Characterization of *Endotrypanum* Parasites Using Specific Monoclonal Antibodies

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A large number of Endotrypanum *stocks (representing an heterogeneous population of strains) have been screened against a panel of monoclonal antibodies (MAbs) derived for selected species of* Endotrypanum *or* Leishmania, *to see whether this approach could be used to group/differentiate further among these parasites. Using different immunological assay systems, MAbs considered specific for the genus* Endotrypanum *(E-24, CXXX-3G5-F12) or strain M6159 of* E. schaudinni *(E-2, CXIV-3C7-F5) reacted variably according to the test used but in the ELISA or immunofluorescence assay both reacted with all the strains tested. Analyses using these MAbs showed antigenic diversity occurring among the* Endotrypanum *strains, but no qualitative or quantitative reactivity pattern could be consistently related to parasite origin (i.e., host species involved) or geographic area of isolation. Western blot analyses of the parasites showed that these MAbs recognized multiple components. Differences existed either in the epitope density or molecular forms associated with the antigenic determinants and therefore allowed the assignment of the strains to specific antigenic groups. Using immunofluorescence or ELISA assay, clone E-24 produced reaction with* L. equatorensis *(which is a parasite of sloth and rodent), but not with other trypanosomatids examined. Interestingly, the latter parasite and the* Endotrypanum *strains cross-reacted with a number of MAbs that were produced against members of the* L. major-L. tropica *complex.*

Key words: *Endotrypanum* - Kinetoplastida - Trypanosomatidae - mammalian reservoirs - molecular characterization - monoclonal antibodies

Endotrypanum parasites are unique among the Kinetoplastida in that they infect erythrocytes of their mammalian host, the two and three-toed sloths (genera *Choloepus* and *Bradypus*, respectively). Inside the erythrocyte the *Endotrypanum* assumes an epimastigote or trypomastigote form, while in the sandfly and during *in vitro* culture the parasite assumes promastigote morphology (Shaw 1992). Similarities at the morphological, molecular and biological levels exist between many trypanosomatids isolated from sylvatic insect and/or vertebrate reservoir hosts that make the identification of the medical importance parasites demanding (Shaw 1985). Moreover, the neotropical tree sloths, which are reservoir of at least six *Leishmania* species pathogenic for human (review in Grimaldi & Tesh 1993), are hosts of *Endotrypanum* (reviewed in Shaw 1992) and several *Trypanosoma* species [*T. cruzi*, *T. rangeli*, *T. leeuwenhoeki*, *T. preguici* and *T. legeri*] (Montero-Gei 1956, Shaw 1969, Zeledón et al. 1975, Herrer & Christensen 1980, Miles et al. 1983).

Identification of *Endotrypanum* strains usually relies on the examination of promastigotes from cultures, and those forms of the parasite are morphological indistinguishable from *Leishmania* (Croft et al. 1980). More precise taxonomic markers for *Endotrypanum*, however, have resulted from the application of molecular techniques (Greig et al. 1989, Lopes et al. 1990, Fernandes et al. 1993, Medina-Acosta et al. 1994, Franco et al. 1996). Among the new approaches for identifying *Endotrypanum* is serodeme analysis using specific MAbs (Lopes & McMahon-Pratt 1989). In order to extend these observations, here we discuss the results of parasite differentiation of *Endotrypanum* (comprised of an heterogenous population of reference strains and Amazonian isolates), as characterized by their reactivities with MAbs and using distinct assay systems.

This work was supported in part by the Brazilian National Council of Scientific Development and Technology (CNPq) and CAPES/BR.
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Received 9 April 1996
Accepted 21 October 1996

MATERIALS AND METHODS

The parasites - The origin (i.e., host species involved or geographical area of isolation) of the 22 *Endotrypanum* stocks examined in this study, which included reference strains (Medina-Acosta et al. 1994) are given in Table I. The stocks were stored as stabilates at -190°C in liquid nitrogen refrigerators, and the freezing medium used was Schneider's medium containing 10% fetal bovine serum (FBS) and 8% dimethylsulfoxide (Grimaldi et al. 1992).

