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Human leukocytic pyrogen: Purification and development of a radioimmunoassay

(fever/endogenous mediator/immunoadsorption/radiolabeling)

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ABSTRACT **Leukocytic pyrogen is a small endogenous protein that mediates fever. Because of the limitations of bioassays, circulating leukocytic pyrogen has not been demonstrated during fever in humans. The pyrogen was produced *in vitro* after phagocytosis of staphylococci by blood monocytes. Antibody against the pyrogen was obtained from rabbits immunized with leukocytic pyrogen and the antiserum was purified by solid-phase immunoadsorbants. Purified antibody to the pyrogen was attached to activated Sepharose 4B and used in conjunction with gel filtration to purify the pyrogen. The pyrogen was labeled with ^{125}I and further purified by gel filtration and ion-exchange chromatography. The final preparation of ^{125}I-labeled pyrogen demonstrated a homogeneous band during isoelectric focusing and other separation procedures. With antibody to pyrogen attached to Sepharose, less than 0.1 of a rabbit pyrogenic dose of human leukocytic pyrogen inhibited the binding of ^{125}I-labeled pyrogen to this immunoadsorbant, and this inhibition was not affected by the presence of human serum. Thus, a radioimmunoassay for human leukocytic pyrogen has been developed that may be used to detect circulating pyrogen during fever in humans.**

Fever is a major host response to many infectious, immunologic, and toxic agents. Although these agents have varied molecular compositions, it is hypothesized that they cause fever by inducing phagocytic leukocytes to synthesize and release a small mediator protein called leukocytic pyrogen (LP) (1). Subsequently, LP raises body temperature by its actions on specialized neurons in the pre-optic region of the anterior hypothalamus. In humans, LP is produced *in vitro* from stimulated phagocytes (2, 3), but circulating LP has yet to be convincingly demonstrated during febrile diseases (4). This may be due to the limitations of the bioassay for human LP, which utilizes the production of fever in rabbits. An immunoassay for human LP should be more sensitive than a bioassay. However, the lability and losses of LP during purification, as well as the lack of a specific antiserum, have hampered the development of an immunoassay. In a recent report (5), we described the production of specific rabbit antibody to human LP. Using this antiserum, we have developed a radioimmunoassay for human LP, which is the subject of this report.

MATERIALS AND METHODS

Human LP was produced *in vitro* after phagocytosis by peripheral blood monocytes of heat-killed staphylococci. The monocytes were obtained from Ficoll–Hypaque gradients of whole blood from normal volunteers (6). Monocyte supernates containing LP were concentrated and the specific activity (protein per rabbit pyrogenic dose) was determined by a two-point dose response curve in six rabbits as described previously (7, 8). A peak rise in rectal temperature of 0.6–0.9° is considered a rabbit pyrogenic dose. Antiserum was obtained from rabbits after multiple injections of crude LP in Freund's adjuvant (5). Immunoadsorbants were made with CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc.) (9). All gel-filtration media were autoclaved for 30 min and run in phosphate-buffered saline, pH 7.4, with 0.02% Na azide. Samples were placed on 0.1% sodium dodecyl sulfate ($NaDodSO_4$)/7.5% polyacrylamide gels (125 × 5 mm) and were electrophoresed in 0.2 M Tris-borate buffer, pH 8.6. Samples were first incubated at 60° for 1 hr in 1% $NaDodSO_4$/10 mM Tris-borate/2 M urea. Gels were stained in 0.2% Coomassie blue and destained in a diffusion destainer (Bio-Rad Laboratories), and the absorbance of the bands was recorded on a scanning device at 525 nm (Helena Laboratories).

Isoelectric focusing was performed in 4.5% polyacrylamide in the presence of 1% Ampholines (LKB, pH 3–10). Gels were cooled at 4° for 18–20 hr and run at 250 V. Gels were cut and eluted in 0.5 ml of H_2O and the pH was measured. ^{125}I-Labeled Bolton–Hunter reagent was obtained from New England Nuclear and proteins were labeled at 0° in the presence of 0.1 M bicine, pH 8.3 (10). Protein concentration was determined at 280 nm, with bovine serum albumin as a standard.

