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Antigenic Lipopolysaccharide Components of *Legionella pneumophila* Recognized by Monoclonal Antibodies: Possibilities and Limitations for Division of the Species into Serogroups

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Legionella pneumophila **accounts for the majority of cases of Legionnaires' disease. By using rabbit antisera, the species has been divided into 14 numbered and 1 unnumbered serogroups. To recognize the antigenic diversity of the lipopolysaccharide (LPS) responsible for this classification, the Dresden *Legionella* LPS MAb panel, containing 98 monoclonal antibodies (MAbs), was created. Each serogroup reference strain possesses at least one specific epitope not found on any other reference strain and therefore designated the serogroup-specific epitope. When the appropriate MAbs were used for serotyping of 1,064 human and environmental isolates, 1,045 (98%) could be placed into the known serogroups. In most cases (97%), this was in agreement with the polyclonal typing. Of the 29 isolates that showed strong cross-reactivities with the rabbit antiserum panel, 11 could be typed easily by MAbs; for the remaining 18, however, only serogroup-cross-reactive epitopes could be determined. Below the serogroup level, monoclonal subtypes were found for 11 serogroups. Altogether, the Dresden *Legionella* LPS MAb panel was able to divide the 1,064 isolates tested into 64 phenons, indicating its usefulness for both serogrouping and subgrouping of *L. pneumophila* strains. In order to compare the identities of patient and environmental isolates, testing their reactivity with MAbs should be the first step, especially if large numbers of colonies are to be typed. Only in cases of identical patterns are the more time consuming and expensive genetic fingerprints necessary. Moreover, the MAbs can also be used for specific antigen detection in respiratory specimens on the serogroup or subgroup level.**

Legionella pneumophila, first characterized in 1977, is recognized as an important pulmonary pathogen (11). Currently, the genus *Legionella* is known to include 42 species (2) and multiple serogroups. Nevertheless, *L. pneumophila* accounts for the majority of cases of Legionnaires' disease and for most of the legionellae isolated from water systems. By using rabbit antisera, *L. pneumophila* can be classified into 14 numbered serogroups and the unnumbered serogroup Lansing 3 (3). The serogroup specificity of *L. pneumophila* is related to its lipopolysaccharide (LPS) characteristics (7). Since many of the serogroups have antigens in common, cross-reacting antibodies should be removed from the polyclonal immune sera by absorption with bacterial suspensions of heterologous serogroups. Nevertheless, the success obtained depends on the individual immune serum, and sometimes the reproducibility is not satisfactory, particularly in comparative studies between different laboratories using different lots of antisera. Monoclonal antibodies (MAbs) therefore have advantages in serotyping assays, especially for subdividing *L. pneumophila* serogroup 1 strains (9). Brindle et al. (4) introduced the classification of *L. pneumophila* by using a panel of 12 MAbs. These MAbs were able to divide 468 isolates into 17 phenons, in the majority of cases agreeing with serogroup divisions. Up to now, a representative study of the antigenic LPS components defining the division into serogroups has not been presented.

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The purpose of this study was to discover MAbs that could be designated serogroup specific because the epitopes they recognized were located on the LPS of only the corresponding reference strains. In addition, MAbs against serogroup-cross-reactive epitopes, which can cause confusion in serotyping by rabbit immune sera, and MAbs for recognizing antigenic differences within serogroups other than the known serogroups 1 and 6 (9, 10) were sought. The Dresden *Legionella* LPS MAb panel, containing 98 MAbs to *L. pneumophila*, was used to recognize the antigenic heterogeneity within 1,064 strains of *L. pneumophila* isolated from patients and water systems.

(The monoclonal antibodies used in this study will be made commercially available. Until that time, researchers interested in performing similar tests may request the reagents from us.)