Preparation of samples - Procedures for growing *Endotrypanum* promastigotes *in vitro* and for preparation of extracts for serodeme analysis with monoclonal antibodies have been reported previously (McMahon-Pratt et al. 1986). Briefly, promastigotes in the log phase of growth in Schneider's medium were harvested by centrifugation (1,500 x g for 10 min at 4°C) and washed twice in phosphate-buffered saline (PBS), pH 7.3. The final pellet was used for preparation of samples. Antigens of each of the strains tested were prepared as promastigote homogenates containing protease inhibitors (Leon et al. 1992). Prior to analysis, the samples were briefly sonicated using a bath sonicator (RAI Research, Model 250 Ultrasonic cleaner) to homogenize the antigens, and then centrifuged (2,000 x g for 10 min, at 4°C). Protein concentration was measured by the protein assay method of Bradford (1976), and all samples were resuspended to the same concentration before analysis.

M*onoclonal antibodies and immunological assay systems* - The MAbs used in this study were produced against membrane components of *Endotrypanum* (Lopes & McMahon-Pratt 1989) or *Leishmania* species (McMahon-Pratt et al. 1986). The following group- or species-specific clones were employed for typing parasites: **E-24**, CXXX-3G5-F12, *Endotrypanum* sp.; **E-2**, CXIV-3C7-F5, *E. schaudinni*; **T-11**, LXIX 2D8-D7, *L. (L.) major*; **T-12**, IS1 2G7-F1, *L. (L.) tropica*; **D-2**, LXXVIII 2E5-A8, *L. donovani* complex; **M-7**, IXVIII 1D7-B8, *L. mexicana* complex; **B-20**, 2 2F7-D3, *Leishmania* (*V.*) sp.; **B-5**, VII-2C5-C5, *L. braziliensis* complex; **B-19**, XLIV-5A2-B9, *L. (V.) guyanensis*; **CR**, G2G4, cross-reactive to all kinetoplastids (Lopes & McMahon-Pratt 1989, WHO 1993, Grimaldi &

TABLE I

Origin and identification of *Endotrypanum* reference strains and 17 isolates from the Amazon Region, Brazil, which were characterized by monoclonal antibodies in this study

Stock code	Designation	Species	Geographic origin	Zymodeme
Reference strains				
E11	MCHO/CR/62/A-9[b]	*E. monterogeii*	Costa Rica	EZ01
E12	MCHO/BR/88/M11602[c]	*E. schaudinni*	Pará	EZ12
E14	MCHO/BR/80/M6159[c]	*E. schaudinni*	Pará	EZ06
E15	MCHO/BR/79/M5725[c]	*E. schaudinni*	Pará	EZ05
E09	MBRA/PA/00/415P01[a]	*Endotrypanum* sp.	Panama	EZ01
Amazonian isolates				
E01	MCHO/BR/89/RO9627[d]	*Endotrypanum* sp.	Rondônia	EZ01
E02	MCHO/BR/89/RO1635[d]	*Endotrypanum* sp.	Rondônia	EZ01
E03	MCHO/BR/89/RO1634[d]	*Endotrypanum* sp.	Rondônia	EZ01
E04	MCHO/BR/89/RO1140[d]	*Endotrypanum* sp.	Rondônia	EZ01
E05	MCHO/BR/89/RO1602[d]	*Endotrypanum* sp.	Rondônia	EZ01
E06	MCHO/BR/89/RO1471[d]	*Endotrypanum* sp.	Rondônia	EZ01
E07	MCHO/BR/89/RO1583[d]	*Endotrypanum* sp.	Rondônia	EZ01
E16	MCHO/BR/85/IM2260[c]	*Endotrypanum* sp.	Pará	EZ07
E17	MCHO/BR/85/IM2382[d]	*Endotrypanum* sp.	Rondônia	EZ09
E18	MCHO/BR/85/IM2384[d]	*Endotrypanum* sp.	Rondônia	EZ01
E19	MCHO/BR/85/IM2389[d]	*Endotrypanum* sp.	Rondônia	EZ10
E21	MCHO/BR/89/IM3605[d]	*Endotrypanum* sp.	Rondônia	EZ04
E22	MCHO/BR/89/IM3606[d]	*Endotrypanum* sp.	Rondônia	EZ03
E31	MCHO/BR/85/IM2259[c]	*Endotrypanum* sp.	Pará	EZ02
E32	MCHO/BR/85/IM2380[d]	*Endotrypanum* sp.	Rondônia	EZ08
E33	MCHO/BR/85/IM2393[d]	*Endotrypanum* sp.	Rondônia	EZ11
E36	MCHO/BR/89/IM3603[d]	*Endotrypanum* sp.	Rondônia	EZ03

