisted of an audit of 47 patients with hypersensitivities to a range of pollutants and foods, which were confirmed at baseline by dermal reactions to intradermal administration. Each patient underwent a course of low-dose immunotherapy for those substances to which he or she was allergic until desensitization was complete.

The AONMREC (Academy of Nutritional Medicine Research Ethics Committee) approved this audit. The study was carried out in accordance with the Declaration of Helsinki.

Procedures

The date of birth, sex, and clinical symptoms for each subject were recorded. A venous blood sample was taken at baseline and heparinized. The samples were sent to Acumen (Tiverton, Devon, UK), for testing by Dr

John McLaren Howard. Lymphocytes were separated on a Histopaque™ gradient (Sigma-Aldrich Leukocyte Separation, Sigma-Aldrich Inc., St Louis, MO, USA) and the cells re-suspended in the patient's plasma at a constant cell density. Aliquots of the suspension were placed in a micro-well analysis plate and samples of the test allergens representing 1 to 5 pg of the allergens (depending on the specific analyte) were added to an appropriate number of wells. Duplicates were also exposed to an electric field which replicated the frequency spectrum measured in a normal (that is, non-Faraday shielded) room. Some wells were also used for sample and reagent blanks. Following incubation of the plate at 37 °C for 10 min, Indo 1-AM cell-permeable calcium-sensing ratiometric fluorescent dye (FluoProbes® BioDirectory of Fluorescence) was added to each well and the plate incubated for a further 10 min. The plate was then washed with phosphate-buffered saline to remove the residual extracellular dye and the emission of the lymphocytes in each well of the microplate was then measured at 480 nm and 410 nm, the ratio between these being directly proportional to the intracellular calcium ion level [9–11]. A correction was made for the calcium ion level recorded for non-treated lymphocytes. The measurements were made using a Thermo Labsystems Multiscan® Multisoft microplate reader.

Low-dose immunotherapy testing began in each patient with a solution of a substance to which allergy was suspected, which was assessed after 10 min. A series of weaker solutions might then be tested, until the correct therapeutic strength was reached. Treatment vaccines were provided for regular use at home. Following successful provocation neutralization treatment with low-dose immunotherapy, when there was no longer a hypersensitivity reaction to the index substance, a further venous blood sample was taken and the above laboratory assays were repeated.

Statistical analysis

Given the balanced and orthogonal experimental design, a two-way repeated measures analysis of variance was carried out for each test substance following analysis of diagnostic plots which in each case included residuals versus fitted values, the square root of the standardized residuals versus fitted values, a normal Q-Q plot, and standardized residuals versus leverage. In order to avoid multiple comparison issues, the third hypothesis was assessed directly from plots, for each test substance, of the mean intracellular calcium ion concentration (including standard errors) versus the two levels of the treatment factor (before and after low-dose immunotherapy). All tests were two-tailed.

The software package used for the statistical analyses was R version 3.0.1, running on an x86_64-w64-mingw32/x64 (64-bit) platform [12].

Results

The mean age (standard error) of the cohort of 47 patients at baseline was 44.0 (2.6) years. Thirty-two (68%) of the cohort were female. The mean time between baseline and follow-up testing was 1.15 (0.14) years.

The mean lymphocyte intracellular calcium ion concentration at baseline and following low-dose immunotherapy, both in the absence and presence of electric field exposure, is shown in Figure 1 for the following sets of test substances: antimony; azo-dyes and precursors; benzoate; formaldehyde; mercury (inorganic); metabisulfite; natural gas; nickel; nitrosamines; organophosphates; petrol (gasoline) exhaust; and salicylate. The corresponding standard errors of the mean are represented by error bars in the figure.

