INFECTION AND IMMUNITY, July 1983, p. 190–196
0019-9567/83/070190-07$02.00/0

Vol. 41, No. 1

Physical and Biological Properties of U.S. Standard Endotoxin EC After Exposure to Ionizing Radiation

GYORGY CSAKO,[1] RONALD J. ELIN,[1]* H. DONALD HOCHSTEIN,[2] AND CHAO-MING TSAI[2]

Clinical Pathology Department, Clinical Center, National Institutes of Health,[1] and the Office of Biologics, Food and Drug Administration,[2] Bethesda, Maryland 20205

Received 29 December 1982/Accepted 1 March 1983

Techniques that reduce the toxicity of bacterial endotoxins are useful for studying the relationship between structure and biological activity. We used ionizing radiation to detoxify a highly refined endotoxin preparation, U.S. standard endotoxin EC. Dose-dependent changes occurred by exposure to ^{60}Co-radiation in the physical properties and biological activities of the endotoxin. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis showed gradual loss of the polysaccharide components (O-side chain and R-core) from the endotoxin molecules. In contrast, although endotoxin revealed a complex absorption pattern in the UV range, radiation treatment failed to modify that pattern. Dose-related destruction of the primary toxic component, lipid A, was suggested by the results of activity tests: both the pyrogenicity and limulus reactivity of the endotoxin were destroyed by increasing doses of radiation. The results indicate that the detoxification is probably due to multiple effects of the ionizing radiation on bacterial lipopolysaccharides, and the action involves (i) the destruction of polysaccharide moieties and possibly (ii) the alteration of lipid A component of the endotoxin molecule.

The general structure of a bacterial lipopolysaccharide (LPS; endotoxin) molecule has been established (6). According to this model, endotoxins consist of a lipid moiety (lipid A) and two primarily carbohydrate-containing regions, the R-core and the O-polysaccharide (Fig. 1). A variety of physical and chemical procedures, such as ionizing radiation, alkylation, acid hydrolysis, treatment with enzymes and detergents, and phthalylation, can reduce the toxic effects of bacterial LPS (2, 7, 13, 14, 19). Since the "detoxification" techniques frequently exert a differential effect on the biological activities of endotoxin, these procedures are useful in studying the relationship between the biological activity and structure of endotoxins (7, 14, 19). Furthermore, the selective removal of the toxic properties from endotoxin while retaining the immunological properties has an obvious practical application as a potential way to enhance host defenses without deleterious side effects (2, 19).

During the past decade, refined bacterial endotoxins have been prepared for the U.S. Food and Drug Administration, first from *Klebsiella pneumoniae* (18) and then from *Escherichia coli* O113 (15), for use as the U.S. standard in bioassays in the pharmaceutical industry and research. When the second standard endotoxin was prepared (designated as U.S. standard endotoxin EC, where EC stands for *E. coli*), special care was taken to produce an LPS with a very low nucleic acid content (15). This minimizes the possibility that the biological properties of the LPS preparation could be ascribed to polynucleotides (5).

Recently, Elin et al. (7) defined the biological activities of the standard endotoxin EC in humans and demonstrated effective detoxification by phthalylation of the parent compound. In this work, we further characterized the national standard endotoxin EC with respect to the alteration of its physical and biological properties after treatment with ionizing radiation.

MATERIALS AND METHODS

Endotoxin. The purification and refinement of the standard endotoxin from *E. coli* O113 have been detailed previously (15). This endotoxin was dissolved at a concentration of 0.1 μg/ml in 0.15 M sodium lactate containing thiomersal (1:10,000) and at 50 μg/ml in pyrogen-free physiological saline and dispensed into ampoules by the Pharmaceutical Development Service of the Pharmacy Department, Clinical Center, National Institutes of Health. The standard endotoxin EC is considered "soluble" since it goes readily into an apparently colloidal aqueous solution, is totally clear at the concentration indicated above, and has no observable tendency to settle on standing (15). The endotoxin-containing vials were frozen at −20°C until use.

