Comparable Endotoxic Properties of Lipopolysaccharides Are Manifest in Diverse Clinical Isolates of Gram-Negative Bacteria

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In general there is a poor correlation between serum lipopolysaccharide (LPS; the biologically active constituent of endotoxin) levels and mortality in septic patients. The objective of this study was to determine if chemical, structural, or biological differences among LPS from different clinical isolates of gram-negative bacteria might explain this discrepancy. LPS preparations were made using the hot phenol-water extraction method from eight clinical isolates of gram-negative bacteria. As a percentage of the total weight of the LPS, the phosphate content ranged from 3.0 to 13.8% (average, 6.7 ± 3.6%), and the 2-keto-3-deoxyoctonate content ranged from 1.9 to 27.4% (average, 8.9 ± 8.5%). These values were not dissimilar to those obtained for a reference endotoxin. In a standard measure of LPS activity, the Limulus amoebocyte lysate assay, there was an approximately twofold difference between the least and most active preparations. The two preparations with the greatest difference in their ability to elicit the secretion of tumor necrosis factor alpha from a mouse peritoneal macrophage cell line were similar in lethality when administered to mice sensitized to the effects of LPS by orally administered galactosamine. These relatively minor differences in LPS activity seem unlikely to explain the generally observed discrepancy between serum endotoxin levels and mortality in patients with gram-negative sepsis.

It is currently estimated that 500,000 cases of sepsis occur in the United States each year. Half of these are caused by gram-negative rods, and septic shock occurs in 50 to 60% of these cases (reviewed in reference 26). The mortality rate remains high despite the use of intensive care units and effective, broad-spectrum antibiotics. Bacterial lipopolysaccharide (LPS; the biologically active constituent of endotoxin) from gram-negative organisms is now well recognized to be a potent microbial toxin that has been postulated to play a critical role in the initiation of the proinflammatory events that contribute to the pathogenesis of this disease. The pathophysiologic mechanism(s) responsible for this illness are thought to result from the noncytotoxic interaction of LPS with host inflammatory mediator cells following its release from the bacterial outer cell membrane, resulting in the production of multiple proinflammatory cytokines, reactive oxygen and nitrogen intermediates, and bioactive lipids. The resultant systemic inflammatory response is thought to lead to multiorgan failure and often death (reviewed in references 14, 26, and 32).

A significant component of the experimental evidence supporting a relationship between endotoxin and sepsis derives from studies in which the administration of purified endotoxic LPS to experimental animals or human volunteers reproduces many of the clinical symptoms seen in patients with gram-negative sepsis. More recently, correlative data from in vitro, ex vivo, and in vivo studies using viable intact gram-negative microorganisms all provide strong indications that the production of cytokines depends, to at least some extent, on the degree to which various antibiotics induce the release of LPS from gram-negative bacteria (3, 5, 6, 15, 29). Furthermore, the amount of LPS released is now recognized to depend, at least in part, on the differential binding of various β-lactam antibiotics to selective penicillin-binding proteins (3, 15). Attempts to correlate circulating levels of LPS and/or cytokine levels with observed mortality in patients with gram-negative sepsis, however, have to date failed to provide an overall convincing case for cause and effect (4, 9, 13, 19, 20, 30). This apparent discrepancy may well be the result of an unappreciated variability in the biological activity and/or chemical structure of the LPS present in clinical bacterial isolates, as well as of differences in its release caused by different kinds of antibiotic treatment. Under such circumstances, it would be virtually impossible to differentiate the pathophysiologic potential of a given endotoxin level detected in the circulation with a projected outcome of a systemic inflammatory episode.

The studies presented here have been designed to test the hypothesis that LPS derived from clinical bacterial isolates is not uniform in its biological potential to induce a proinflammatory response in vitro. This hypothesis contrasts with the generally accepted underlying assumption made in most studies that LPS derived from different microbiological sources is of equal potency in biological systems. There exists at least some evidence to support a diversity of LPS biological activity, based upon observations that LPS activity can vary substantially depending upon both the type of organism and the method by which the LPS is extracted (23), the presence of outer membrane proteins in association with the LPS (22), the specific subunit composition of the LPS (i.e., presence of O-antigen subunits) (33), and the presence of O-antigen carbohydrates that are linked to the LPS (24). To date, these findings have not been extended to the full range of microorganisms usually encountered in clinical gram-negative bacterial sepsis.