RESULTS

Purification of Antiserum. Antiserum against human LP contained precipitating antibodies to several substances present in crude leukocyte supernates, as demonstrated in agar diffusion. To remove unwanted antibodies we used solid-phase immunoabsorption by attaching an antigen mixture to Sepharose 4B. These antigens consisted of (*i*) leukocyte supernates obtained after 30 min of incubation with serum and staphylococci, a time period not adequate to produce LP (3); (*ii*) leukocyte supernates obtained after 18 hr of incubation in 2.5 μg of cycloheximide per ml, which inhibits the synthesis of LP (8); and (*iii*) fresh human AB serum. After concentration of these materials, bioassay in rabbits confirmed the absence of pyrogen. These antigens were then attached to activated Sepharose 4B and the globulin fraction of crude antiserum was applied to this immunoadsorbant. The ability of antiserum to neutralize the pyrogenicity of human LP was recovered in the unbound protein, which eluted at pH 7.4. After eight separate passages, there were no visible precipitation lines in agar diffusion between the purified globulin and crude leukocyte supernates. However, the globulin retained its ability to neutralize the pyrogenicity of LP.

Use of Specific pH to Elute LP from Anti-LP Immunoadsorbants. Purified anti-LP was next attached to Sepharose. Crude LP bound to the anti-LP immunoadsorbant at pH 7.4

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Abbreviations: LP, leukocytic pyrogen; $NaDodSO_4$, sodium dodecyl sulfate.

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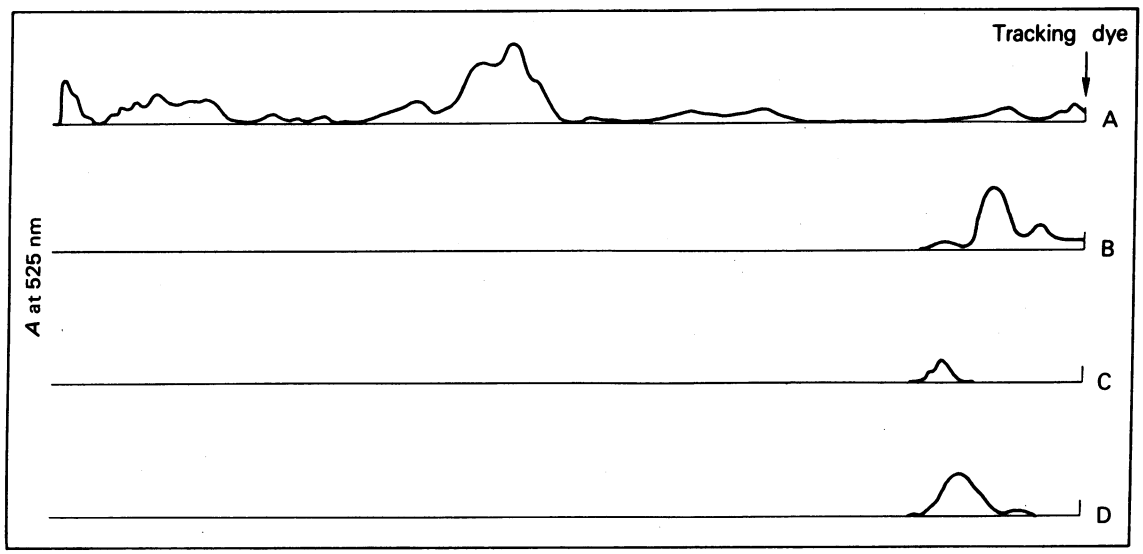

FIG. 1. Tracings of stained bands from $NaDodSO_4$/polyacrylamide gels during sequential purification of human LP. (*A*) Crude LP. Protein (133 μg) equivalent to 0.33 of a rabbit pyrogen dose was applied to gels. (*B*) LP eluted from gel-filtration column. One hundred doses of crude LP were chromatographed over Sephadex G-50 (fine) (165 × 5.6 cm). The 15,000 molecular weight pyrogen peak was isolated, concentrated, dialyzed, and lyophilized. Protein (240 μg) was recovered and 24 μg was applied to the gels. (*C*) LP eluted from gel-filtration column and immunoadsorbant. One hundred doses of the 15,000 molecular weight. LP shown in *B* were eluted from an anti-LP immunoadsorbant at pH 3.2 and then neutralized with 0.1 M NaOH, concentrated, dialyzed, and lyophilized. This material was applied to the gels. Accurate protein determinations were difficult to ascertain because of low protein concentration, adherence to dialysis tubing, and nonspecific substances which elute from Sepharose and interfere with the absorbance at 280 nm. (*D*) Cytochrome *c* (25 μg) used as a standard (molecular weight 12,382).

