Proinflammatory Cytokine Genes Are Constitutively Overexpressed in the Heart in Experimental Systemic Lupus Erythematosus: A Brief Communication

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The heart is one of a number of organs that may be affected in systemic lupus erythematosus (SLE), a prototypic autoimmune disease. Potential anatomical sites of involvement include the myocardium, pericardium, endocardium, valves, conduction system and blood vessels that subserve the heart. Typically, the severity of cardiovascular disease in lupus correlates with the degree of systemic inflammation, which is mirrored by the level of C-reactive protein (CRP) in the plasma. C-reactive protein, in turn is regulated by proinflammatory cytokines, such as interleukins (ILs) 1β and 6. These cytokines have been found in functionally and/or structurally damaged areas of the heart and have been implicated in disease pathogenesis. It has been assumed that the source of these putatively pathogenetically relevant cytokines in the compromised heart is infiltrating mononuclear cells. This study tests the hypothesis that cardiomyocytes *per se* may contribute to proinflammatory cytokine production in the setting of systemic inflammation. Using as the experimental model MRL/MpJ-*Tnfrs6*^{lpr} (MRL-*lpr/lpr*) mice, which spontaneously manifest an autoimmune syndrome that has clinical features of SLE, we show that ventricular homogenates and ventricular cardiomyocytes constitutively overexpress genes encoding the proinflammatory cytokines IL-1β, IL-6, IL-10, and gamma interferon. The results suggest the possibility that proinflammatory cytokines emanating from the heart may actually contribute to the high levels of CRP that appear to aid in predicting subsequent cardiac events. Viewed in this setting, CRP becomes a footprint of an ongoing pathogenic process mediated, in part, by the heart muscle itself.
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Introduction

Systemic lupus erythematosus (SLE), is a multisystem autoimmune disease of unknown etiology (1–4) accompanied by cardiac involvement in approximately 50% of cases (5). Although all-cause mortality due to SLE-related illness has declined during the last 2 decades, the risk of cardiovascular death in lupus has remained unchanged (6–8). Cardiac involvement in lupus may affect the endocardium, myocardium, pericardium, heart valves, conduction system, and coronary arteries (9, 10). Independent of the site of anatomical involvement or its etiology, inflammation is thought to play a pivotal role in the pathogenesis of cardiovascular disease in SLE (reviewed in 11). Thus, myocarditis, endocarditis, pericarditis, and coronary arteritis typically occur in the setting of highly active lupus (12), and coronary heart disease in SLE is more often observed in patients with poorly controlled disease (13).

Peripheral inflammation is mirrored in part by the production of acute phase reactants such as C-reactive protein (CRP) and serum amyloid A (SAA). Elevated systemic levels of these inflammatory markers (14) are associated with unfavorable prognoses in unstable angina and appear to predict subsequent cardiac events (15–17). The synthesis of both CRP and SAA by hepatocytes is transcriptionally upregulated by proinflammatory cytokines, most notably interleukin-6 (IL-6), IL-1β, and tumor necrosis factor (TNF) α (18–22). These and other cytokines have been found in functionally and structurally damaged areas

of the heart and have been implicated in disease pathogenesis. The origin of these putative pathogenetically relevant cytokines remains to be fully defined.

In diseases characterized by leukocytic infiltration, such as myocarditis, monocytes, macrophages, and T cells are conventionally thought to account for the spectrum of cytokines found in the heart (23). In atherosclerotic cardiovascular disease, mononuclear cells are also potential sources of cytokine production. Thus, immunohistochemical analysis of atherosclerotic lesions revealed leukocytic infiltrates consisting of ~80% monocytes or monocyte-derived macrophages (Mϕ) and 5%–20% of T cells (24). These Mϕ cells may be recruited to the lesion by interaction with adhesion molecules on endothelial cells and oxidized low-density lipoprotein (LDL) from the fatty core. T-cell clones from atherosclerotic lesions have been shown to produce interferon gamma (IFNγ), and Tellides *et al.* (25) reported that IFNγ could induce arteriosclerotic changes in the absence of detectable lymphocytes in a posttransplant graft atherosclerosis model using subacute combined immunodeficient (SCID) mice as the host. T-cell clones isolated from atheroclerotic lesions have been shown to recognize oxidized LDL (26) and antigen recognition may be accompanied by cytokine production. Oxidization of LDL by activated macrophages may be enhanced by the TH2 cytokines IL-4 and IL-13 (27), which de Boer *et al.* (28) have detected in atherogenic plaques. These observations indicate that atherosclerotic lesions may contain mononuclear cell infiltrates, which may produce cytokines and further contribute to the atherogenic process.

