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Inflammation-related effects of adjuvant influenza A vaccination on platelet activation and cardiac autonomic function

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Abstract. Lanza GA, Barone L, Scalone G, Pitocco D, Sgueglia GA, Mollo R, Nerla R, Zaccardi F, Ghirlanda G, Crea F (Istituto di Cardiologia; and Università Cattolica del Sacro Cuore, Roma; Italy). Inflammation-related effects of adjuvant influenza A vaccination on platelet activation and cardiac autonomic function. *J Intern Med* 2011; **269**: 118–125.

Background. Inflammation, platelet reactivity and cardiac autonomic dysfunction increase the risk of cardiovascular events, but the relationships between these prognostic markers are poorly defined. In this study, we investigated the effect of an inflammatory stimulus (influenza A vaccine) on platelet activation and cardiac autonomic function.

Methods. We measured serum C-reactive protein (CRP) and interleukin-6 levels, monocyte–platelet aggregates, platelet activation and cardiac autonomic dysfunction very low-frequency amplitude showing the most significant change (34.6 ± 11.8 and 31.0 ± 10.2 ms 48 h before and after vaccination, respectively; $P = 0.002$). A significant correlation was found between percentage changes in CRP levels and in most HRV variables, with the most significant correlations between changes in CRP levels and changes in standard deviation of all normal RR intervals ($r = 0.43$; $P = 0.02$).

Conclusions. Together with an inflammatory reaction, influenza A vaccine induced platelet activation and sympathovagal imbalance towards adrenergic predominance. Significant correlations were found between CRP levels and HRV parameters, suggesting a pathophysiological link between inflammation and cardiac autonomic regulation. The vaccine-related

and interleukin-6 levels, monocyte–platelet aggregates (MPAs) and monocyte/platelet receptor expression before and after adjuvant influenza A vaccination in 28 patients with type II diabetes (mean age 62.1 ± 8 years, 18 men). Twenty-four-hour Holter electrocardiogram was recorded 24 h before and after vaccination; heart rate variability (HRV) was assessed as a measure of cardiac autonomic function.

Results. Inflammatory cytokines, MPA formation and monocyte/platelet receptor expression increased after vaccination. CRP was 2.6 ± 2.8 and 7.1 ± 5.7 mg L^{-1} 48 h before and after vaccination, respectively ($P < 0.0001$). HRV parameters decreased after vaccination compared to baseline, with platelet activation and cardiac autonomic dysfunction may transiently increase the risk of cardiovascular events.

Keywords: autonomic nervous function, heart rate variability, inflammation, influenza A vaccine, platelet reactivity.

Abbreviations: ANS, autonomic nervous system; CRP, C-reactive protein; HF, high-frequency amplitude; HRV, heart rate variability; IL-6, Interleukin-6; LF, low-frequency amplitude; MPA, monocyte–platelet aggregate; SDNN, standard deviation of all normal RR intervals; SDNNi, mean of the standard deviations of normal RR intervals of all 5-min segments in the entire recording; VLF, very low-frequency amplitude.

Introduction

Numerous studies have shown that inflammation has a relevant role in the pathogenesis of atherosclerosis as well as of acute coronary syndromes [1–3]. Moreover, markers of inflammation predict cardiovascular events in several disease states [4–6]. Increased platelet activation [7, 8] and sympathovagal

imbalance of cardiac autonomic nervous system (ANS) activity [9–12] are also well-known predictors of acute coronary events.

Inflammatory stimuli are believed to induce a prothrombotic state [13, 14], but there is a relative lack of data about their effects on platelet activation in the clinical setting [15]. Similarly, a relation between ANS activity and inflammation has recently been reported in experimental studies [16–18] as well as in several diseases [19–22], but the exact relation between ANS activity and inflammation in the clinical setting remains unclear. In particular, how inflammatory stimuli affect sympathovagal balance in humans has not been explored.

Influenza A vaccination is globally recommended each year for patients with increased risk of life-threatening complications from influenza A disease, including patients with diabetes [23]. The aim of this study was to investigate the effects of the inflammatory reaction induced by influenza A vaccination on platelet activation and on cardiac ANS activity in a group of patients with type II diabetes.