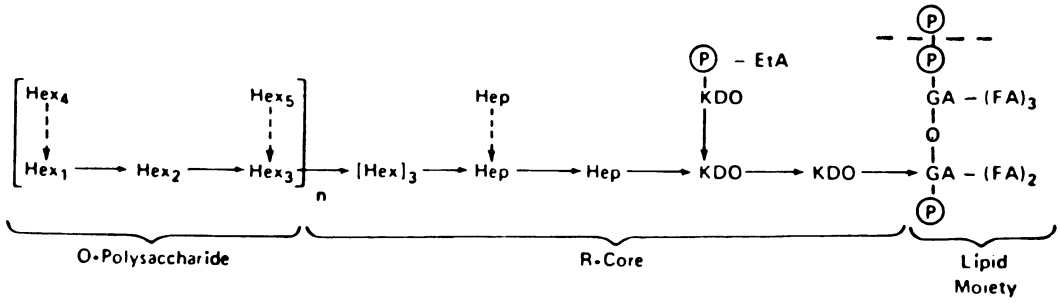

FIG. 1. General structure of bacterial endotoxins. Lipid A exhibits minor variations in composition and is thus a common component of all bacterial endotoxins, whereas the R-core and the O-side chain possess considerable polymorphism in carbohydrate composition—the former appears to be unique to the genus, and the latter ("repeating unit") is characteristic for the species (O-antigenic specificity). Abbreviations: Hex, hexose; Hep, L-glycero-D-mannoheptose; P, phosphate; KDO, 2-keto-3-deoxyoctonate; EtA, ethanolamine; GA, glucosamine; FA, fatty acids. The broken line in lipid A indicates variable occurrence of pyrophosphate at that location. (Figure modified from reference 6 and used with the permission of the Chemical Rubber Co., CRC Press, Inc.

Lipid A. The lipid A fraction of endotoxin prepared from an *E. coli* K-12 strain PL2 (3) was a generous gift of Loretta Leive of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. Lipid A was solubilized in water containing triethylamine (1 μl/ml).

Radiation treatment. The glass vials containing 2.2 ml of standard endotoxin EC at concentrations of 0.1 μg/ml and 50 μg/ml were exposed to γ radiation from a ^{60}Co source. The radiation dose was delivered at a rate of 0.003 Mrad/min at room temperature.

SDS-PAGE. LPS samples were desalted by dialysis on Millipore filter disks (10). The samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by Laemmli's technique in 14% gel (9). Patterns were visualized with a modified silver stain as detailed before (20). Since the detection of multiple bands even with the highly sensitive silver stain requires the electrophoresis of endotoxin in the microgram range (20), the standard endotoxin EC for this assay was irradiated at a higher concentration (50 μg/ml).

Spectroscopy. Samples containing 50 μg of standard endotoxin EC per ml were scanned in 1-cm quartz cuvettes in a Beckman model 25 Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Pyrogen assay. New Zealand albino male rabbits weighing 1.8 to 2.0 kg were used for fever studies. Endotoxin or saline was injected in a volume of 1.5 ml per rabbit via the marginal ear vein. Sham experiments that included the recording of temperatures after intravenous injection of saline were performed 1 day before the testing of endotoxins. Temperatures were recorded by means of a thermistor probe that was coated with petrolatum jelly, inserted 7 cm rectally, and connected to an electronic thermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). The febrile responses were followed for up to 3 h and quantified by three parameters: the maximum temperature (T_{max}); the maximum change in temperature from the baseline (ΔT); and the fever index, expressed in degrees Celsius per hour and calculated from the area obtained by planimetry above the baseline temperature (15). The minimum pyrogenic dose for LPS was defined as the dose producing a temperature rise of 0.6°C (15).

LAL test. The reactivity of endotoxin in specimens was determined by gelation of lysate from the amebocytes of the horseshoe crab, *Limulus polyphemus* (1, 4, 5, 15). The Limulus amebocyte lysate (LAL) was prepared as described previously (4, 5). The test was performed in 10 by 75-mm tubes by the addition of 0.1 ml of LAL to 0.1 ml of various dilutions of the sample prepared in pyrogen-free distilled water. Tubes were incubated in a 37°C water bath for 1 h, and the results were scored as follows: negative, clear, free-flowing liquid; 1+, some flocculation or small "clots"; 2+, soft gel that dislodges from the bottom of the tube when inverted; and 3+, firm gel that adheres to the bottom of the tube when inverted. The lysate used in this study could detect <1 ng of standard endotoxin EC per ml (see Fig. 5a). Results are expressed as follows: (i) LAL titer that is the reciprocal of the highest dilution giving a positive response (1+ or more) and (ii) LAL score that is the sum of scores of the reaction in each tube of the dilution series made up in duplicate.