and nearly 100% of activity was recovered during citric acid elution at pH 2.5 (5). However, $NaDodSO_4$/polyacrylamide gel electrophoresis revealed that several proteins eluted at pH 2.5. Therefore, we attempted to elute LP at different pH ranges, assuming the binding of LP could be displaced at a specific pH. Approximately 80% of the LP activity was recovered during elution at pH 3.2. $NaDodSO_4$/polyacrylamide gel electrophoresis revealed that after the elution of LP other proteins continued to elute at pH 2.5. Thus, in subsequent experiments, LP was eluted at pH 3.2.

Effect of Gel Filtration and Immunoadsorption on Purification of LP. Concentrated LP was chromatographed on Sephadex G-50 and the 15,000 molecular weight pyrogen peak was isolated and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. Fig. 1 (tracings *A* and *B*) compares the proteins present in crude LP with those isolated after gel filtration. This 15,000 molecular weight pyrogen peak was then applied to the anti-LP immunoadsorbant and the eluted pyrogen was subjected to electrophoresis. Fig. 1 (tracing *C*) reveals the presence of a homogeneous staining band.

With the combination of gel filtration and immunoadsorption, protein concentration of the pyrogen was low, and it was difficult to accurately calculate specific activity. Therefore, we labeled the 15,000 molecular weight pyrogen peak with ^{125}I and then applied this preparation to the anti-LP immunoadsorbant. Table 1 presents the data from three experiments in which the labeled protein was applied to the anti-LP immunoadsorbant. The radioactivity per dose of pyrogen that eluted at pH 3.2 was 0.1% of the radioactivity of the nonbinding protein. This suggests that at this stage of purity the specific activity of human LP is approximately 50 ng of protein or less per rabbit pyrogenic dose.

Table 1. Elution of ^{125}I-labeled LP from anti-LP immunoadsorbant

Exp.	μg protein/ pyrogenic dose*	μCi ^{125}I added/ pyrogenic dose	Radioactivity/pyrogenic dose: Before immunoadsorbant†	Radioactivity/pyrogenic dose: After immunoadsorbant‡
A	50	7	1.4×10^6	5×10^2
B	50	7	7×10^5	1.5×10^2
C	70	25	9×10^5	7×10^2

* Source of human LP: 15,000 molecular weight pyrogen peak from Sephadex G-50 gel filtration of crude LP.
† cpm after removal of unbound ^{125}I.
‡ cpm in pyrogen eluted by citric acid (pH 3.2).

Direct Labeling of Human LP with ^{125}I. In the experiments shown in Table 1, we were unable to increase significantly the radioactivity of the pyrogen by using three times the number of μCi per pyrogenic dose. Therefore, we directly labeled the pyrogen molecule as follows. LP that had been eluted from the anti-LP immunoadsorbant and then subsequently chromato-

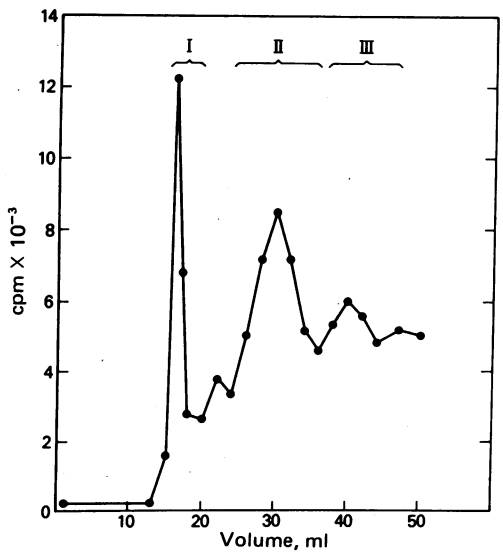

FIG. 2. Sephadex G-15 gel filtration (40 × 1.5 cm) of ^{125}I-labeled LP. Four hundred rabbit pyrogen doses were purified by passage over an anti-LP immunoadsorbant followed by Sephadex G-50 gel filtration. The sample was concentrated and lyophilized before being labeled with 1 mCi of ^{125}I as Bolton–Hunter reagent.

