ORIGINAL ARTICLE

In vitro initial immune response against *Leishmania amazonensis* infection is characterized by an increased production of IL-10 and IL-13

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Submitted on: 3/5/2010
Approved on: 8/2/2010

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This study received financial support from the CAPES (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* from Portuguese) - PROCAD 0018/01-5, and CNPq (*Conselho Nacional de Desenvolvimento Científico e Tecnológico* from Portuguese).

ABSTRACT

The initial encounter of *Leishmania* with its host's immune system is important in the outcome of infection. Previous studies have shown that PBMCs from healthy volunteers (HV) exposed to *Leishmania* differ in IFN-γ production. We have expanded such observations evaluating the profile and kinetics of cytokines (IFN-γ, IL-12p70, IL-10, IL-13), chemokines (CCL5, CCL3, CCL4, CXCL10), and chemokine receptors (CCR1,CCR5, CXCR3, CCR4) *in vitro L. amazonensis*-stimulated of HV´s PBMCs. HVs were divided in groups of high (HR) or low (LR) IFN-γ responders. In both groups, HR and LR, after *L. amazonensis* infection there was a predominance of IL-10 and IL-13 over IFN-γ production, while IL-12 was produced in similar amount. Regarding chemokines, a more striking difference was observed for CCL3 expression that was lower at 12 hours and 48 hours post infection in LR than in HR. Interestingly, a downregulation of CCR5 and a greater expression of CCR4 were found in low IFN-γ responders. These data suggest that early after *L. amazonensis* infection there is a cytokine *milieu* dominated by IL-13 and IL-10, and despite of this environment, IFN-γ is produced, supporting the complexity of the response. It is noteworthy that the pattern of immune response is mounted in first hours after *Leishmania* stimulation, with the definition of the differentiation of Th1 versus Th2 cells. It remains to be determined if such an *in vitro* difference has an *in vivo* counterpart in terms of susceptibility to infection.

Keywords: *Leishmania amazonensis*, interleukin-10, interleukin-13.
[Braz J Infect Dis 2010;14(5):476-482]

INTRODUCTION

Leishmania parasites preferentially reside as an amastigote in phagocytic cells such as macrophages, resulting in diverse clinical manifestations. The severity of disease is dependent on both causative species of parasite and immunological status of the host.[1] *L. amazonensis* is the etiologic agent of anergic diffuse cutaneous *leishmania*sis in the New World, a condition that is associated with specific impairment of the cell-mediated immune response.[2,3]

Protection and healing in human and experimental cutaneous *leishmania*sis correlates to the development of Th1 immune response, and IFN-γ is considered the pivotal molecule in such process.[4,5] In contrast, a Th2 response with IL-4 and IL-10 production often results in disease progression.[5] Of note, IFN-γ production following *Leishmania* contact differs among naive individuals; some are high IFN-γ responders, whereas others produce low amounts of this cytokine.[6] This observation has been documented with several species of *Leishmania*, as well as in individuals naturally infected, in the initial phase of the infection.[7,8,9,10]

Understanding the initial steps of the human immune response against *Leishmania* may be useful in design rational approaches to vaccination against the disease. Several approaches have been used in developing a vaccine against *leishmania*sis, including some which lead to considerable IFN-γ production, however, up to now, there are no effective vaccines.[11,12] Thus, the understanding of the early events besides IFN-γ production are of utmost importance, and the *in vitro* priming system using human cells may be useful in such attempts.

Chemokines and chemokine receptors have a fundamental role in the development and regulation of immune and inflammatory responses and have received a great deal of attention in both human and experimental *leishmania*sis.[13] There is strong evidence that some chemokines are also involved in polarized

immune responses.[14] Chemokine receptors are differentially expressed on polarized Th cells. Typically, CXCR3 and CCR5 are preferentially expressed on polarized Th1 cells, whereas CCR3, CCR4 and CCR8 have been associated with the Th2 phenotype.[14] However, little is known about the expression profiles of these mediators in *L. amazonensis*-infected individuals and in the human *in vitro* priming system.

In order to better understand the early events on the host-parasite interactions, in this paper, we evaluated the production of proinflammatory (IFN-γ, IL-12p70, CCL3, CCL4, CCL5, CXCL10) and of anti-inflammatory (IL-10, IL-13) cytokines, chemokines and chemokine receptors taking advantage of the *in vitro* priming system using peripheral blood mononuclear cells (PBMCs) from healthy individuals stimulated by live promastigotes of *L. amazonensis*.