RESULTS

Changes in the molecular composition of standard endotoxin EC after treatment with ionizing radiation. After SDS-PAGE, a sensitive silver stain uncovered the presence of more than 20 bands in the untreated LPS preparation (Fig. 2, lane A). In addition to two fast-migrating major bands, several faint bands occurred throughout the gel, and stronger-staining minor bands formed a cluster in the slow-migrating area. Treatment of the LPS with an ionizing radiation dose as little as 0.18 Mrad destroyed most of the minor bands, including the more intensively staining ones in the slow-moving region (Fig. 2, lane B). Increasing radiation doses caused further destruction of the bands, eliminating first

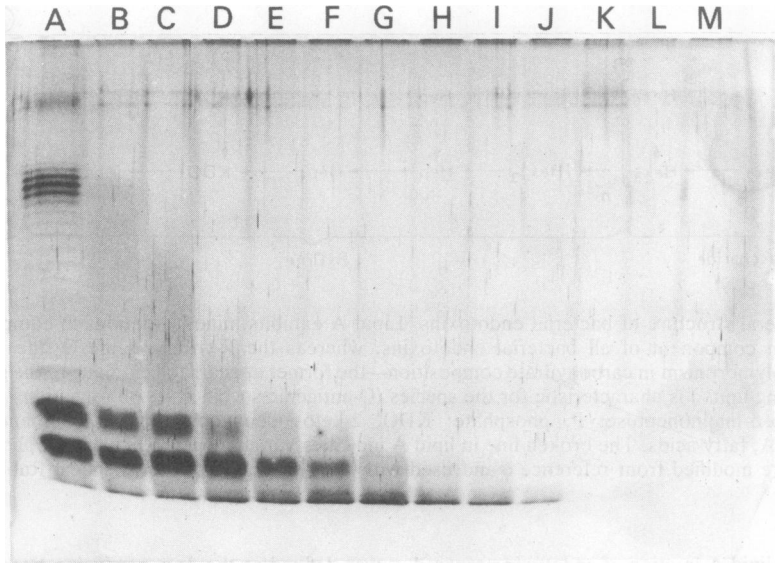

FIG. 2. SDS-PAGE analysis of the standard endotoxin EC before and after treatment with ionizing radiation. Samples of 2.5 μg of LPS were applied to each site. Lanes: A, LPS with no treatment; B, LPS irradiated with 0.18 Mrad; C through L, 0.36 to 3.60 Mrad in 0.36-Mrad increments; M, 4.32 Mrad. The faint double lines across the upper part of the gel in all samples were contaminants from the buffer used for electrophoresis.

the ones with lower migration ability. The fastest-moving major band appeared to resist degradation for the longest period of time, being totally destroyed only by radiation doses exceeding 2.5 Mrad (Fig. 2, lane I). No bands could be demonstrated by silver stain in the gel after irradiating the LPS with doses higher than 3 Mrad (Fig. 2, lanes K, L, and M).

Spectral properties of irradiated standard endotoxin EC. When tested at a concentration of 50 μg/ml, native or irradiated standard endotoxin EC did not exhibit any measurable absorbance upon scanning in the visible range. However, in the near-UV and far-UV region, a complex absorption pattern was obtained with both the untreated and irradiated standard endotoxin EC preparations by scanning them against pyrogen-free 0.9% saline (Fig. 3). Nevertheless, no difference could be demonstrated between the spectral properties of native endotoxin and endotoxin exposed to increasing doses (up to 4.32 Mrad) of radiation. Thus, the regions corre-