MATERIALS AND METHODS

Legionella **strains.** *L. pneumophila* type strains were obtained from the American Type Culture Collection (ATCC) or the National Collection of Type Cultures (Table 1). Clinical and water isolates were characterized as being *L. pneumophila* on the basis of their ability to grow on buffered charcoal-yeast extract agar supplemented with 1% alpha-ketoglutarate (BCYE agar) and their reactivity with a species-specific fluorescein isothiocyanate-labeled MAb (Fresenius, Oberursel, Germany). *Legionella* strains were grown on BCYE agar for 48 to 72 h at 37°C with 2.5% carbon dioxide. Organisms were harvested from the plates with a glass rod and suspended in phosphate-buffered saline (PBS). The concentrations of the bacteria were measured by nephelometry with the McFarland standard as a reference. A total of 165 clinical and 899 environmental isolates were examined. About 70% of the isolates came from Germany; others were from Austria, Denmark, the Czech Republic, England, France, Italy, Scotland, Slovakia, Spain, and Sweden.

MAbs. BALB/c mice were immunized by the method of Cianfriglia et al. (6) with the living *L. pneumophila* reference strains listed in Table 1. In addition, three environmental isolates (16453-92, 10-1, and R10) not typeable by our

TABLE 1. MAbs selected for recognition of serogroup-specific and serogroup-cross-reactive epitopes of *L. pneumophila* by ELISA and IIFT

Serogroup	Strain	Reference	MAb(s) recognizing serogroup-specific epitope with strong reaction[a] (isotype)	Reactivity[a] of MAbs recognizing serogroup-cross-reactive epitopes (isotype)													No. of cross-reactive epitopes
				32/3 (G1)	5/4 (M)	7/3 (G3)	9/1 (M)	13/2 (M)	18/2 (M)	40/1 (G3)	42/4 (M)	52/1 (G3)	52/7 (G3)	54/1 (G3)	54/2 (G2b)	54/3 (G2a)	
1	Philadelphia 1[b]	ATCC 33152	8/5 (IgM)	o	o	o	o[e]	o	o	o	o	o	o	o	o	o	0[f]
2	Togus 1	ATCC 33154	7/1 (IgG3)	++	++	o	o	o	o	o	o	o	o	o	+	o	3
3	Bloomington 1	ATCC 33155	4/7 (IgG3)	++	++	o	o	o	o	o	o	o	++	o	o	o	3
4	Los Angeles 1	ATCC 33156	7/4[c] (IgG1)	++	o	o	o	++	++	o	++	++	o	o	+	o	5
	Portland 1	4	40/4[d] (IgG3)	++	o	o	o	o	++	++	++	o	++	o	o	o	5
5	Cambridge 1	NCTC 11191	27/1[d] (IgG3)	++	o	o	o	o	++	+	+	o	++	o	o	o	5
	Dallas 1E	ATCC 33216	5/2[c] (IgG3)	+	o	++	o	++	o	o	o	o	o	++	o	++	5
6	Chicago 2	ATCC 33215	9/2 (IgG2b)	++	o	o	++	o	o	o	o	o	o	o	+	o	3
7	Chicago 8	ATCC 33823	6/3 (IgM)	o	o	o	o	o	o	o	o	o	o	o	o	o	0
8	Concord 3	ATCC 35096	13/1 (IgG3)	+	o	++	++	o	o	o	++	++	o	++	+	++	8
9	IN-23-G1C2	ATCC 35289	37/2 (IgG3)	++	o	o	o	++	++	o	o	o	+	o	o	o	4
10	Leiden 1	ATCC 43283	17/2 (IgG3)	++	o	++	o	++	o	o	+	++	o	++	+	o	5
11	797-PA-H	ATCC 43130	32/1 (IgG2b)	o	o	o	++	o	o	o	o	o	o	o	o	o	1
12	570-CO-H	ATCC 43290	33/3 (IgG3)	++	o	o	o	o	o	o	o	o	o	o	++	o	2
13	82-A-3105	ATCC 43736	41/3 (IgG2a)	o	o	++	+	++	o	o	o	o	o	o	o	o	2
14	1169-MN-H	ATCC 43703	42/3 (IgG3)	++	o	++	o	o	o	o	++	++	o	o	++	o	5
Lansing 3	Lansing 3	ATCC 35251	51/1 (IgG3)	++	o	o	o	o	++	++	o	o	++	o	o	o	4