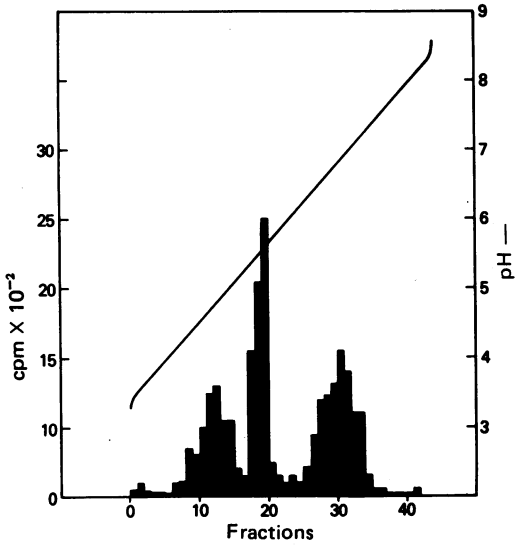

FIG. 3. Isoelectric focusing of ^{125}I-labeled LP obtained from pool I of Sephadex G-15 gel filtration (Fig. 2). The gels were sliced every 2 mm.

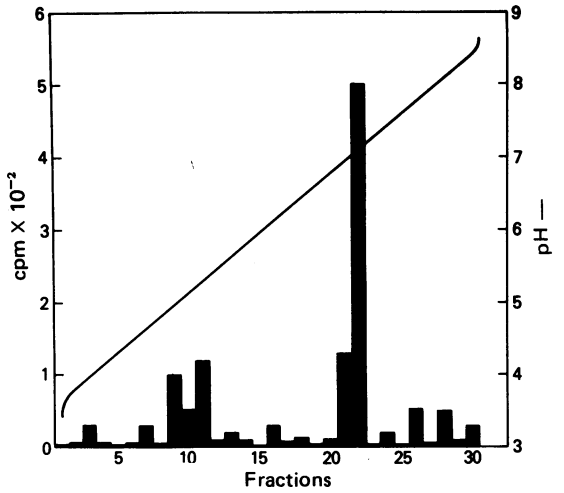

FIG. 5. Isoelectric focusing of pool I (Fig. 4) obtained from ion-exchange chromatography of ^{125}I-labeled LP. Gels were sliced every 2.5 mm.

graphed over Sephadex G-50 was lyophilized and labeled with 0.5–1.0 mCi of ^{125}I per 400 rabbit pyrogen doses. To remove unbound ^{125}I, we used gel filtration. Fig. 2 depicts a typical experiment in which the labeled LP preparation was applied to a Sephadex G-15 column, and demonstrates three peaks of radioactivity. These peaks were pooled separately, and pyrogen activity was recovered only in pool I. $NaDodSO_4$/polyacrylamide gel electrophoresis of pool I revealed a single localization of radioactivity occurring before the marker protein, cytochrome *c*. However, isoelectric focusing of pool I revealed three separate proteins (Fig. 3). The pI values of these three proteins were 4.7, 5.6, and 7.0.

Ion Exchange of Labeled LP. It was clear that the homogeneous band of the pyrogen consisted of at least three proteins, as demonstrated by isoelectric focusing; thus, they should be separable during ion-exchange chromatography. Human LP elutes at low salt concentrations from DEAE-cellulose at pH 8.1 (7). Fig. 4 demonstrates DEAE-cellulose ion-exchange chromatography of pool I from the Sephadex G-15 gel filtration. The first major peak of radioactivity eluting at 3–5 mmho (pool I, Fig. 4) contained the pyrogen activity, as had been reported previously (7). The recovery of biologically active pyrogen from the crude state to that following ion exchange was approximately 1%. LP isolated after ion exchange produced brief monophasic fevers in rabbits; heating this material at 80° for 15 min destroyed its pyrogenicity. When the pyrogen-containing peak from the DEAE-cellulose ion-exchange step was focused in polyacrylamide gels, a single major band of radioactivity was found at pH 7.0–7.1. (Fig. 5). Further evidence that labeled LP was homogeneous was confirmed by chromatography on Sepharose CL-6B in 6 M guanidine-HCl, on hydroxylapatite in 0.1% $NaDodSO_4$, and under a high pressure gradient of CH_3CN/10 mM KH_2PO_4 using a Bondapak C_{18} column (Waters Associates). In each case, a single peak of radioactivity was observed. In addition, the 15,000-dalton LP trimerized into a 45,000-dalton molecule with a pI at 5.5. The existence and pyrogenicity of this larger molecular weight LP in crude leukocyte supernates has been previously demonstrated (6).