Designations: Host [M= Mammalia: BRA= *a: Bradypus infuscatus*; CHO= *Choloepus* sp. (*b*: *C. hoffmanni*; *c*: *C. didactylus*; *d*: *C. juruanus*)] /country of origin/year of isolation/original code. Zymodeme (= strain variant); EZ = *Endotrypanum* zymodeme, classified by enzyme electrophoresis according to their mobility profiles (Franco et al. 1996).

McMahon-Pratt 1996).

Typing of the *Endotrypanum* with MAbs was performed with an indirect immunofluorescence assay (IFAT), using whole fixed promastigotes as antigen (McMahon-Pratt et al. 1986) or by distinct immune binding assay systems such as indirect radioimmune assay (RIA) (Grimaldi et al. 1992), the ELISA technique (Jaffe & McMahon-Pratt 1987), or dot-blot ELISA (Hawkes et al. 1982), using whole parasite lysates as antigen (Grimaldi et al. 1992). Characterization of the molecules associated with the specific antigenic determinants was performed by Western blot analysis (Jaffe & McMahon-Pratt 1983). Briefly, the soluble antigen extracts were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% slab gels in non-reducing conditions, and electrophoretically transferred to nitrocellulose paper (Schleicher and Schuell, Keene, New Hampshire, USA). The techniques have been described in detail previously (Leon et al. 1992).

RESULTS AND DISCUSSION

In this paper, the characterization of representative strains of *Endotrypanum*, based on their reactivity patterns using specific MAbs to *Endotrypanum* (Lopes & McMahon-Pratt 1989) or *Leishmania* (WHO 1993, Grimaldi & McMahon-Pratt 1996) is presented. The genus *Endotrypanum*, analyzed in this study (Table I), may represent an heterogeneous complex of parasite species or strain variants, as classified by numerical zymotaxonomy (Franco et al. 1996).

Reactivity with monoclonal antibodies -Two MAbs (E-2, CXIV-3C7-F5 and E-24, CXXX-3G5-F12) tested in these experiments were produced against membrane-enriched preparations of *Endotrypanum* species. In studies using a dot-blot radioimmune assay, these MAbs appear to be specific for the genus *Endotrypanum*, but monoclonal E-2 reacted exclusively with strain M6159 of *E. schaudinni;* no cross-reaction was observed when *Leishmania* species or other trypanosomatids were examined (Lopes & McMahon-Pratt 1989). In this study, although the two clones reacted variably according to the test used (Fig. 1; Tables II-IV), in the ELISA (Table III) as well as IFAT (Table IV) both reacted with all *Endotrypanum* strains tested.

TABLE II

A comparison of the binding[a] of *Endotrypanum*-specific monoclonal antibodies to reference strains and *Endotrypanum* isolates from the Amazon Region, Brazil

Stock code	Species	Monoclonal antibodies[b] E-2	E-24	CR
Reference strains				
E11	*E. monterogeii*	10.7	18.3	6.5
E12	*E. schaudinni*	10.4	14.6	6.9
E15	*E. schaudinni*	9.1	8.3	6.6
E14	*E. schaudinni*	13.6	3.4	8.4
Amazonian isolates				
E01	*Endotrypanum* sp.	6.4	5.3	6.1
E02	*Endotrypanum* sp.	3.6	6.3	15.0
E03	*Endotrypanum* sp.	7.1	5.5	6.1
E04	*Endotrypanum* sp.	7.6	11.6	8.7
E05	*Endotrypanum* sp.	7.1	3.4	9.7
E06	*Endotrypanum* sp.	4.0	2.3	4.6
E07	*Endotrypanum* sp.	8.3	8.6	8.7
E16	*Endotrypanum* sp.	8.4	9.3	6.6
E17	*Endotrypanum* sp.	14.5	6.5	5.7
E18	*Endotrypanum* sp.	8.0	10.0	6.0
E19	*Endotrypanum* sp.	4.8	2.6	5.8
E21	*Endotrypanum* sp.	10.9	26.4	9.6
E22	*Endotrypanum* sp.	9.0	5.2	10.0
E31	*Endotrypanum* sp.	8.6	6.1	17.1
E32	*Endotrypanum* sp.	3.5	2.8	7.6
E36	*Endotrypanum* sp.	7.2	7.0	9.8

a: results shown express the ratio of cpm bound monoclonal antibodies/cpm bound control; values > 3 were considered positive.