[a] ++, strong reaction ($OD_{492} \geq 0.5$ and good or bright fluorescence by ELISA and IIFT, respectively); +, weak reaction ($0.2 < OD_{492} < 0.5$ and weak fluorescence by ELISA and IIFT, respectively); o, no reactivity.
[b] The same reaction pattern was obtained for Allentown 1 (ATCC 43016), Benidorm 030E (ATCC 43108), Knoxville 1 (ATCC 33153), France 5811 (ATCC 43112), OLDA (ATCC 43109), Oxford 4032E (ATCC 43110), Heysham 1 (ATCC 43107), Camperdown 1 (ATCC 43113), and Bellingham 1 (ATCC 43111).
[c] MAbs 7/4 and 5/2 do not recognize the strain Portland 1 (serogroup 4) and the NCTC serogroup 5 reference strain, respectively.
[d] MAb shows only weak reaction with the ATCC serogroup reference strain.
[e] Positive results were obtained for the serogroup 1 strains Benidorm 1E and Knoxville 1.
[f] Benidorm 1E and Knoxville 1 have an epitope in common with Chicago 2, Concord 3, 797-PA-H, and 82-A-3105.

MAbs or by polyclonal antisera were used for immunization. Cellular fusions were carried out with P3X63-Ag8/653 mouse myeloma cells as described elsewhere (12). Supernatants of hybridoma cultures were screened for specific antibodies by enzyme-linked immunosorbent assay (ELISA). In cases of positive reactions, hybridoma cells were subcloned three times. MAb 3, described by Joly et al. (9), was obtained from the ATCC (1767-CRL). Isotype determination of MAbs was done with ImmunoType (Sigma, St. Louis, Mo.). The concentration of mouse immunoglobulin G (IgG) in culture supernatants used for ELISA was determined by anti-mouse IgG ELISA (Boehringer, Mannheim, Germany).

ELISA. Immuno-Plates (Greiner, Frickenhausen, Germany) were coated with heat-killed (10 min, 95°C) bacteria (McFarland 1, corresponding to approximately 3×10^8 cells per ml) in PBS overnight at 4°C. In order to prove that the epitopes recognized were LPS components, bacteria were sonicated and treated with proteinase K (2 μg/ml; 60 min at 60°C followed by 15 min at 95°C) and were also used for coating. After being washed three times with PBS containing 0.05% Tween 20 (PBS-T), the wells were blocked with PBS-T containing 10% fetal calf serum (PBS-T-FCS) for 1 h at 37°C. The plates were washed once, and then MAbs were added as a culture supernatant diluted in PBS-T-FCS, giving a concentration of approximately 2 μg of IgG/ml. A dilution of 1:20 was used for MAbs belonging to the isotype IgM. After incubation for 90 min at 37°C and three washes, the bound MAbs were detected by anti-mouse (polyvalent)-horseradish peroxidase (Sigma) diluted in PBS-T-FCS. The plates were incubated for 90 min at 37°C and washed three times. Then the substrate solution containing *o*-phenylenediamine (1 mg/ml) and H_2O_2 (0.003%) in 0.1 M phosphate-citrate buffer (pH 5.0) was added. The reaction was stopped by 2 M sulfuric acid after 30 min. Optical densities at 492 nm (OD_{492}) of blanks (either without antigen for coating or without MAb) did not exceed 0.075. The strengths of reactivity were described semiquantitatively as "++" ($OD_{492} \geq 0.5$) and "+" ($0.2 < OD_{492} < 0.5$).

IIFT. *Legionella* bacteria were scraped from BCYE agar; fixed in 1% formalin overnight; resuspended in 0.5% normal yolk sac-PBS, giving a concentration of about 3×10^8; and employed for indirect immunofluorescence testing (IIFT) (15). The MAbs were used as undiluted cell culture supernatants. Goat anti-mouse fluorescein isothiocyanate conjugate (polyvalent) was obtained from the Institut für Immunpräparate und Nährmedien, Berlin, Germany.

Serotyping with polyclonal antibodies. Serological typing was carried out with absorbed *Legionella* antisera by IIFT. The sera were prepared by immunization of rabbits with the ATCC type strains of *L. pneumophila* serogroups 1 to 14 and strain Lansing 3 according to the method of Cherry and McKinney (5). A pool of heterologous serogroups of *Legionella* bacteria was used for absorption of the rabbit antisera. The rabbit antisera were defined as serogroup specific when IIFT produced bright fluorescence with the homologous antigen and no fluorescence with heterologous ones. Isolates were classified as belonging to a given serogroup if they reacted strongly with one serogroup-specific absorbed antiserum. If they reacted against multiple antisera, strains were designated as cross-reactive.