An understanding of the range of biological diversity among the LPS of clinical isolates of gram-negative bacterial is clearly a necessary prerequisite to rational interpretation of the results of clinical endotoxin-cytokine assays in septic patients. To ad-
dress these issues, we have undertaken a comprehensive series of studies in which LPS has been extracted and purified from a variety of clinical isolates of gram-negative bacteria. These various LPS have then been compared to one another in a variety of standard in vitro and in vivo assays that have traditionally been utilized to assess LPS biological activity. Qualitative structural analyses were carried out using silver staining after fractionation of LPS subunits using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Biological activity in vitro was assessed by the quantitative *Lamblia* amoebocyte lysate assay and by the mortality of the LPS preparations to initiate the secretion of tumor necrosis factor alpha (TNF-α) from a macrophage-like cell line. The in vivo potency of LPS was assessed using a sensitive mouse model of lethality.

**MATERIALS AND METHODS**

Clinical isolates of gram-negative microorganisms. Eight clinical isolates of gram-negative microorganisms representing four major genera commonly encountered in gram-negative infections (7, 8, 25) were obtained from the clinical pathology laboratory at the University of Kansas Medical Center. These included two isolates each of *Citrobacter diversus*, *Escherichia coli*, and *Serratia marcescens* and one sputum and one urine isolate of *Proteus mirabilis*. All cultures were grown on Trypticase soy broth to late log phase, harvested by centrifugation, and washed several times in sterile saline, and the LPS was extracted as described below. (Three isolates of *Klebsiella* sp. were also selected. LPS preparations from these isolates were analyzed by SDS-PAGE and silver staining as described below, but results of other analyses for the *Klebsiella* LPS preparations are not included here because of a concern about substantial contamination of the *Klebsiella* LPS preparations with capsular polysaccharide.)

**Extraction and purification of LPS.** The standard hot phenol-water extraction method described by Westphal and Ludzertiz (34, 35) was used to extract LPS from approximately 5 to 10 g (wet weight) of bacteria. The late-log-phase bacteria were sedimented by centrifugation and then suspended in pyrogen-free distilled water to a concentration of approximately 10^9 CFU/ml. Bacteria were then dialyzed extensively against double-distilled H_2O at 4°C and lyophilized. Nucleic acids were removed by reconstitution of the LPS-enriched extracts to 10 mg/ml in 0.1 M acetic buffer with 0.02% MgSO_4 and 0.4% chloroform and digestion with RNase (0.4 mg/ml; Sigma Chemical, St. Louis, Mo.) and DNase (20 μg/ml; Sigma) by incubation at 37°C overnight. Contaminating protein was then removed by the addition of protease K (20 μg/ml; Sigma) in 0.1 M Tris (pH 8.0), followed by heating at 60°C for 1 h and then incubation overnight at 37°C. Enzyme-digested LPS samples were then dialyzed extensively against multiple changes of deionized H_2O. The resulting purified LPS was lyophilized, dried over P_2O_5 (Sigma), reconstituted in pyrogen-free distilled water at 1.0 mg/ml, and stored in aliquots at −70°C. LPS from *E. coli* O111:B4 prepared by phenol-water extraction was obtained from List Biological Laboratories, Campbell, Calif. * “Rough” or R-chemotype LPS from *Neisseria meningitidis* 6257 was kindly provided by C. M. Tsai, Food and Drug Administration, Rockville, Md.

**Analytical procedures.** Studies to characterize common chemical constituents of these oligosaccharide-lipid A were carried out by using standard analytical procedures. Phosphate content was determined by the colorimetric method of Ames and Dubin (1); 2-keto-3-deoxyoctonate (KDO) was determined according to the thiobarbituric acid method of Karkhanis et al. (17). Protein contamination was estimated by using a Coomassie Protein Reagent Kit (Pierce, Rockford, Ill.).