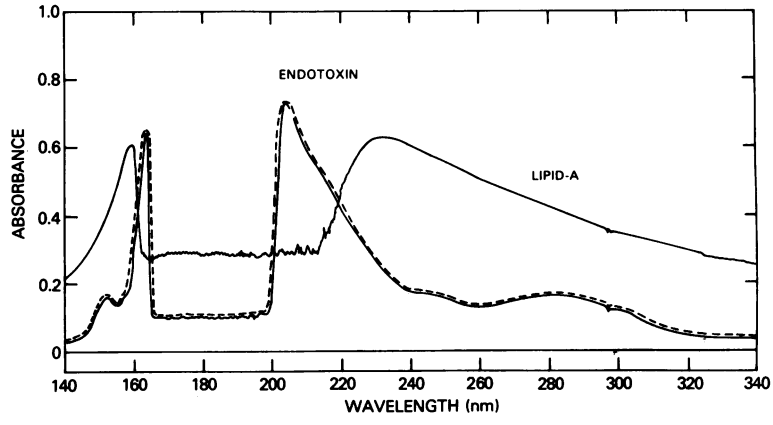

FIG. 3. Spectroscopic analysis of the standard endotoxin EC after treatment with ionizing radiation. Native (solid line) and irradiated (0.18 to 4.32 Mrad) endotoxin (broken line) were scanned at a concentration of 50 μg/ml, and lipid A was tested at a concentration of 236 μg/ml.

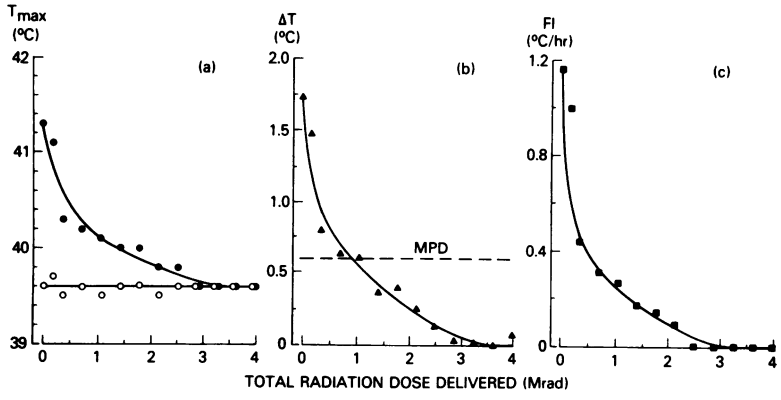

FIG. 4. Dose-dependent effect of ionizing radiation on the pyrogenicity of the standard endotoxin EC in rabbits. All points correspond to the average of measurements obtained in three animals. Open circles indicate the means of preinjection temperature of the three rabbits.

sponding to lipid A absorbance were also without an obvious change after radiation treatment of standard endotoxin EC.

Effect of ionizing radiation on the pyrogenicity and LAL activity of standard endotoxin EC. Native standard endotoxin EC caused a strong pyrogenic response (on average, 41.25°C [n = 3]) when given intravenously at a dose of 150 ng per rabbit (~75 ng/kg). Figure 4 shows that the pyrogenicity of the LPS preparation, described by either of the fever parameters T_{max}, ΔT, or fever index, was progressively reduced by ^{60}Co radiation. The abrogation of the pyrogenic potency of LPS followed an exponential curve, being the "smoothest" when the fever index was calculated. Even the smallest radiation dose tested (0.18 Mrad) produced an easily recognizable decrease in the pyrogenic activity of endotoxin, and 3 Mrad totally eliminated pyrogenic activity. A radiation dose of about 1 Mrad reduced the pyrogenicity of the standard endotoxin EC to the minimum pyrogenic dose, which corresponds to the effect of about 0.71 ng of the native standard endotoxin EC per rabbit (15). This indicates a decrease of approximately 200-fold by 1 Mrad radiation in the pyrogenicity of the LPS (150 versus 0.71 ng).

Similar results were obtained for the LAL activity of the irradiated standard endotoxin EC. There was a dose-dependent, exponential decrease in the LAL activity of the LPS after treatment with ionizing radiation (Fig. 5). The effect was already apparent at a dose of 0.18 Mrad, and 3 Mrad completely abrogated it. Furthermore, the LAL score (Fig. 5b) that incorporated both the endpoint (titer) and strength of the reaction resulted in better data fitting for the altered endotoxin reactivity than the LAL titer alone (Fig. 5a).