RESULTS

Recognition of epitopes specific for serogroup reference strains. Twenty-three fusion experiments were carried out to produce MAbs to *L. pneumophila* serogroup reference strains. A total of 81 clones were obtained. MAbs were tested by both ELISA and IIFT. In comparing the two methods, no significant differences in epitope recognition were seen. Good or bright fluorescence corresponded to an ELISA OD_{492} of ≥0.5, and an OD_{492} of 0.2 to 0.5 was obtained in cases of weak fluorescence. All of the epitopes recognized by these MAbs were components of the LPS as demonstrated by ELISA reactivity against pronase-treated antigens. A panel of the MAbs was selected that recognized only the homologous serogroup type strains 1 to 14 and Lansing 3 (Table 1). The corresponding epitopes were described as serogroup specific. Because serogroups 4 and 5 are known to be heterogeneous, two different strains of these serogroups (serogroup 4, Los Angeles 1 and Portland 1; serogroup 5, Dallas 1E and Cambridge 1) were used for the immunization of mice. No major common LPS epitope was found in the two serogroups. The major antigenic components of serogroup 4 strain Los Angeles 1 (MAb 7/4) or serogroup 5 strain Dallas 1E (MAb 5/2) were not present on Portland 1 (serogroup 4) and Cambridge 1 (serogroup 5), respectively. On the other hand, MAbs 40/4 and 27/1 reacted very strongly with strains Portland 1 and Cambridge 1, respectively, but only weakly with the other reference strain of the same serogroup (Table 1).

TABLE 2. Serogrouping of 1,064 *L. pneumophila* isolates by serogroup-specific MAbs

Serogroup	MAb	No. of isolates	Correlation with typing by rabbit antisera[a]
1	8/5	419	Yes
2	7/1	39	Yes
3	4/7	95	Yes
4	7/4, 40/4	44	Yes
5	5/2, 27/1	48	Yes
6	9/2	213	Yes
7	6/3	8	Yes
8	13/1	14	3
9	37/2	15	Yes
10	17/2	80	A
11	32/1	5	Yes
12	33/3	35	2
13	41/3	8	Yes
14	42/3	21	4
Lansing 3	51/1	1	Yes
None by MAbs		19	B

[a] Yes, complete correlation; A, MAb recognized 23 isolates which appeared to belong to serogroups 4 (1 strain), 5 (17 strains), 8 (4 strains), or 9 (1 strain) by polyclonal typing and 2 strains which could not be typed definitively; B, 1 isolate was typed as serogroup 13 by rabbit antisera. Numerals indicate the number of isolates recognized by the MAb which could not be typed definitively by rabbit antisera.

MAbs recognizing epitopes common to several serogroup reference strains. Thirty-four of the 98 MAbs raised against the LPS of *L. pneumophila* recognized two or more serogroups. The reactivity patterns of the serogroup reference strains indicated that the LPS of *L. pneumophila* possessed at least 13 different serogroup-cross-reactive epitopes (Table 1). Only one cross-reactive epitope or none was found for serogroups 1, 7, and 11, whereas the reference strains of serogroups 4, 5, 8, 10, and 14 carried five or more different epitopes in common with other serogroups (Table 1).

Assignment of *L. pneumophila* isolates to serogroups by MAbs. A total of 1,064 isolates were included in this study (Table 2). All of the isolates assigned to serogroups 1 to 7, 9, 11, 13, and Lansing 3 by using the serogroup-specific MAbs listed in Table 2 were classified by rabbit sera to the same serogroup (Table 2). In contrast, MAb 17/2, which is specific for serogroup 10, also recognized 23 isolates that exhibited stronger reactivity with rabbit antisera to either serogroup 4, 5, 8, or 9 than to serogroup 10. Table 2 also contains 29 isolates that showed cross-reactivity to serogroups 4, 5, 8, 10, and 14 with rabbit antisera, which made it impossible to assign them to definitive serogroups by using those reagents. With the serogroup-specific MAbs listed in Table 1, recognition of serogroup-specific epitopes was attained for 11 of the 29 isolates. No single serogroup classification by MAbs, however, was obtained for the remaining 18 isolates (see below) or for one strain typed by rabbit antisera as serogroup 13.