On theoretical grounds, cytokine production in the heart may originate not only from infiltrating leukocytes but also from cardiac myocytes. In this connection, multiple cytokine cascades have been shown to be activated within the myocardium in experimental pathological states, such as myocardial infarction (29), and in an experimental model of congestive heart failure, cardiac myocytes from both the atria and ventricle were found to produce leukemia inhibitory factor, a cytokine capable of inducing cardiac hypertophy (30). To explore the possibility that cardiomyocytes may be involved in cytokine production in the setting of inflammation, we examined constitutive gene expression in the hearts of MRL/MpJ-*Tnfrs6*^{lpr} (MRL-*lpr/lpr*) mice, a murine model of SLE. MRL-*lpr/lpr* mice spontaneously develop an autoimmune syndrome characterized clinically by arthritis and immune-complex glomerulonephritis (31), immunologically by deregulated production of cytokines in multiple tissues (32–34), and serologically by autoantibodies to Smith antigen and native DNA, which are pathognomonic of human lupus (35). As is true of most inbred mouse strains, MRL-*lpr/lpr* mice resist atherosclerosis unless given a diet high in saturated fat. Our results indicate that in systemic autoimmunity, the heart, *per se*, may be a source of proinflammatory cytokines.

Materials and Methods

Male and female MRL-*lpr/lpr* (H-2^k) and CBA/J (H-2^k) mice were purchased from the Jackson Laboratory (Bar Harbor, ME), and maintained in our American Association of Laboratory Animals-approved animal facility. Mice were examined at 6, 20, and 26 weeks of age.

RPMI 1640, Ca^{2+}-free minimal essential medium (MEM), Hanks balanced salt solution (HBSS), Tris-HCl, and agarose were obtained from Gibco BRL (Grand Island, NY). Penicillin/streptomycin and glutamate were purchased from Quality Biological, Inc. (Gaithersburg, MD). dNTPs were obtained from Amersham (Arlington Heights, IL). Fetal calf serum (FCS) was purchased from Hyclone (Logan, UT). [^{32}P]CTP was purchased from NEN (Boston, MA). Taq polymerase was obtained from Promega (Madison, WI). Other chemicals were purchased from Sigma (St Louis, MO). Cytokine primers for reverse transcription–polymerase chain reaction (RT-PCR) were obtained from Biosource International (Camarillo, CA).

For histological analyses, the hearts of 6- and 26-week-old CBA/J and MRL-*lpr/lpr* mice were fixed with 10% buffered formaldehyde solution and subjected to hematoxylin-eosin (HE) staining. To detect the presence of fatty streaks oil red O-isopropanol method was employed (36).