b: the hybridoma clones indicated above the lanes and their specificities are shown in Materials and Methods. CR (clone G2G4) is a cross-reactive monoclonal antibody used as a positive control throught these experiments.

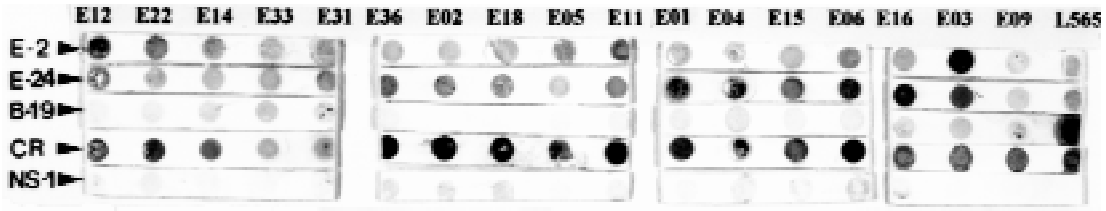

Fig. 1: specificity of monoclonal antibodies, E-2 (CXIV-3C7-F5) and E-24 (CXXX-3G5-F12) for the *Endotrypanum* strains, as shown by dot blot enzyme binding assay. The stock codes of the *Endotrypanum* strains analyzed are indicated above the lanes, and their origins are shown in Table I. Stock L565 is a *Leishmania guyanensis* reference strain (MHOM/BR/75/M4147) and clone B-19 (XLIV-5A2-B9) is considered specific for this parasite species. Clone CR (G2G4) is cross-reactive MAbs and was used as a positive control; NS-1, hybridoma culture supernatant (negative control).

The analysis showed antigenic variation occurring among the *Endotrypanum* strains. Significant differences between the reactive patterns with specific MAbs could be observed among stocks (Tables II-III), but no qualitative or quantitative pattern could be consistently related to parasite origin (i.e., host species involved) or geographical area of isolation. These differences can be related with strain variation in the level of expression of certain antigenic determinants, as recognized by these MAbs. Variation in the sensitivity of the test may also occur due to the type of screening assay used.

IFAT studies were carried out to confirm the specificity and binding of the MAbs to the *Endotrypanum* surface determinants on intact parasites (Table IV). Each clone reacted in a continuous pattern on the surface of the promastigotes. Interestingly, clone E-24 produced the same immunofluorescent pattern of reaction (data not shown) with a parasite of sloth and rodent, *L. equatorensis* (Grimaldi et al. 1992). As noted with *L. colombiensis* (Kreutzer et al. 1991), two strains of *L. equatorensis* cross-reacted with MAbs T1 (clone XLVI-5B8-B3), T2 (XLVI, 4H12-C2), T3 (XLVI-5A5-D4), T4 (LXVIII-1A4-G1) and T8 (LXVII-3E12-F8) (Grimaldi et al. 1992), which were originally produced against parasites in the *L. major/L. tropica* complex (Jaffe & McMahon-Pratt 1983). Moreover, using either IFAT (data not shown) or ELISA assay (Table III) the *Endotrypanum* strains reacted with MAbs specific to *Leishmania*, particularly with clones IS1-2C8-C7 (T-12) and LXIX-2D8-D7 (T-11) which are *L. major*- or *L. tropica*-specific respectively (WHO 1993). Our data confirm a previously reported study (Shaw 1992) showing that a number of *Endotrypanum* strains cross-reacted with these MAbs (Jaffe & McMahon-Pratt 1983) or monoclonal antibody (WIC 79.3) which recognizes lipophosphoglycan components of *L. major* (Handman et al. 1984). Based on this results, it appears that close antigenic links may exist between *Endotrypanum* and *Leishmania*. Work is now in progress to better define the phylogenetic relationship between these parasites. Whatever the explanation for the existence of these cross-reactive epitopes, these results indicate caution should be taken by all researchers working with field isolates.