Comparison of changes in the physical and

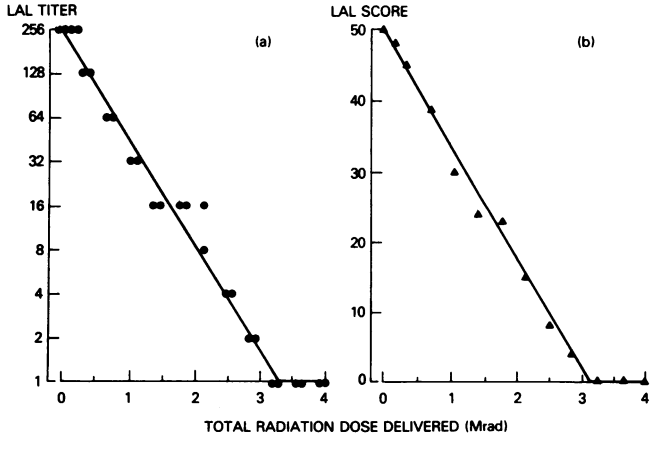

FIG. 5. Dose-dependent effect of ionizing radiation on the LAL activity of the standard endotoxin EC.

biological activities of endotoxin after ^{60}Co irradiation. In general, a good correlation could be observed between the changes occurring in the physical and biological activities of LPS after exposure to increasing doses of ionizing radiation. The comparisons are summarized at three different radiation dose levels which appeared to be especially important regarding the changes in the physical or biological properties (or both) of the endotoxin preparation.

The lowest radiation dose tested (0.18 Mrad) eliminated all the slow-migrating bands on SDS-PAGE (Fig. 2, lane B), and both the pyrogenic (Fig. 4) and the LAL reactivities (Fig. 5) of the endotoxin were slightly diminished.

One megarad of the ^{60}Co radiation left staining reactivity in only one molecular species of the endotoxin preparation, namely, in the fastest-migrating band on SDS-PAGE (Fig. 2, lane E). A considerable decrease in the pyrogenicity to the level of the minimum pyrogenic dose (~200-fold decrease) (Fig. 4b) occurred, but only an eightfold decrease in the titer of LAL activity was found (Fig. 5a).

Finally, a radiation dose of 3 Mrad eliminated all stainable bands on SDS-PAGE (Fig. 2, lanes J and K) and led to no measurable pyrogenic (Fig. 4) or LAL activities (Fig. 5) in the endotoxin preparation.

DISCUSSION

The molecular heterogeneity of endotoxin preparations has been clearly demonstrated by recent studies (8, 11, 20). According to results obtained by analysis of LPS on SDS-PAGE, the size heterogeneity is due to differences in the number of antigenic side chain units (O-polysaccharide; Fig. 1) per molecule rather than variations in the lipid A or R-core oligosaccharide region (or both). With *E. coli* and *Salmonella typhimurium* strains that are deficient in synthesizing various components of the endotoxin molecule (8, 11), it has been shown that the fastest-migrating wide band on SDS-PAGE represents LPS molecules that are devoid of the O-side chain, thus consisting only of lipid A and R-core. The second, fast-migrating major band has been identified as a structure composed of lipid A, R-core, and a single unit of the repeating O-polysaccharide. Further bands appear to contain LPS molecules with increasing numbers of O-side chain units up to about 40, lending progressively decreased mobility to the molecules. In addition, the distribution of LPS molecules with increasing numbers of repeating units is unequal. The majority of these molecules have 19 to 34 units, and only a few occur with 2 to 18 repeating units per LPS molecule. It is noteworthy that lipid A alone has not yet been identified on SDS-PAGE (20).

As shown by a new sensitive silver stain, native standard endotoxin EC also shows a considerable size heterogeneity on SDS-PAGE. The dectection of two fast-migrating major bands and several minor bands with the cluster of a few, somewhat stonger staining bands in the slow-moving region is in good agreement with previous findings on various endotoxin preparations (8, 11, 20). With knowledge of the SDS-PAGE pattern for native endotoxin, it is possible to interpret the changes occurring after its exposure to ionizing radiation. Electrophoretic analysis of irradiated endotoxins suggested that even very small doses of radiation are able to cleave the repeating O-side chain units from the lipid A–R-core complex. Consequently, the SDS-PAGE pattern of endotoxin was reduced to the existence of two fast-moving wide bands containing no or only one repeating unit. Eventually these bands were also destroyed by high radiation doses, probably breaking down all of the LPS molecules at least into the basic subunits. During the irradiation process, the low electrophoretic mobility (long O-side chain) LPS molecules might have been first converted into high-mobility products with fewer or no repeating units, contributing to the relatively long persistence of the fastest-moving band(s).