Mice were sacrificed by cervical dislocation. The hearts were excised and rinsed in ice-cold Hanks balanced salt solution (HBSS). RNA was isolated from the heart as previously described (37). Five micrograms of RNA were then reverse transcribed using SuperScript reverse transcriptase (Gibco BRL). Polymerase chain reaction was performed on various amounts of 1:50 and 1:150 dilutions of the newly synthesized cDNA from each sample with β-actin primers for standard curve titration using a modified protocol of Cai *et al.* (38). Briefly, PCR was performed in a 20-μl reaction mixture consisting of 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 100 μg/ml bovine serum albumin (BSA), 50 μ*M* of each deoxynucleotide triphosphate (dNTP), 250 n*M* of β-actin-specific primer, and 1.0 units of Taq DNA polymerase. Following initial denaturation at 94°C for 2 mins, PCR was performed for 30 cycles of denaturation at 94°C for 15 secs, annealing at 60°C for 20 secs, and primer extension at 72°C for 1 min. There followed a final extension at 72°C for 5 mins in order to ensure completion of all reactions. Polymerase chain reaction products were separated on 1.0% agarose gels, and negative images were taken with positive/negative film (Polaroid Corp., Cambridge, MA). Band intensities were obtained using a ScanJet 3c scanner (Hewlett Packard, Palo Alto, CA), and relative values were calculated using the National Institutes of Health Image Analysis 1.58 computer program. Following normalization, PCR was performed using specific primers. Polymerase chain reaction products were separated on a 1.0% agarose gel and, in the case of IL-1β, IL-6, IL-3, TNFα, and TGFβ1, visualized by ethidium bromide staining. For the cytokines IL-2, IL-10, and IFNγ,

gels were further subjected to Southern analyses as described earlier (37, 39) using 30-mer probes. The sequences of primers used in this study have previously been reported (40, 41).

Cardiac myocytes from adult mice were isolated as described by Hilal-Dandan *et al.* (42) with some modifications. Fifteen minutes after injecting heparin intraperitoneally (10,000 U/Kg; Upjohn, Kalamazoo, MI), the mouse was anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg). The heart was then excised, rendered free of noncardiac tissues, and cannulated via the aorta. The cannulated heart was transferred to the perfusion setup and perfused (5 ml/min) with Ca^{2+}-free media (MEM without Ca^{2+} plus 23.8 m*M* $NaHCO_3$) for 5 mins at 37°C, followed by perfusion for 5 mins with 0.2 mg/ml of collagenase type 2 (>280 μ/mg; Worthington Biochemical Corp., Lakewood, NJ) in Ca^{2+}-free media plus 2% BSA and 10 m*M* 2,3-butanedione monoxime (BDM; Sigma, St. Louis, MO). Following perfusion, the atria were removed, the ventricles were cut into small pieces then incubated in 3 ml of collagenase solution (same as above) at 37°C for 3 mins in a shaking water bath. Supernatants containing digested cardiac myocytes were collected, and the incubation was repeated 5 to 7 times. The cells were then washed twice and resuspended in Ca^{2+}-free media plus 2% BSA and 10 m*M* BDM. $CaCl_2$ was then added stepwise from a 1 *M* stock solution with gentle shaking to provide a final concentration of 1 m*M*. Cardiac myocytes were considered viable when they had a rod-shaped appearance, clear striations, sharp edges, no evidence of granulation or blebs, and excluded trypan blue dye. The viability of cardiac myocytes was consistently greater than 90% in all preparations reported on. For mRNA extraction, cells were collected by centrifugation and washed twice with Hanks balanced salt solution with Ca^{2+}. The experiment was repeated in three separate sets of mice.

Results

Initially, we compared cytokine gene expression in whole ventricular homogenates from immunologically normal, H-2 identical ($H\text{-}2^K$), CBA/J and autoimmune MRL-*lpr/lpr* mice. mRNA from young (6 weeks old) and old (26 weeks old) mice was reverse transcribed, and cDNAs were amplified by semiquantitative PCR using cytokine primers for IL-1β, IL-2, IL-3, IL-6, IL-10, IFNγ, TNFα, and TGFβ1. For analyses of IL-2, IL-10, and IFNγ, amplified cDNA was subjected to Southern analyses. The expression of genes encoding the cytokines IL-2, IL-10 and IFNγ was found to be markedly increased in ventricular homogenates of 6-week-old, autoimmune MRL-*lpr/lpr* mice compared with that in those of age-matched, CBA/J control mice (Fig. 1A). The levels of mRNA for IL-2, IL-10, and IFNγ were observed to be increased further in ventricular homogenates of 26-week-old MRL-*lpr/lpr* mice compared with 6-week-old autoimmune mice (Fig. 1A), and gene