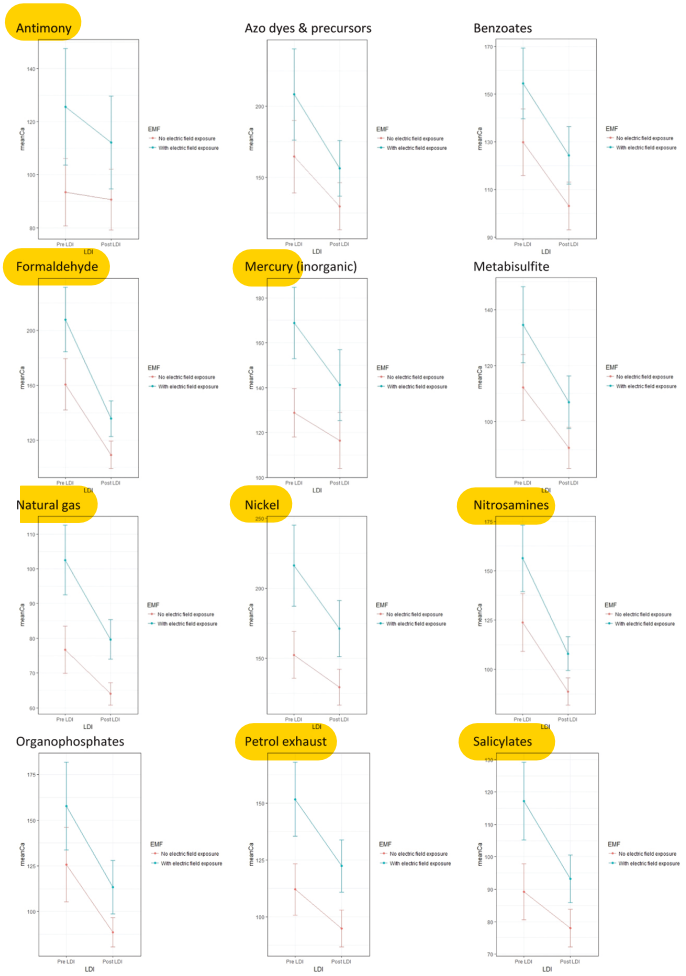

Figure 1: The mean lymphocyte intracellular calcium ion concentration in nM (meanCa) at baseline (pre LDI) and following low-dose immunotherapy (post LDI), both in the absence and presence of electric field exposure, for 12 sets of test substances. The error bars represent standard errors of the mean. Graphs created by B.K.P. using R v. 3.0.1.

In respect of the initial three hypotheses, from Figure 1 it can be seen that: (i) desensitization by low-dose immunotherapy is associated with reduced influx of calcium ions into lymphocytes in the presence of electric field exposure; (ii) the effect of low-dose immunotherapy on intracellular calcium ion concentration does not depend on electric field exposure; and (iii) both before low-dose immunotherapy, and after low-dose immunotherapy, the intracellular calcium ion concentration is amplified by electric field exposure. Statistical test results supporting the first two hypotheses are given in Table 1.

Table 1: Mean lymphocyte intracellular calcium ion concentrations pertaining to sensitivities before and after corresponding provocation neutralization treatment with low-dose immunotherapy (LDI) in the absence and presence of electric field exposure (EMF) (standard errors are given in parentheses); main effects of LDI and EMF; and interaction between LDI and EMF.

Allergen	Baseline [Ca^{2+}] (nM)	Follow-up [Ca^{2+}] (nM)	*F*	*df*	p-value
Antimony					
No EMF	93.44 (12.69)	90.63 (11.47)			
EMF	125.63 (21.97)	112.19 (17.49)			
LDI main effect			0.2442	1,60	0.6230
EMF main effect			2.6723	1,60	0.1073
LDI*EMF			0.1044	1,60	0.7477
Azo-dyes and precursors					
No EMF	164.58 (25.52)	129.58 (16.59)			
EMF	208.33 (32.15)	156.25 (19.50)			
LDI main effect			3.2404	1,44	0.0787
EMF main effect			2.1188	1,44	0.1526
LDI*EMF			0.1247	1,44	0.7257