Only one previous work has dealt with the spectroscopic properties of irradiated endotoxin; Previte et al. (13), however, did not study the UV range. In the infrared spectrum, they found no qualitative change for irradiated *S. typhimurium* LPS, but the patterns published in their article indicate some quantitative differences between the parent endotoxin and the irradiated derivative. Moreover, they reported on a color change from a white opalescence to yellow, as well as a general increase in optical density in the visible range after irradiation of the endotoxin preparation. These authors also described a quantitative relationship between the increment in optical density at 400 nm and the radiation dose delivered to the LPS. In our study, the LPS preparation was colorless, and we could not detect light absorbance with the standard endotoxin EC in the visible spectrum. The discrepancy, however, may be simply because of the relatively low concentration of LPS in our preparation (microgram-per-milliliter range) as opposed to the LPS concentration in the preparation used for spectroscopy by Previte et al. (milligram-per-milliliter range) (13). However, scanning endotoxin in the UV range, we found a complex absorption pattern that did not change upon increasing exposure of endotoxin to ionizing radiation.

Regarding the biological activities of irradiated endotoxin, our results show effective, dose-dependent detoxification when the standard en-

dotoxin EC in water is exposed to ionizing radiation at ambient temperature. Reduced pyrogenicity has already been documented for radiodetoxified endotoxins (2, 13), but a similar decrease in the LAL activity has not been established in previous reports on irradiated endotoxins. The former observations that endotoxin irradiated in a dry state (17) or in water frozen to −184°C (13) exhibits little or no loss of toxicity indicate the importance of free radical formation in a mobile state for inactivation of the toxic determinants of LPS. Consistent with that observation appears to be our finding on the high potency of radiation doses, which are low in comparison to the ones utilized in previous studies, to decrease the toxic properties of endotoxin. Since, in contrast to other investigators (2, 13), we used LPS made up in water at a very low concentration, even with low radiation doses the relative proportion of active radicals formed to each LPS molecule could be high enough to alter their structure, resulting in reduced toxicity. In addition, the endotoxin used here is a highly refined product and the elimination of the possible "absorbing-shielding" effect of impurities might have been another factor for the increased efficiency of ionizing radiation.

The decreased pyrogenicity and LAL reactivity of standard endotoxin EC upon exposure to ionizing radiation were associated with molecular changes, notably, with the removal of polysaccharide components (O-side chain and R-core) (SDS-PAGE analysis). Obviously, destruction by radiation treatment of the primary toxic component (lipid A) alone would explain the reduced toxicity, but it has also been suggested that intact lipid A alone may not be as effective a toxic agent as the parent LPS having the polysaccharide moieties. In one study, for instance, about 100 times less activity was obtained with lipid A on a weight-for-weight basis in an LAL-chromogenic substrate (S2222) test than with the intact LPS (16).

Our results showing the loss of biological functions as a result of degradation by ionizing radiation of LPS into smaller-molecular-weight components are also consistent with the original findings of Ribi et al. (14). Using analytical ultracentrifugation for the study of endotoxin subjected to acid hydrolysis, these authors demonstrated that dissociation of endotoxin into particles of the size of haptenic polysaccharide parallels the decline in biological potency. They concluded that "a macromolecular complex of a critical size is one of the major requirements for endotoxin to elicit its characteristic effects in the mammalian host."

The chemical modification of bacterial endotoxins revealed that their multiple biological properties can be dissociated (19). The existence of various structures that are responsible for lethal, immunological, and pyrogenic activities is also supported by results obtained on endotoxins exposed to ionizing radiation (2, 12, 13). The use of ionizing radiation thus provides a promising technique to produce endotoxin that has lost the noxious properties while retaining the beneficial ones such as adjuvanticity to increase the body's natural defense (2, 12, 13).

ACKNOWLEDGMENT

We thank Honterial Henderson for secreterial assistance in manuscript preparation.

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