Methods

Patients

Thirty patients followed at the Diabetic Center of our

MF59C.1 adjuvant (Fluad; Novartis Vaccines and Diagnostics srl, Siena, Italy).

A second 24-h Holter ECG recording was started on day 3 (i.e., 24 h after vaccination), and venous blood samples were collected 24 and 48 h after vaccination.

Part of each collected blood sample was centrifuged at 1078 ***g*** for 20 min, and three aliquots of 500 μL serum were stored at −80° C until assayed. Part of each sample collected 48 h before and after vaccination was used for the assessment of levels of monocyte–platelet aggregates (MPAs; see later).

Inflammatory serum markers

C-reactive protein (CRP) was measured using a high-sensitivity nephelometric method (BN100; Behring Diagnostic, Milan, Italy) with a detection range of 0.175–1100 mg L^{-1} (coefficient of variation < 5%). In the study group, patients' CRP level was measured both 24 and 48 h before and after vaccination.

Interleukin-6 (IL-6) serum levels were measured 48 h before and after vaccination using an enzyme-linked immunoassay kit (Pierce Biotechnology, Rockford, IL, USA) with a detection range of 0–149 pg mL^{-1} (coefficient of variation < 10%).

hospital for an established diagnosis of type II diabetes mellitus who underwent influenza A vaccination in the autumn of 2008 were initially included in the study. This number of patients was planned according to the short period of enrolment (during the few autumn months of the vaccination programme) and the limited availability of recorders for 24-h ambulatory Holter electrocardiogram (ECG) monitoring.

Patients were excluded in cases of overt cardiovascular disease and/or acute or chronic inflammatory disease, or if they were taking beta-adrenergic blocking agents. Written informed consent for participation in the study was obtained from patients, and ethical approval of the study was obtained from our Institute Review Board.

Study protocol

The day before vaccination (day 1), patients underwent a basal evaluation including a 24-h Holter ECG recording and blood sample collection from an antecubital vein. On day 2, patients underwent influenza A vaccination using an inactivated virus with

Monocyte and platelet activation

Monocyte and platelet activation was assessed by measuring MPA formation and membrane receptor expression by flow cytometry. Within 10 min of collection, blood (100 μL) was labelled for 15 min at room temperature with saturating concentrations of peridin-chlorophyll protein-conjugated CD14 (lipopolysaccharide receptor), phycoerythrin (PE)-conjugated CD40, PE-conjugated CD142 (tissue factor receptor) and PE-conjugated CD162 (P-selectin GP ligand-I) for monocyte assessment, and with fluorescein isothiocyanate-conjugated glycoprotein IIb/IIIa (GP IIb; CD41) for platelet assessment (assay kits from Becton Dickinson, Milan, Italy).

Following incubation, erythrocytes were lysed with buffered ammonium chloride, and samples were analysed by FACScan. MPAs were identified using the logical gating facility by combination of the binding characteristics of anti-CD14 (monocyte marker) and of anti-CD41 (platelet marker) antibodies.

A minimum of 3000 monocytes were counted for each test. MPA formation was measured as percentage of

monocytes binding to platelets, whereas monocyte/platelet receptor expression in the MPA gate was measured as mean fluorescence intensity (mfi).

Holter recording and heart rate variability

Twenty-four-hour Holter ECG was recorded using three-channel digital recorders (Oxford Medilog FD5) with bipolar chest leads CM5, CM1 and modified aVF, and data were analysed using the Oxford Excel 3 system (Oxford Instrument, Abingdon, UK). Cardiac autonomic function was assessed throughout the recording by the analysis of heart rate variability (HRV), using the dedicated software associated with the system.

Variables of both time-domain and frequency-domain HRV were obtained. Time-domain variables included the standard deviation of all normal RR intervals (SDNN) and the mean of the standard deviation of normal RR intervals of all 5-min segments in the entire recording (SDNN-i). Frequency-domain HRV analysis was performed using a fast Fourier transform algorithm with spectral resolution of 0.0005 Hz. The amplitude of the following variables was obtained: very low frequency (VLF, 0.0033–0.04 Hz), low frequency (LF, 0.04–0.15 Hz) and high frequency (HF, 0.15–0.40 Hz). The LF/HF ratio was also calculated.

assess the changes in CRP levels at 24 and 48 h, compared to baseline. Correlation analysis was performed using the Pearson or Spearman test, as indicated. A value of $P < 0.05$ was required for statistical significance. Data are reported as mean ± SD.