**SDS-PAGE and silver staining of LPS.** SDS-PAGE and silver staining were performed as described by Tsai and Frasch (31). A modified Laemmli SDS-PAGE system was used with the Mini-Protean II gel apparatus (Bio-Rad, Hercules, Calif.) and 1.0-mm-thick gels.

**LAL assays.** *Lamblia* amoebocyte lysate (LAL) assays were carried out using a chromogenic assay (the QCL 1000 Kit purchased from BioWhittaker, Walkersville, Md.). The kit included vials of standard LPS (*E. coli* O111:B4) containing a defined number of endotoxin units (EU). The standard was reconstituted in pyrogen-free distilled H_2O. A standard curve ranging from 0 to 1.0 EU/ml was constructed for each assay by plotting the optical density at 410 nm (OD_410) versus EU per milliliter. Sample LPS preparations were assayed over a range of 0.01 to 0.1 mg/ml and concentration was plotted against OD_410. From the standard curve, the OD_410 corresponding to 0.5 EU/ml was determined. This value was substituted into the formula for the line describing the sample LPS to determine the concentration (in nanograms per milliliter) of sample LPS corresponding to 0.5 EU/ml. The EU nanogram values for each LPS were calculated from these data. Each LPS was assayed three times. Standards and samples were analyzed in duplicate, and absorption was measured at 410 nm by using a Dynatech 5000 microplate reader.

**Secretion of TNF-α from LPS-stimulated mouse macrophages.** J774A.1 cells, a mouse macrophage cell-like line (American Type Culture Collection, Rockville, Md.) were cultured in canted neck flasks in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 4 mM glutamine (Sigma), penicillin (100 U/ml; Sigma), and streptomycin (100 μg/ml; Sigma) and 10% heat-inactivated (50°C, 30 min) fetal bovine serum (endotoxin content of <0.06 ng/ml; Sigma) in a humidified incubator at 37°C with 5% CO_2. Adherent cells were removed with a rubber policeman and used to make a suspension of 10^6/ml in RPMI. Then, 0.5-ml aliquots were distributed to the wells of a 48-well tissue culture plate and allowed to adhere overnight. The cells were then stimulated with the purified LPS from the various clinical isolates or the standard *E. coli* O111:B4 LPS (range, 0.5 to 20 ng/ml) for 15 h. Mouse TNF-α was measured in the supernatants by enzyme immunoassay (Genzyme, Cambridge, Mass.).

**RESULTS**

**Extraction and chemical characterization of LPS from clinical isolates of gram-negative organisms.** LPS was extracted from eight clinical isolates of gram-negative bacteria using the standard hot phenol method of Westphal et al. (34, 35). Yields of between 10 and 20 mg of LPS were obtained from about 5 g (wet weight) of bacterial pellets. Commercially prepared LPS from *E. coli* O111:B4 was included in these studies as a reference standard.

To assess the purity of the LPS preparations, experiments were carried out to detect contaminating nucleic acids and protein. Protein contamination was <2% for all but two of the LPS preparations (Table 1). A determination of the UV spectrum of each LPS preparation indicated the lack of a significant absorption peak at 260 nm, a finding consistent with the LPS preparations at 1.0 mg/ml having less than 1% nucleic acid by weight. Thus, we conclude that the LPS preparations were relatively pure.

Our initial efforts were designed to determine the relative amounts of common chemical constituents usually found in the inner core and lipid A region of the LPS macromolecule. Phosphate is a constituent of lipid A (substituting the 1 and 4 positions of the diglucosamine backbone). It is also associated with phosphate serves as a measure of the relative amounts of common chemical constituents usually found in the inner core and lipid A region of the LPS macromolecule. Phosphate is a constituent of lipid A (substituting the 1 and 4 positions of the diglucosamine backbone). It is also associated with the nature of lipid A.