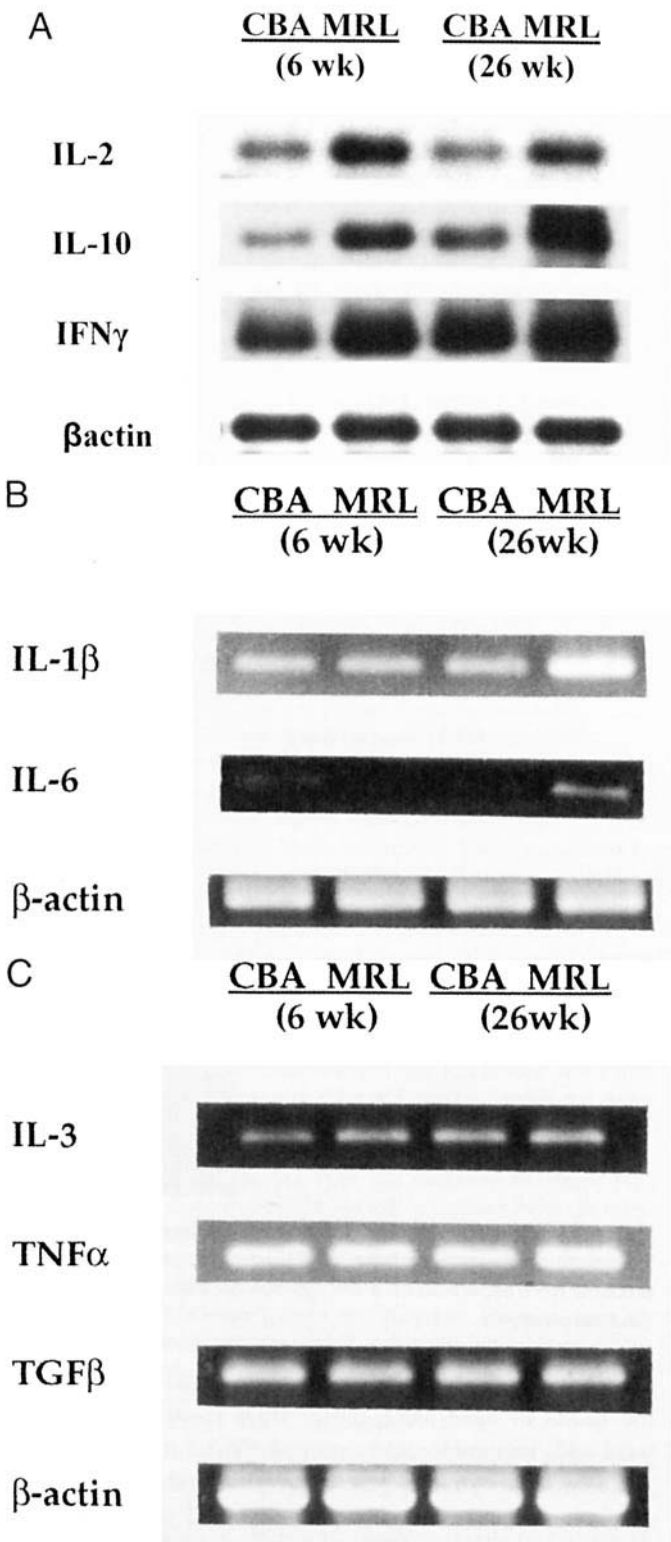

Figure 1. Cytokine gene expression in the ventricles of autoimmune MRL-*lpr/lpr* and normal CBA/J mice. The ventricles were isolated from the hearts of young (6-week-old) and old (26-week-old) mice. cDNA was prepared, normalized, and amplified using cytokine-specific primers; polymerase chain reaction products were (B and C) separated on a 1% agarose gel then stained with ethidium bromide or (A) transferred to nylon membranes and subjected to Southern analysis.