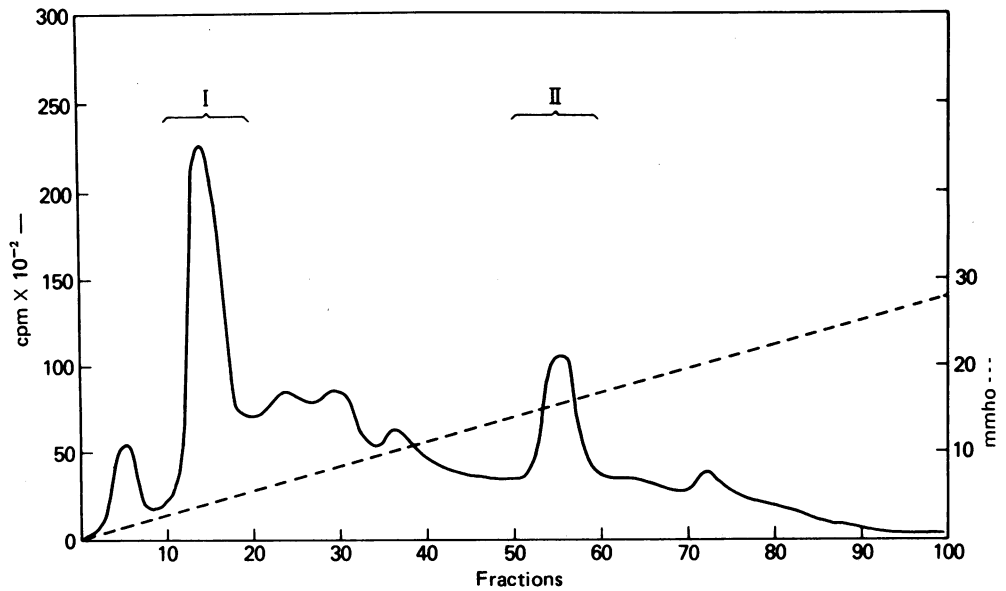

FIG. 4. Ion exchange of ^{125}I-labeled LP obtained from pool I of Sephadex G-15 gel filtration (Fig. 2). The sample was dialyzed against 20 mM Tris, pH 8.1, before being applied to a 8 × 1.5-cm DEAE-cellulose (DE-52; Whatman, Ltd.) column equilibrated in the sample buffer and run at 4°. NaCl was used as the salt gradient.