Benzoate					
No EMF	129.84 (13.96)	103.06 (9.97)			
EMF	154.52 (14.78)	124.35 (12.07)			
LDI main effect			4.9219	1,120	0.0284
EMF main effect			3.2083	1,120	0.0758
LDI*EMF			0.0174	1,120	0.8952
Formaldehyde					
No EMF	160.63 (18.69)	109.22 (10.05)			
EMF	207.97 (23.56)	135.63 (13.01)			
LDI main effect			13.0369	1,124	0.0004
EMF main effect			4.6303	1,124	0.0334
LDI*EMF			0.0174	1,124	0.5424
Mercury (inorganic)					
No EMF	128.82 (10.83)	116.47 (12.52)			
EMF	168.82 (15.89)	141.18 (15.75)			
LDI main effect			2.0653	1,64	0.1556
EMF main effect			5.4045	1,64	0.0233
LDI*EMF			0.3019	1,64	0.5846
Metabisulfite					
No EMF	112.27 (11.72)	90.61 (7.34)			
EMF	134.70 (13.62)	106.97 (9.34)			
LDI main effect			5.2549	1,128	0.0235
EMF main effect			3.2405	1,128	0.0742
LDI*EMF			0.0791	1,128	0.7790
Natural gas					
No EMF	76.67 (6.79)	64.00 (3.25)			
EMF	102.50 (10.03)	79.67 (5.69)			
LDI main effect			6.6401	1,116	0.0112
EMF main effect			9.0743	1,116	0.0032
LDI*EMF			0.5446	1,116	0.4620
Nickel					
No EMF	152.50 (16.67)	129.38 (12.80)			
EMF	216.25 (28.92)	171.41 (20.08)			
LDI main effect			2.7477	1,124	0.0999
EMF main effect			6.6554	1,124	0.0111
LDI*EMF			0.2806	1,124	0.5973
Nitrosamines					
No EMF	123.75 (14.60)	88.75 (6.91)			
EMF	156.25 (16.81)	107.92 (8.52)			
LDI main effect			11.2693	1,44	0.0016
EMF main effect			4.3319	1,44	0.0433
LDI*EMF			0.2885	1,44	0.5939
Organophosphates					
No EMF	125.67 (20.38)	88.67 (8.05)			
EMF	157.67 (24.01)	113.33 (14.52)			
LDI main effect			5.2177	1,56	0.0262
EMF main effect			2.5328	1,56	0.1171
LDI*EMF			0.0424	1,56	0.8376
Petrol exhaust					
No EMF	112.03 (11.33)	94.84 (8.12)			
EMF	151.72 (16.28)	122.34 (11.55)			
LDI main effect			3.6593	1,124	0.0581
EMF main effect			7.6190	1,124	0.0067
LDI*EMF			0.2507	1,124	0.6175
Salicylate					

No EMF	89.22 (8.58)	77.97 (5.79)			
EMF	117.19 (12.02)	93.28 (7.35)			
LDI main effect			4.0428	1,124	0.0465
EMF main effect			6.1274	1,124	0.0147
LDI*EMF			0.5239	1,124	0.4705

Discussion

The results of this study offer support for all three hypotheses and are consistent with our previous findings [1]. Significant main effects for low-dose immunotherapy were observed for: benzoate; formaldehyde; metabisulfite; natural gas; nitrosamines; organophosphates; and salicylate. Trend-level main effects for low-dose immunotherapy were observed for: azo-dyes and precursors; nickel; and petrol (gasoline) exhaust. Significant main effects for electric field exposure were observed for: formaldehyde; mercury (inorganic); natural gas; nickel; nitrosamines; petrol (gasoline) exhaust; and salicylate; while trend-level main effects were found for: benzoate; and metabisulfite. The lack of significant main effects for both antimony and for azo-dyes and their precursors may have at least partly resulted from the small number of patients tested for these substances, as reflected in the corresponding degrees of freedom given in Table 1. In none of the cases was there a significant interaction between low-dose immunotherapy and electric field exposure, in line with our second prior hypothesis.

Our present results suggest that patients may experience increased sensitivity to allergens as a result of exposure to everyday electric fields. We noted in our previous recent paper that while resting lymphocytes maintain a low cytosolic calcium ion concentration, antigen receptor signaling results in calcium ion influx, predominantly via store-operated calcium (SOC) channels, and inhibition of lymphocyte calcium ion channel expression has been found to prevent experimental asthma [1, 13, 14]. Our new findings may be associated with the finding that electromagnetic fields activate voltage-gated calcium ion channels; Pall has recently suggested that such activation may produce adverse effects [15, 16].

Interestingly, the close association between chemical sensitivity and electromagnetic factors was postulated earlier by both Monro and Smith [17, 18]. Smith has pointed out that it is very rarely the case that a patient who suffers from electrical hypersensitivity does not also have chemical sensitivities, and, in his words, "Even the exception may be just a case of inadequate chemical sensitivity diagnosis" [17, 19]. Meanwhile, Monro and Smith have demonstrated that there exists an absolute concordance between the neutralizing vaccines used in low-dose immunotherapy and electromagnetic fields [17, 18]. It should be noted that these electromagnetic field effects can occur with weak field strengths; increased field strength is not necessarily associated with a magnification of the effects [17, 18].

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