TABLE III

Results of ELISA test[a], employing monoclonal antibodies produced against *Endotrypanum* or *Leishmania* species, to reference strains and selected *Endotrypanum* isolates from the Amazon Region, Brazil

Stock		Monoclonal antibodies[b]								
code	Species	E-2	E-24	CR	T-12	T-11	D-2	M-7	B-5	B-20
Reference strains										
E11	*E. monterogeii*	2/1	1/1	1/1	2/2					
E12	*E. schaudinni*	3/2	2/2	2/2	1/1	1/2	1/1	1/1	1/1	2/2
E15	*E. schaudinni*	2/2	2/2							
E14	*E. schaudinni*	2/2	2/2	2/2	1/1	1/1	1/1	1/1	1/1	1/1
E09	*Endotrypanum* sp.	1/2		1/	1/2					
Amazonian isolates										
E01	*Endotrypanum* sp.	2/2	2/1	2/2		4/4				
E02	*Endotrypanum* sp.	1/1	1/1	1/1						
E03	*Endotrypanum* sp.	2/2	1/2	1/1						
E04	*Endotrypanum* sp.	2/2	1/1	1/1	1/2					
E06	*Endotrypanum* sp.	2/2	1/1	2/2	1/2					
E07	*Endotrypanum* sp.	1/1	2/3	3/5		7/7				
E16	*Endotrypanum* sp.	1/1	1/1	1/	1/1					
E18	*Endotrypanum* sp.	2/2	1/1	1/1						
E19	*Endotrypanum* sp.	1/1	1/1	1/1						
E21	*Endotrypanum* sp.	1/1	1/1	1/	3/3	1/3				
E22	*Endotrypanum* sp.	1/2	2/1	1/1						
E31	*Endotrypanum* sp.	6/7	3/2	2/2	5/7	1/2	2/2	1/2		
E32	*Endotrypanum* sp.	3/3	2/2	1/1	2/4					
E36	*Endotrypanum* sp.	3/3	1/1	1/	2/2					

a: the tabulated numbers (duplicate tests) correspond to the OD ranges at absorbance 405 nm. Values ³1 were considered positive (all blanks equal negative results).
The range were: 0=0 to 0.130; 1=0.131 to 0.317; 2=0.318 to 0.504; 3=0.505 to 0.691; 4=0.692 to 0.878; 5=0.879 to 1.065; 6=1.066 to 1.252; 7=1.253 to 1.439.
b: the hybridoma clones indicated above the lanes and their specificities are shown in Materials and Methods.

TABLE IV

Specificity of monoclonal antibodies, E-2 (CXIV-33C7-F5) and E-24 (CXXX-3G5-F12) for the *Endotrypanum* strains by indirect immunofluorescence[a]

Stock code	Species	Monoclonal antibodies	
		E2	E24
Endotrypanum reference strains			
E11	*E. monterogeii*	++	+++
E12	*E. schaudinni*	++	+++
E15	*E. schaudinni*	++	+++
E14	*E. schaudinni*	++++	++
E09	*Endotrypanum* sp.	++	++
Endotrypanum isolates			
E01	*Endotrypanum* sp.	+++	+++
E02	*Endotrypanum* sp.	+++	++
E03	*Endotrypanum* sp.	++	+++
E04	*Endotrypanum* sp.	++	+++
E05	*Endotrypanum* sp.	++	++
E06	*Endotrypanum* sp.	++	+++
E07	*Endotrypanum* sp.	+	++
E16	*Endotrypanum* sp.	++	++
E17	*Endotrypanum* sp.	+++	+++
E18	*Endotrypanum* sp.	++	++++
E19	*Endotrypanum* sp.	++	+++
E21	*Endotrypanum* sp.	+	++
E22	*Endotrypanum* sp.	+	++
E31	*Endotrypanum* sp.	++	+++
E32	*Endotrypanum* sp.	+	+
E33	Endotrypanum sp.	++	+++
E36	*Endotrypanum* sp.	++	++

a: scored: (-) = negative; (+) = positive; (++) = strong reaction; (+++) and (++++) = very strong reaction.