- **TABLE 1. Phosphate, KDO, and protein contents of various LPS preparations**

<table>
<thead>
<tr>
<th>LPS prep</th>
<th>% Phosphate</th>
<th>% KDO</th>
<th>% Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. diversus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10.4 (0.6)</td>
<td>27.4 (1.7)</td>
<td>&lt;2.1</td>
</tr>
<tr>
<td>2</td>
<td>8.4 (0.1)</td>
<td>7.6 (2.4)</td>
<td>&lt;2.1</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O111</td>
<td>3.0 (1.4)</td>
<td>19.0 (2.0)</td>
<td>&lt;2.1</td>
</tr>
<tr>
<td>1</td>
<td>6.1 (2.7)</td>
<td>4.5 (0.14)</td>
<td>&lt;2.1</td>
</tr>
<tr>
<td>2</td>
<td>13.8 (0.7)</td>
<td>5.6 (2.4)</td>
<td>&lt;2.1</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.9 (0.18)</td>
<td>4.6 (0.44)</td>
<td>6.2</td>
</tr>
<tr>
<td>2</td>
<td>4.7 (0.78)</td>
<td>4.2 (0.59)</td>
<td>3.5</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.2 (1.3)</td>
<td>1.9 (0.6)</td>
<td>&lt;2.1</td>
</tr>
<tr>
<td>2</td>
<td>4.0 (0.14)</td>
<td>5.7 (0.4)</td>
<td>&lt;2.1</td>
</tr>
</tbody>
</table>

* Values in parentheses represent the standard deviations.
molybdate assay, and KDO was quantitated by measuring thio-
barbituric acid as described in Materials and Methods. 

Characterization of LPS subunit composition. It is well rec-
nized that purified LPS from most strains of gram-negative 
bacteria is heterogeneous with respect to the composition of 
individual LPS subunits that comprise the LPS macromolecule. 
Evidence for heterogeneity is readily detected by analysis by 
using SDS-PAGE, followed by silver staining to detect individu-

Activity of LPS preparations in the LAL assay. The LAL 
assay is recognized as one of the most sensitive detection 
systems for determining the presence of LPS, and it is gener-
ally accepted as the method of choice for determining the 
protein concentration of LPS in parenterally administered drugs and of 
endotoxin in body fluids. Since this assay has been used to 
establish correlations with outcome in cases of sepsis, it was 
fee to be important to use this measure of activity to assess the 
relative potency of the different LPS preparations.

The LAL activity of the various sample LPS preparations 
was determined in chromogenic LAL assays in comparison to 
a known standard. The standard LPS was provided by the LAL 
supplier and consisted of vials of E. coli O111:B4 LPS contain-
ing a defined number of EU. Sample LPS preparations were 
compared to the standard curve obtained with LPS from E. coli 
O111:B4, and the data are expressed in EU per nanograms of 

Determination of LD_{50} values. In earlier published experi-
ments to assess the lethal effects of LPS in mice, Galatos et al. 
(111) have shown that, in contrast to LPS alone, simultaneous 
intraperitoneal injection of d-galactosamine with LPS renders 
the mice up to 10,000-fold more sensitive to LPS. This effect is 
mediated by an increased sensitivity of the mouse to TNF- 
a elaborated by LPS-stimulated host inflammatory cells in vivo 
(10). Therefore, this is a useful model for examining the rela-
tionship between the in vitro results of LPS-induced TNF- 
alpha from J774A.1 cell line, and we compared these results to their potency in the 
LAL assay (Table 2). As shown by the data in this table, there 
is clearly a large variability in the amount of TNF-alpha produced 
by the different LPS preparations. Of the five LPS preparations 
assayed for their ability to stimulate the release of TNF-alpha, 
E. coli 2 and S. marcescens 1 had the least activity in the LAL 
assays. However, E. coli 2 was the most effective at releasing 
TNF-alpha from J774 cells, and S. marcescens 1 was the least 
effective. Therefore, we conclude that the ability of an individu-
al LPS preparation to induce the secretion of TNF-alpha from 
J774A.1 cells does not necessarily correlate to a precise 1:1 
ratio with its potency in the LAL assay.