Results

Thirty patients underwent basal evaluation. However, Holter ECG recordings could not be obtained after vaccine administration in two patients. Thus, complete data were available for 28 patients (18 men, 10 women; mean age 62.1 ± 8 years); this constituted the final study group. The main clinical characteristics of these patients, together with those of the control group, are summarized in Table 1. No patient reported any side effects following vaccination.

The main results in the study group are summarized in Table 2. Compared to basal serum levels (2.68 ± 2.9 mg L^{-1}), CRP showed a progressive and significant increase at 24 (4.15 ± 3.0 mg L^{-1}, $P = 0.04$ vs. baseline) and 48 h after vaccination (7.26 ± 5.9 mg L^{-1}, $P < 0.001$ vs. both baseline and 24 h) (Fig. 1). A significant increase in IL-6 serum levels was found 48 h after vaccination ($P = 0.001$). Fur-

Table 1 *Main clinical findings of patients included in the final*

Control group

To confirm the stability of outcome variables in the absence of vaccination, subclinical inflammatory state and cardiac autonomic function were assessed during the same period in a control group of 12 patients with type II diabetes who did not undergo influenza A vaccination and were similar with regard to gender, age and clinical history to patients in the study group. As before, the number of controls was planned according to the short period of enrolment and the limited availability of recorders for 24-h Holter ECG monitoring. In these patients, serum CRP levels were measured, and 24-h ECG Holter monitoring for HRV analysis was carried out on admission to the study and 48 h later.

Statistical analysis

Statistical analysis was performed using the statistical software SPSS 12.01 (SPSS Inc., Florence, Italy). Changes in IL-6, MPA and HRV variables after vaccination were assessed by paired *t*-test or Wilcoxon test, as indicated. Friedman ANOVA was applied to

analyses

	Study group	Controls	*P*
Number of patients	28	12	
Gender (M:F)	18:10	7:5	0.74
Age (years)	62.1 ± 8	60.0 ± 8	0.45
Body mass index ($kg\ m^{-2}$)	29.5 ± 3.8	28.6 ± 3.9	0.50
Fasting plasma glucose ($mg\ dL^{-1}$)	121 ± 23	118 ± 24	0.71
Smoking	4 (14%)	3 (25%)	0.41
Hypertension	15 (54%)	9 (75%)	0.30
ACE inhibitors/ARBs	14 (50%)	9 (75%)	0.18
Diuretics	6 (21%)	3 (25%)	1.00
Calcium antagonists	2 (7%)	1 (8%)	1.00
Statins	7 (25%)	3 (25%)	1.00
Metformin	16 (57%)	10 (83%)	0.16
Glycosylated haemoglobin (%)	7.1 ± 1.5	6.6 ± 0.8	0.28

ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker.

Table 2 *Results of HRV parameters, inflammatory markers and MPAs in the study group*

	Baseline	Postvaccine	*P*
Inflammatory markers			
C-reactive protein (mg L^{-1})	2.8 ± 2.9	7.26 ± 5.9[a]	<0.001
Interleukin-6 (pg mL^{-1})	0.82 ± 0.9	1.58 ± 1.3	0.001
HRV parameters			
RR intervals (ms)	785 ± 77	753 ± 72	<0.001
SDNN (ms)	120 ± 41	115 ± 37	0.10
SDNN-i (ms)	40.4 ± 15	36.7 ± 13	0.015
VLF (ms)	34.6 ± 12	31.0 ± 10	0.002
LF (ms)	18.2 ± 8.9	16.2 ± 7.1	0.02
HF (ms)	11.9 ± 5.8	10.7 ± 6.1	0.12
LF/HF ratio	1.59 ± 0.5	1.60 ± 0.5	0.87
Monocyte–platelet variables			
MPA (%)	28.5 ± 3.7	30.5 ± 4.1	<0.001
CD-41 (mfi)	24.6 ± 4.1	27.0 ± 4.2	<0.001
CD-40 (mfi)	17.4 ± 5.4	21.1 ± 7.6	0.002
CD-162 (mfi)	413.8 ± 90.2	453.4 ± 79.1	<0.001
CD-142 (mfi)	14.0 ± 3	16.5 ± 3.4	<0.001

[a] Forty-eight hours after vaccination. HRV, heart rate variability; MPA, monocyte–platelet aggregate; mfi, mean fluorescence intensity. See text for definition of HRV parameline, with VLF amplitude showing the most significant change (31.0 ± 10 vs. 34.6 ± 12 ms, $P = 0.002$).