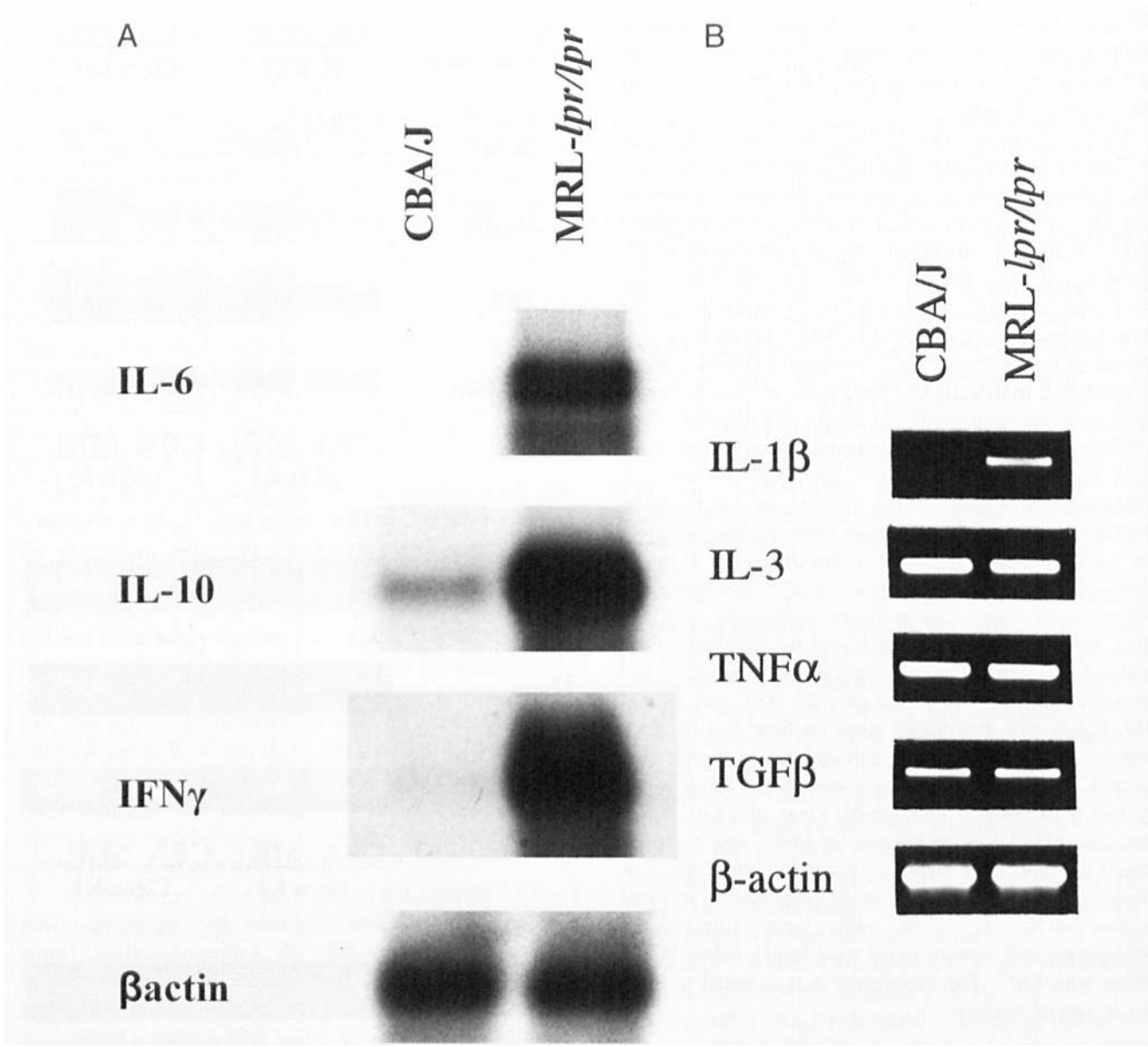

Figure 2. Cytokine gene expression in cardiomyocytes of autoimmune MRL-*lpr/lpr* and normal CBA/J mice. Cardiomyocytes were isolated from the hearts of 20-week-old mice. cDNA was prepared, normalized, and amplified using cytokine-specific primers; polymerase chain reaction products were separated on a 1% agarose gel then (B) stained with ethidium bromide or (A) transferred to nylon membranes and subjected to Southern analysis.