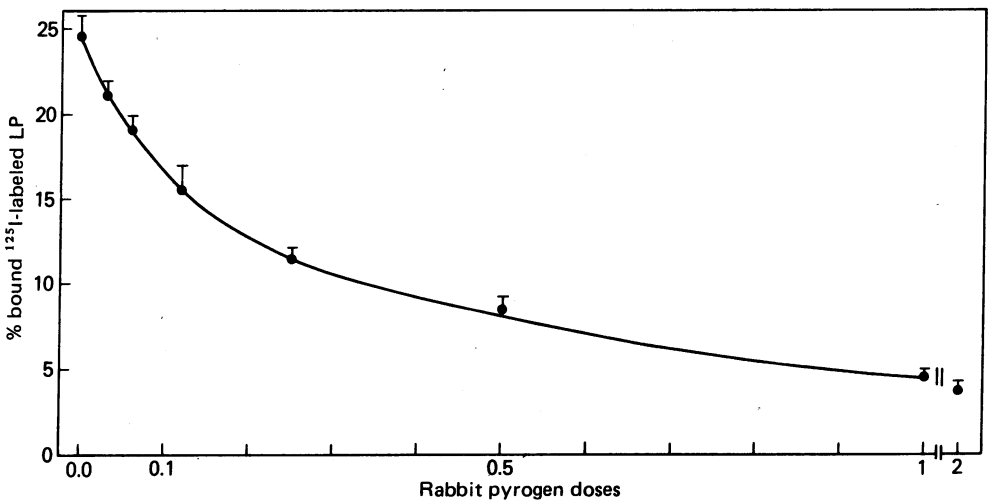

FIG. 6. Radioimmunoassay of crude LP. To each 12 × 75-mm polystyrene tube 1 ml of the anti-LP immunoadsorbant (equivalent to 1 mg dried weight), 100 μl of pooled human AB serum, and 1–100 μl of crude human LP were added. After 24 hr at room temperature, approximately 2000 cpm of ^{125}I-labeled LP was added to each tube; 24 hr later, the percent of bound radioactivity was determined. All samples were in quadruplicate; the means (±SEM) are shown.

Radioimmunoassay. Purified anti-human LP was attached to Sepharose 4B and suspended in phosphate-buffered saline with 0.1% casein (Hammersten). A source of human LP made from monocytes was assayed in rabbits, and increasing amounts of this LP preparation were added to the immunoadsorbant. After 24 hr at room temperature, ^{125}I-labeled LP (stored in 0.1% casein) was added to each tube; after an additional 24 hr at room temperature, these were centrifuged at 1000 × *g* and washed once in H_2O. Radioactivity in the supernate, wash, and pellet was determined with a gamma counter, and the percent bound per total counts added was plotted against the pyrogenicity of LP in rabbits. Fig. 6 depicts a significant inhibition of the binding of ^{125}I-labeled LP by less than 0.1 of a rabbit pyrogenic dose. The presence of normal human serum in these assays had no effect on the binding of labeled LP or on inhibition of binding by unlabeled LP in several experiments.

DISCUSSION

The association of fever and a circulating endogenous or leukocytic pyrogen has been well described in experimental animals (1). There have been reports in which the rabbit or human bioassay has been adapted to study human LP during clinical fevers (4, 11); however, because of limitations of a bioassay, our knowledge of this molecule during fever has remained speculative. The development of a radioimmunoassay for human LP should provide answers to some perplexing problems concerning the pathogenesis of fever in humans.

In a previous study we reported the difficulties encountered in the purification of human LP obtained from peripheral neutrophils (7). The purification of human LP in the present study was aided by the following: (*i*) the use of blood monocytes to produce LP *in vitro* since these cells release 20–40 times more LP per cell than neutrophils (6); (*ii*) the quantity of contaminating protein per rabbit pyrogenic dose is less in supernates from monocytes than from neutrophils; (*iii*) the use of a purified anti-LP immunoadsorbant, which provided us with greater recovery and purity of LP than previously described; and (*iv*) the use of a ^{125}I-labeling technique, which did not destroy biologic activity and which allowed us to identify LP during further purification techniques. Since the labeling of protein with ^{125}I-labeled esters of hydroxysuccinimide is not dependent on the presence of tyrosine, but rather on the terminal and free amino groups (10), we have assumed that all protein is labeled by this method. Thus, during the later purification procedures, we were confident that radioactivity associated with biologic activity indicated the presence of LP and radioactivity not associated with biologic activity was contaminating protein. The low recovery of biologic activity (1%) is not uncommon during multiple purification procedures, but fortunately the amount of radiolabeled LP needed in the radioimmunoassay is far less than that necessary to produce fever in rabbits.

In this report the quantity of LP is expressed in rabbit pyrogenic doses. We are currently investigating the quantity of protein necessary to produce a rabbit pyrogenic dose. Nevertheless, the potential superiority of the radioimmunoassay over the bioassay is shown in this paper by the ability to detect less than 0.1 of a rabbit pyrogen dose in crude human LP. Thus, the radioimmunoassay should be able to replace the rabbit bioassay as a measure of human LP produced in the laboratory. Furthermore, since human serum did not interfere with the *in vitro* immunoassay, clinical studies now are possible.

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