No significant changes in CRP levels or in HRV parameters were observed in diabetic controls (Table 3).

Correlation amongst variables

At baseline, IL-6 was not detectable in the serum in nine patients (32%), making impossible to calculate the percentage change after vaccination. Therefore, correlation analyses were performed only for serum CRP levels.

The percentage change in serum CRP levels at 48 h after vaccination in the study group was found to be significantly correlated with the percentage change in several HRV variables (Table 4), with the most significant association being found with the percentage changes in SDNN for time-domain variables and in VLF amplitude for frequency-domain variables ($r = 0.43$, $P = 0.02$; $r = 0.39$, $P = 0.03$, respectively; Fig. 2).

No statistically significant correlations could be demonstrated between the changes in MPA formation and in monocyte/platelet receptor expression and either serum CRP levels or HRV variables (Table 5).

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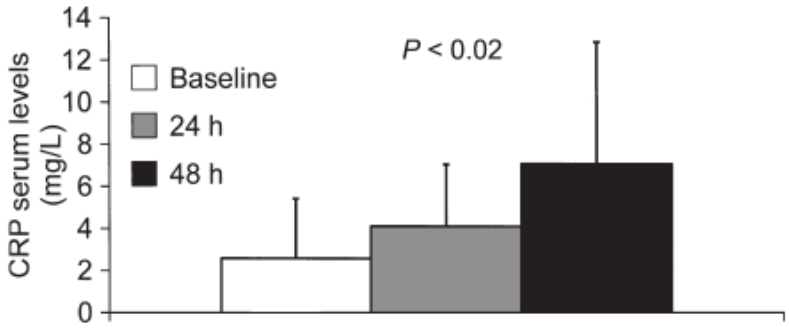


Fig. 1 *Changes in serum C-reactive protein levels 24 and 48 h before and after adjuvant influenza A vaccine administration.*

thermore, significant increases in MPA formation and in monocyte/platelet membrane receptor expression were observed in response to vaccine administration (Table 2).

By contrast, several HRV parameters showed a significant reduction after vaccination compared to base-

Discussion

To our knowledge, this is the first study to directly assess the effects of an inflammatory stimulus on plate-

Table 3 *HRV parameters and C-reactive protein serum levels in control patients*

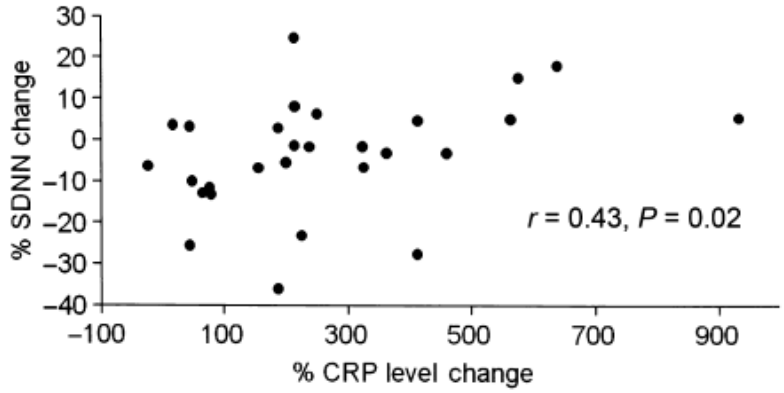
	Baseline	48 h	*P*
C-reactive protein (mg L^{-1})	2.73 ± 2.0	3.01 ± 2.2	0.39
HRV parameters			
RR intervals (ms)	780 ± 54	775 ± 72	0.72
SDNN (ms)	122 ± 33	122 ± 37	0.96
SDNN-i (ms)	45.3 ± 13	46.0 ± 18	0.74
VLF (ms)	37.7 ± 8	37.8 ± 11	0.98
LF (ms)	24.9 ± 15	22.2 ± 13	0.40
HF (ms)	13.1 ± 6	13.2 ± 6	0.85
LF/HF ratio	1.88 ± 0.6	1.67 ± 0.4	0.10

HRV, heart rate variability. See text for definition of HRV parameters.