Isolates lacking a typical serogroup-specific epitope. For typing of the 19 isolates not recognized by serogroup-specific MAbs, reactivity with serogroup-cross-reactive MAbs was evaluated. Of the 13 serogroup-cross-reactive MAbs listed in Table 1, two (MAbs 32/3 and 54/2) recognized all 19 isolates. Five other MAbs were necessary to classify them into 10 different reactivity patterns (Table 3). To see if there were additional major epitopes not located on the LPS of the reference strains (Table 1), three isolates exhibiting different reactivity patterns were used as immunogens for the production of MAbs. Of the 13 MAbs produced in three cellular fusion experiments, 11

TABLE 3. LPS phenons of *L. pneumophila* recognized by MAbs

Serogroup	No. of phenons	No. of isolates	MAb(s) needed for recognition of:	
			Serogroup-specific epitope	Monoclonal subgroup
1	15	419	8/5	3/1, 8/4, 9/1, 10/6, 12/2, 20/1, 26/1, 26/2, 30/4, 3[a]
2	2	39	7/1	5/4
3	4	95	4/7	5/4, 52/7, 29/3
4	5	44	7/4, 40/4	13/2, 9/1, 39/3
5	6	48	27/1, 5/2	7/3, 18/2, 54/5
6	5	213	9/2	4/5, 18/2, 54/2
7	2	8	6/3	39/3
8	3	14	13/1	6/1, 13/2
9	1	15	37/2	
10	2	80	17/2	6/1
11	1	5	32/1	
12	1	35	33/3	
13	3	8	41/3	7/3, 9/1
14	3	21	42/3	7/3, 54/3
Lansing 3	1	1	51/1	
None by MAbs	10	19		13/2, 32/3, 40/1, 40/6, 52/7, 54/2, 54/3
Total	64	1,064	17[b]	26[b]

[a] MAb 3, described by Joly et al. (9), was obtained from ATCC.
[b] Total number of MAbs.

recognized epitopes of at least two serogroup type strains but two (MAbs 52/4 and 52/2) reacted only with the isolate used for immunization.

Recognition of epitopes to discriminate between monoclonal subgroups below the serogroup level. The Dresden MAb panel contained nine MAbs that recognized different epitopes of serogroup 1 strains. Together with MAb 3, obtained from the ATCC, they allowed discrimination into 15 subgroups. With the exceptions of serogroups 9, 11, 12, and Lansing 3, monoclonal subgroups were obtained for all the serogroups (Table 3).

Antigenic diversity of *L. pneumophila* LPS recognized by MAbs. The antigenic diversity of the *L. pneumophila* LPS is summarized in Table 3. Seventeen MAbs were needed to recognize the serogroup-specific epitopes, and another 26 MAbs were needed for the recognition of monoclonal subgroups. With this panel, the 1,064 isolates in this study could be divided into 64 LPS phenons.

DISCUSSION

In recent years, genetic methods for typing legionellae have become epidemiologically important. Nevertheless, genotyping below the species level is time consuming and often the results are not easy to interpret. In the case of *L. pneumophila*, which accounts for the majority of cases of Legionnaires' disease (13), assignment to serogroups is still the predominant method used. Because of problems of cross-reactivity and atypical reactions with polyclonal antisera (8, 14), we raised 98 MAbs that recognize *L. pneumophila* LPS epitopes. Two or more MAbs were found for many of the epitopes recognized. It can be assumed, therefore, that all the major antigenic LPS components of *L. pneumophila* which are able to trigger an immune response in the murine system were recognized. Both IIFT and ELISA can be used for serotyping with our MAbs. In the laboratory praxis, the ELISA technique used in this study was more reproducible, especially for the recognition of both serogroup-cross-reactive and subgroup-specific MAbs.

MAbs specifically recognizing the serogroup reference strains are listed in Table 1. Finding such serogroup-specific MAbs was uncomplicated for serogroups 1, 2, 3, 6, 7, 9, 11, and 13, indicating the existence of respective major antigenic components. As expected from the difficulty of typing serogroups 4, 5, 8, 10, 12, and 14 by rabbit immune sera, the production of specific MAbs to these serogroups was more difficult. Most of the MAbs produced reacted with several serogroups. Furthermore, no major common serogroup-specific epitopes could be detected for serogroups 4 and 5, for each of which two MAbs are required for recognition.