Properties of LPS from Clinical Isolates

FIG. 1. Silver stain of LPS fractionated over SDS-PAGE. Lane 1 is blank, 
and lanes 2 to 13 contain 3 μg of LPS. Lane 2, E. coli 1; lane 3, E. coli 2; lane 4, 
C. diversus 2; lane 5, C. diversus 2 prepared on a separate occasion; lane 6, C. 
diversus 1; lane 7, S. marcescens 2; lane 8, S. marcescens 1; lane 9, P. mirabilis 1; 
lane 10, P. mirabilis 2; lanes 11 to 13, LPS from three different isolates of K.

pneumoniae; lane 14, 200 ng of “rough” (R) chromotype LPS from N. meningitidis 
from strain 6275; lane 15, 3 μg of LPS from E. coli O111:B4.
a standard of comparison, LPS from *E. coli* O111B:4 was determined to have an LD<sub>50</sub> value of 11. *E. coli* 2, with the greatest TNF-α inducing ability, had an LD<sub>50</sub> value of 12, while *S. marcescens* 1, with the least TNF-α inducing ability, had an LD<sub>50</sub> value of 17. The LD<sub>50</sub> values for LPS from *E. coli* 2 and *P. mirabilis* 2 (with, respectively, the least and greatest LAL activity of the five LPS preparations assayed for TNF-α inducing ability) were 12 and 15. These are considered minor differences for this assay. In this small sample, LPS preparations with differing activity in the TNF-α producing and LAL assays have virtually identical toxic effects in mice sensitized to LPS by D-galactosamine.

**DISCUSSION**

In order to investigate the possibility that differences in the inflammatory properties of LPS from gram-negative bacteria might account for the often observed discrepancy between detected levels of serum LPS in patients with sepsis and clinical outcome, we have tested the hypothesis that LPS from diverse clinical isolates of gram-negative bacteria differ significantly in several standard measures of LPS activity and LPS-induced inflammation. Our results show that there is little evidence to suggest that there are substantial differences among such LPS preparations when extracted from clinical isolates of bacteria using standard chemical methods.

Specifically, we were unable to detect large differences in the activity of the LPS preparations in chromogenic *Limulus* amoebocyte assays, a standard measure for detecting the presence of LPS in serum. Moreover, the differences determined in the ability of these preparations to elicit the release of TNF-α, thought to be a key mediator of inflammation, from macrophage-like cells did not correlate with the in vivo model of lethality in which mice are sensitized to TNF-α produced in response to LPS. Because the differences among the LPS preparations in the biological parameters studied in these experiments are small, they are not useful in understanding the apparent discrepancy between serum endotoxin levels and mortality.

The modified hot phenol-water extraction method of Lud-
eritz and Westphal (34) worked well in purifying LPS from the clinical isolates as demonstrated by the low level of protein and nucleic acid contamination. In general, the phosphate and KDO contents of the LPS preparations were within the expected range, based on a comparison to other LPS. The silver-staining patterns of the LPS preparations demonstrated the typical ladder-like pattern characteristic of S-LPS. Thus, these preparations were suitable for the studies performed here. However, as a percentage of the total weight, two LPS preparations had KDO compositions that were substantially higher than the rest: C. diversus 1 and the standard LPS, E. coli O111:B4 (27.4 and 19.0% compared to a mean of 4.9% for the other six LPS preparations). The reasons for and significance of this finding are not clear. However, it is probable that other deoxy sugars present in the LPS were not adequately corrected for in the chemical analysis; nevertheless, since neither of the LPS preparations with a relatively high KDO content was characterized by especially high or low activity in any of the studies done here, we have not placed more significance upon this finding.

As pointed out above, it is generally accepted that the LAL assay is one of the most sensitive methods for detecting the presence of LPS, and it has therefore been the primary means for detection of LPS in plasma from patients with sepsis. However, it is not known if LPS activity in this assay varies among pathogenic strains of gram-negative bacteria isolated from clinical specimens or if activity in this assay correlates with the ability of LPS to induce inflammation or cause death. Within the limits of reproducibility of the assay, we were unable to detect a measurable difference in LAL activity among the eight LPS preparations studied.

TNF-α is considered a leading mediator of the septic response induced by gram-negative organisms during infection. Therefore, as a measure of their in vitro inflammatory potential, we chose to compare the ability of different LPS preparations to induce the release of TNF-α from a mouse macrophage-like cell line. We did not identify a strong positive 1:1 correlation between activity of individual LPS preparations in the LAL assay and the in vitro TNF-α induction studies. For example, P. mirabilis 2 had twice the activity of E. coli 2 in the LAL assay but produced one-third less TNF-α. Subtle relationships between LAL activity and TNF-α inducing ability might be obscured by variations inherent to the LAL assay and TNF-α studies.