Table 4 *Correlations between the percentage variations of serum C-reactive protein levels (at 48 h compared to baseline) and those of HRV parameters following vaccine administration*

	r	*P*
RR intervals (ms)	0.14	0.46
SDNN (ms)	0.43	0.02
SDNN-i (ms)	0.30	0.08
VLF (ms)	0.39	0.03
LF (ms)	0.32	0.07
HF (ms)	0.25	0.17
LF/HF	−0.08	0.65

HRV, heart rate variability. See text for definition of HRV parameters.



wards reduced vagal activity and a relative sympathetic predominance.

No significant relation was found, however, between the increase in platelet activation and the degree of inflammation as assessed by serum CRP level. On the other hand, significant correlations were found between the changes in CRP level and in HRV variables, suggesting some pathophysiological link between the inflammatory and the cardiac autonomic responses to vaccine administration.

Inflammation and platelet activation

Influenza A vaccination in our patients induced a significant increase in MPA formation and in expression of monocyte and platelet membrane receptors, suggesting that vaccine-related monocyte activation led to a secondary increase in platelet activation. Of note, no significant association was found between serum CRP levels and MPA, suggesting that the increased platelet activation was not significantly dependent on the degree of the humoral reaction to the inflammatory stimulus.

The observation of increased MPA formation and monocyte/platelet receptor expression following influenza A vaccination may be clinically relevant, as these changes suggest the development of a pro-

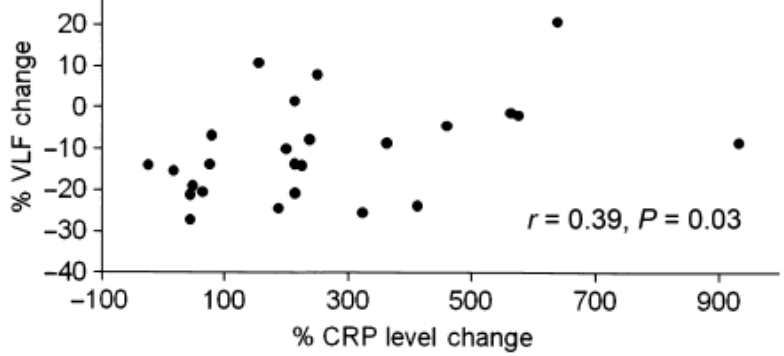


Fig. 2 *Correlation between percentage changes in serum C-reactive protein levels 48 h after vaccine administration, compared to baseline, and percentage changes in the heart rate variability variables standard deviation of all normal RR intervals and very low-frequency amplitude.*

let activation and cardiac autonomic activity, as well as the correlation between these variables, in patients. Our findings, obtained from patients with type II diabetes, show that, together with the expected rise in inflammatory cytokines and monocyte activation, influenza A vaccine induced an increase in platelet activity and changes in cardiac autonomic tone to-

ies, MPAs have been shown to be associated with acute coronary events [8, 24].

Infectious diseases [26, 27], including influenza [28], have been associated with an increased occurrence of acute cardiovascular events, in particular in patients with evidence of increased risk of cardiovascular disease. Several abnormalities induced by infections can favour acute cardiovascular events, including endothelial dysfunction, prothrombotic changes in the blood and direct damage to coronary plaques [29, 30].

Vaccination is recommended each year as a valid way to reduce morbidity and mortality related to seasonal influenza A disease in moderate- to high-risk patients, including those with diabetes [23]. However, the ability of the influenza A vaccination to reduce the risk of acute coronary events remains debated, as discordant results have been reported [31–33]. The increased platelet activation observed after vaccination in our study might transiently increase the risk of thrombosis in high-risk patients. This might significantly counteract the benefits on the cardiovascular

Table 5 *Correlations of the percentage variation in MPA formation and in monocyte and platelet receptor expression with the variation in HRV parameters after vaccine administration*