expression of IL-1β and IL-6 was substantially elevated in the hearts of aged MRL-*lpr/lpr* mice compared with 26-week-old, immunologically normal CBA/J mice (Fig. 1B). Differences in cytokine gene expression in the ventricles of autoimmune and normal strains appeared to be selective since comparable expression of the genes encoding IL-3, TNF-α, and TGF-β1 was observed (Fig. 1C). Analyses of the hearts of 6- and 26-week-old MRL-*lpr/lpr* mice revealed no leukocytes, fatty streaks, or plaques (data not shown). Thus, proinflammatory cytokine gene expression was increased in the hearts of autoimmune mice in the absence of either atherosclerotic lesions or mononuclear infiltrates.

To eliminate a possible contribution or spillover of cytokine mRNA from peripheral blood mononuclear cells to the above results, we isolated cardiac myocytes from 20-week-old MRL-*lpr/lpr* mice and age- and sex-matched immunologically normal CBA/J mice. mRNAs were extracted and reverse transcribed and cDNAs were amplified with primers as described above. The results (Fig. 2A) confirmed that constitutive gene expression of IL-1β, IL-6, IL-10, and IFNγ was increased in cardiac myocytes from aged MRL-*lpr/lpr* mice compared with immunologically normal CBA/J mice. Also consistent with our data in ventricular homogenates, no significant differences in the expression of genes encoding IL-3, TNFα, or TGFβ1 were observed (Fig. 2B).

Discussion

Our results show for the first time that ventricular myocytes from autoimmune MRL-*lpr/lpr* mice express high levels of genes that encode proinflammatory cytokines in the absence of histological evidence of either atherosclerosis or mononuclear cell infiltrates. Implicit in our findings is the suggestion that under conditions of chronic systemic inflammation, as occur in many connective tissue diseases, mediators derived from the myocardium may participate in the pathogenesis of heart disease.

It is notable that the gene encoding TNFα was not among those that were overexpressed in MRL-*lpr/lpr* cardiomyocytes. Tumor necrosis factor α drives inflammation in rheumatoid arthritis (RA), has been causally associated with vascular injury in chronic inflammatory states, and may induce the production of both IL-1β and IL-6 (reviewed in Ref. 43). Although the gene encoding TNFα was equally expressed in autoimmune and normal cardiomyocytes, enhanced expression of IL-1β and IL-6 was observed only in the setting of autoimmunity. One possible explanation for this finding is that TNFα mRNA is differentially regulated posttranscriptionally in autoimmune and normal hearts. However, to fully understand the discordance between the levels of TNFα, IL-1β, and IL-6 mRNAs, as well as the significance of deregulated gene expression by autoimmune cardiomyocytes, concomitant cytokine protein measurements will need to be performed.

Our findings may be particularly relevant to diseases such as SLE and RA, which are typically accompanied by elevated plasma levels of proinflammatory cytokines when clinically active (44). These connective tissue diseases are associated with a significant incidence of cardiovascular disease, which occurs at a younger age than in the normal population (45). The observations described herein suggest that systemic inflammation, a consequence of poorly controlled disease activity in SLE and RA, may be accompanied by the production of proinflammatory cytokines by cardiac myocytes. Theoretically, these cytokines may act in a paracrine or autocrine fashion to promote the generation of pro-oxidant molecules, such as nitric oxide and superoxide, and ultimately generate damaging free radicals, such peroxynitrite and hydroxyl ion. In these patients, proinflammatory cytokines emanating from the heart, such as IL-1β and IL-6, if produced in sufficient quantities may enter the periphery and transcriptionally upregulate hepatocytes to produce CRP. Viewed in this setting, CRP becomes a footprint of an ongoing pathogenic process mediated, in part, by the heart muscle itself.

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