CF-1 mice sensitized to LPS with d-galactosamine according to the method of Galanos et al. (10, 11) were used to determine if differences existed in the toxic properties of the different LPS preparations. Of the five LPS preparations tested for TNF-α inducing capacity, those with the largest differences in LAL activity (E. coli 2 versus P. mirabilis 2) manifested only minor differences in LD₅₀. Of these five LPS, the two preparations with the greatest difference in TNF-α inducing ability (E. coli 2 and S. marcescens 1) also demonstrated minor differences compared to each other. Therefore, we were unable to detect meaningful differences in lethality among the LPS preparations with the greatest disparity in both LAL activity and TNF-α inducing ability by using a murine model of LPS lethality. However, these findings are not inconsistent with the results recently published by Amura et al. (2), who reported a lack of correlation between in vivo lethality of LPS and in vitro ability to induce TNF-α production.

Although we were unable to define a difference in the bioactivity or toxicity among the LPS preparations studied in these experiments, it would nevertheless be premature to conclude that differences do not exist among LPS produced by gram-negative bacteria in clinical situations. This is because the LPS prepared for these studies was a chemically extracted and purified preparation virtually devoid of cell-associated membranes, proteins, and nucleic acids. Although this method of preparation has been a powerful tool in the study of LPS, the direct relevance of such preparations to the in vivo setting is not well known. The in vivo release of LPS may occur by a variety of mechanisms, including natural shedding of LPS from “blebs” formed on the surface of bacteria and through the process of bacterial lysis mediated either by humoral factors such as complement and antibody or by exogenous factors such as antibiotics. Indeed, antibiotics that inhibit penicillin-binding protein 3 result in disordered cell wall synthesis, elongation of bacteria into filamentous forms, a substantial increase in cell biomass, and subsequent release of large quantities of LPS from gram-negative bacteria (reviewed in reference 16). The precise composition and structure of antibiotic-released LPS is unknown, but it is clear that relevant questions should include whether other cellular components, such as proteins, lipids, or other cell wall and outer membrane structures might be associated with released LPS and also what role they might play in the inflammatory properties of such LPS preparations. For example, it is well established that isolation of LPS from gram-negative bacteria is often associated with coisolation of microbial outer membrane proteins, usually tightly bound to the LPS molecular complex (21). Further, the demonstrated biological activity of such proteins in stimulating cells of the immune-inflammatory system is well documented (12, 18, 22). Evidence from animal models of infection with gram-negative bacteria indicates that antibiotics which release greater quantities of LPS may result in higher mortality (5). Thus, there would appear to be a number of compelling reasons to explore in more detail the “natural state” of endotoxin generated under simulated situations likely to be operative in clinical sepsis.

Currently, there are few studies comparing the activity of LPS released from bacteria following interaction with antibiotics and LPS chemically extracted using the modified hot phenol-water method. The findings reported here, therefore, demonstrating that there is little variation between purified LPS of clinical isolates from different species, provide a solid foundation upon which such questions can be addressed experimentally. We are actively investigating this question by using the clinical isolates of gram-negative bacteria used for the experiments described here. By incubating these bacteria with different β-lactam antibiotics and isolating the LPS-associated subcellular components released into the growth medium by filtration and centrifugation, we will be able to compare the activity of antibiotic-released LPS with the LPS prepared by hot phenol-water extraction for the experiments reported here.

In summary, we have prepared LPS from clinical isolates of gram-negative bacteria by using the modified hot phenol-water method and characterized them chemically, structurally, and physiologically. There were only small differences among these LPS preparations with respect to their activity in the LAL assay, their ability to elicit the secretions of TNF-α from a macrophage-like cell line, or their toxicity in mice sensitized to the effects of LPS by d-galactosamine. These differences are insufficient to explain the lack of correlation between serum endotoxin levels and mortality in sepsis.

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