	CD 41		CD 40		CD 162		CD 142		MPA	
	r	*P*	*r*	*P*	*r*	*P*	R	*P*	R	*P*
CRP	0.34	0.06	0.20	0.27	−0.04	0.82	0.16	0.39	0.08	0.67
RR interval	−0.05	0.79	0.29	0.12	−0.04	0.79	−0.07	0.7	0.26	0.16
SDNN	−0.06	0.74	0.21	0.25	0.13	0.47	0.11	0.56	0.11	0.55
SDNN-i	−0.04	0.82	0.37	0.04	−0.09	0.62	−0.07	0.69	0.18	0.33
VLF	0.13	0.49	0.38	0.03	−0.02	0.88	0.09	0.61	0.26	0.16
LF	−0.03	0.85	0.41	0.02	−0.26	0.16	−0.14	0.44	0.2	0.28
HF	0.01	0.98	0.31	0.08	−0.23	0.21	−0.15	0.41	0.12	0.52
LF/HF	−0.01	0.95	−0.17	0.35	0.14	0.45	0.19	0.29	0.43	0.82

CRP, C-reactive protein; HRV, heart rate variability; MPA, monocyte–platelet aggregate. See text for definition of HRV parameters.

system deriving from the prevention of influenza disease by vaccination, contributing to explain the controversial results about the protection of influenza A vaccine against acute coronary events.

Inflammation and cardiac autonomic function

Many studies have also shown that even an alteration of cardiac ANS activity towards a predominance of adrenergic tone is associated with an increased risk

A relation between impaired cardiac parasympathetic function and a subclinical inflammatory state has recently been reported in several clinical studies [18–21]. Such a relation implies that impairment of the autonomic modulation of inflammation, because of significant autonomic dysfunction, may favour an increase in subclinical inflammation.

To obtain insight into the complex link between the ANS and inflammation, we recently assessed the ef-

of cardiac events in several disease settings [9–12], including diabetes [34].

It is noteworthy that, in recent years, several experimental studies have shown a link between autonomic nervous function and inflammation. Early studies in rats showed that vagotomy increased, whereas vagus nerve stimulation decreased, mortality related to endotoxin-induced sepsis [35]. The capacity of parasympathetic stimulation to limit the response to inflammatory stimuli was confirmed in several other studies [16, 36] and seems to be mainly mediated by the inhibition of tissue macrophage activation through acetylcholine-mediated stimulation of alpha-7 nicotinic receptors [37].

Other studies have shown that inflammation in peripheral tissues can be 'sensed' by afferent nerve fibres stimulated by inflammatory cytokines. Sensing of inflammation triggers autonomic vagal activation that determines anti-inflammatory effects with a probable attempt to avoid tissue damage as a consequence of excessive local inflammatory reactions (the 'inflammatory reflex') [38].

fects of beta-blockade on HRV and CRP levels in a small group of patients with type 1 diabetes. As expected, beta-blockade improved HRV, but, at the same time, also reduced serum CRP levels, suggesting that the improvement of sympathovagal balance by beta-blockers may translate into anti-inflammatory effects [39].

In this study, we investigated the cardiac autonomic response to an inflammatory stimulus in a group of patients with type II diabetes without overt cardiovascular disease. Our results show that vaccine administration induced a cardiac sympathovagal imbalance towards relative sympathetic predominance, as expressed by the reduction in HRV parameters, which probably resulted from systemic changes induced by inflammation (including possible mild increase in body temperature and peripheral vasodilation). Of note, the changes in most HRV parameters correlated with those of CRP levels, confirming the evidence from previous studies of a pathophysiological link between subclinical inflammation and cardiac sympathovagal balance [16, 19–22].

The increase in MPA, however, did not show any significant correlation with HRV changes, suggesting that the acute cell-mediated inflammatory response to viral vaccine did not result in appreciable effects on cardiac autonomic activity. Accordingly, no significant relation was found between HRV changes and platelet activation in this context.

Conclusions

In this study, we have shown that influenza A vaccination in patients with type II diabetes induces, together with the expected inflammatory reaction, an increase in platelet activation and a cardiac sympathovagal imbalance. Overall, the vaccine-induced changes in platelet activity and autonomic nervous activity may transiently increase the risk of cardiovascular events in vaccinated patients.

Conflict of interest statement

Nothing to declare.

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