Altogether, 1,064 *L. pneumophila* isolates were screened for serogroup-specific epitopes by using the MAbs listed in Table 1. A deviation from the polyclonal antiserum typing results was found only in the case of serogroup 10. The epitope recognized by MAb 17/2 was also detected in some strains that typed by rabbit sera as serogroup 4, 5, 8, or 9, whereas the corresponding serogroup-typical epitopes were not found. On the other hand, 11 of 29 isolates that could not be typed by rabbit sera possessed typical serogroup-specific epitopes recognized by our MAbs.

In addition to MAbs which reacted with the reference strains of *L. pneumophila* (Table 1), we produced two MAbs (52/2 and 52/4) against the strain 16453-92, isolated in Spain. MAbs 52/2 and 52/4 recognized an epitope not present on any of the reference strains but present on the immunizing strain and on one other unserogroupable strain isolated in Germany. Studies with rabbit immune sera are indicated to determine whether the antigenic potential of this LPS component is sufficient for the definition of a new serogroup.

Joly et al. (9) developed a standard subgrouping scheme for serogroup 1. Use of this international panel makes it possible to classify serogroup 1 strains into at least 12 subgroups (1). The MAbs presented in this study combined with MAb 3 from the ATCC allow the subgrouping of serogroup 1 into 15 phenons. This discriminative potential of MAbs has not been evaluated against other serogroups; however, the present study showed for the first time that MAbs are able to distinguish subgroups in the majority of serogroups (Table 3). If the MAbs recognizing the serogroup-specific epitopes and their subgroups were used, 39 LPS phenons could be distinguished for serogroups 2 to 14 and Lansing 3. In addition, the 19 unserogroupable strains could be divided into 10 additional phenons by using seven MAbs from our panel. Taken together, the isolates not belonging to serogroup 1 could be divided into 49 LPS phenons. Considering that fewer than 50% of *L. pneumophila* isolates do not belong to serogroup 1, the discriminative power is much higher for these strains than for serogroup 1. Serogroup 6, however, is an exception, as it exhibits only three variants.

The use of MAbs to *L. pneumophila* in routine and reference clinical laboratory work allows specific identification as well as typing both at and below serogroup level. It is not uncommon to isolate different legionellae from a single environmental specimen. Moreover, legionellosis cases due to more than one strain are also documented. Therefore, a sufficient number of colonies on the primary isolation medium must be tested for identification of the causative agent. MAbs provide the most useful simple procedure for screening large numbers of colonies. Only in cases of identical MAb panel patterns should time-consuming and expensive genetic fingerprint methods for confirmation of the identity of patient and environmental isolates be necessary. In our hands, the working time needed for testing 25 isolates with a panel of 25 MAbs by ELISA is about

4 h. By contrast, the pulsed-field gel electrophoresis (PFGE) of cleaved DNA takes about 25 working hours and the results are not available for a week. In our experience, the majority of isolates belonging to different LPS types were shown by PFGE to have different patterns of cleaved DNA. Nevertheless, there are rare cases in which differences in serogrouping were not reflected in differences in PFGE patterns with the enzymes *Sfi*I and *Not*I (data not shown).

Besides the testing of patient isolates, MAbs are suitable for identification of *L. pneumophila* directly in bronchoalveolar lavages, tracheal aspirates, and postmortem lung tissue by IIFT (1). If *L. pneumophila* has been identified by a species-specific MAb, the specimens should be retested by MAbs for rapid typing of the causative agent at least to the serogroup level. Furthermore, if the cultural isolation was not successful, the LPS pattern demonstrated by IIFT is the only evidence for identification of the infection source. Preliminary data obtained in our laboratory showed that serogrouping by our MAbs was successful in all but one case for 18 clinical specimens demonstrated by the commercial species-specific MAb to contain *L. pneumophila.*

Considering these applications of MAbs in routine and clinical laboratory work, it is recommended that the traditional polyclonal testing be replaced by the use of MAbs. MAbs have the advantage of being well defined, and the typing of isolates on the basis of their LPS epitope patterns is highly reproducible.

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