Identification of Endotrypanum antigens by MAbs - Fig. 2 shows the results of the Western blot analysis identifying the molecules associated with the antigenic determinants recognized by MAbs E-2 and E-24. While E-2 recognized two weak components with an apparent molecular mass of 24 kD and 30 kD, the latter MAb identified multiple molecular components with apparent relative mobility (Mr) values ranging from 25 to more than 80-kDa. These results are consistent with previous studies using these MAbs (Lopes & McMahon-Pratt 1989). However, variation occurred between strains analyzed in this study, in either the Mr, intensity or number of components observed. These observations indicated that differences existed either in the epitope density or molecular forms associated with the specific antigenic determinants and therefore allowed the assignment of the strains to specific antigenic groups. These strains formed a polymorphic population according to their enzyme profiles and were grouped into 12 zymodemes. However, all of the isolates from the same species of sloth (*C. juruanus*) and geographical origin (Rondônia, Brazil) had the same enzymic profile (Franco et al. 1996). In contrast, this group of parasites (zymodeme EZ01) when characterized by serodeme analysis, appeared to be a heterogenous population. An explanation for these differences could be related to any of the following reasons: (1) the possibility of cross-contamination or mixed-up of samples in our laboratory, (2) a mixed infection, and (3) the fact that the two techniques measure different properties of parasites. Ultimately, cloning procedures, as well as genetic differences (DNA) may need to be established for these parasites.

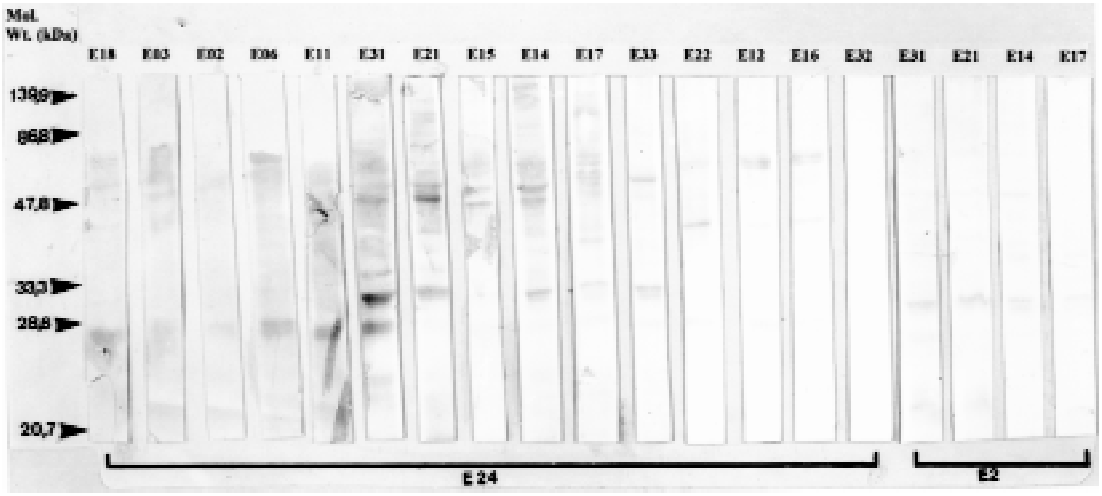

Fig. 2: western blot analyses of promastigotes homogenates from representative strains of *Endotrypanum* using monoclonal antibodies E-2 (CXIV-3C7-F5) and E-24 (CXXX-3G5-F12). The stock codes of the strains analyzed are indicated above the lanes, and their origins are shown in Table I. Molecular weights are indicated in kDa beside the figure.

In conclusion, we have classified a total of 17 Amazon *Endotrypanum* isolates (from the states of Rondônia and Pará) through the application of techniques employing specific MAbs in comparison with standard reference strains. Characterization of *Endotrypanum* with specific MAbs is an additional approach useful to group/differentiate further among these parasites, but problems related to the identification of stocks (the described species are indistinguishable) were encountered using the MAbs employed in this study. Our results confirm previous studies (Lopes et al. 1990, Franco et al. 1996) reporting heterogeneity in this genus.

ACKNOWLEDGMENTS

To Dr JJ Shaw (Evandro Chagas Institute, PA, BR) for the donation of reference strains.

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