MATERIALS AND METHODS

Human cells

Fourteen buffy coats were obtained from healthy individuals by *Centro de Hemoterapia e Hematologia do Ceará* (HEMOCE), Fortaleza, Ceará, Brazil. All individuals had a proliferation index of ≤ 5 when stimulated with *Leishmania* antigens. Serology results were negative for *leishmania*sis, Chagas' disease, hepatitis, and human immunodeficiency virus. The study was approved by the Human Research Ethics Committee of the *Universidade Federal do Ceará*, Brazil.

In vitro sensitization of human cells to *Leishmania*

In vitro sensitization to *Leishmania* was performed by using the protocol described by Pompeu *et al.*,[6] with some modifications. Briefly, PBMCs were obtained by using a Ficoll-Hypaque gradient (Sigma Aldrich) at 1,500 rpm, 21°C, per 30 minutes. The cells were washed three times, and resuspended in RPMI 1640 medium supplemented with 10mM HEPES, 100 U/mL penicillin, 200 μg/mL streptomycin, 2 mM L-glutamine (all from Sigma Aldrich), 50 μg/mL gentamicin (Gibco BRL), and 10% AB inactivated human serum. PBMCs were placed in 24-well plates (Costar, Corning Inc., Acton, MA) at a final concentration of 5 x 10^6 cell/mL (1 mL/well) and incubated for 5 days with and without live promastigotes (final concentration of 5 x 10^6 cells/mL) at 37°C at 5% CO_2.

Lymphocytes proliferation assay

PBMCs were cultivated in triplicates at a concentration of 5 x 10^5 cell/well and stimulated with live promastigotes (5 x 10^5 cell/well), or 20 μg/mL of concanavalin A (Sigma Aldrich), or no stimulated in 96-plates well (Costar). Five days after initiation of the cultures, 0.2 μCi[3] H-thymidine were added to assays and incubated for the overnight. Cells were collected on glass-fiber filters (Skatron Inst., Sterling, VA) using a Cell Harvester (Cambridge Technology Inc., USA) and quantified in liquid scintillation counter (Beckmann LS 6000). Only the samples with index proliferation ≤ 5 were used for the other tests.

Measurement of cytokines by ELISA

The supernatants were harvested after 12, 48, and 120 hours for determination of IFN-γ, IL-10, IL-12p70, and IL-13 production and stored at -20°C until use. The cytokine production was quantified by ELISA (BD Biosciences PharMingen, San Jose, CA) according to the manufacturer's instructions. The sensitivity of the ELISA was 1 pg/mL (IFN-γ and IL-13), 4 pg/mL (IL-12p70), and 2 pg/mL (IL-10). Results are presented as differences in the cytokine production between cells stimulated with *L. amazonensis* and controls without any stimulation.

Oligonucleotide primers

Oligonucleotide primers specific for human chemokines and chemokine receptors were used to amplify cDNA by PCR. β-actin primers were used as a control to evaluate the expression of a housekeeping gene. The primers for chemokines, chemokine receptors and β-actin (Table 1) were prepared from Invitrogen Life Technologies (São Paulo, SP, Brazil).

Table 1. Primer sequences and the sizes of PCR products

Oligonucleotide	Sense primer (5´-3´)	Antisense primer (3´-5´)	Product size (bp)
β-actin	ATG TTT GAG ACC TTC AAC AC	CAC GTC ACA CTT CAT GAT GG	495
CCL3/MIP-1α	ACC ATG GCT CTC TGC AAC CA	TTA AGA AGA GTC CCA CAG TG	391
CCL4/MIP-1β	CCT GCT GCT TTT CTT CAC CC	CAC CTA ATA CAA TAA CAC GGC	336
CCL5/RANTES	TCA TTG CTA CTG CCC TCT GC	CGT CGT GGT CAG AAT CTG GG	373
CXCL10/IP-10	CCT GCT TCA AAT ATT TCC CT	CCT TCC TGT ATG TGT TTG GA	229
CCR1	CCT TCT GGA TCG ACT ACA AGT T	GTA GCA GAT GAT CAT GAC CAA C	396
CCR4	GGA TAT AGC AGA TAC CAC CCT C	AAA TCA TCT TGC ACA GAC CTA G	329
CCR5	GGC TGT GAC GCT TAT CTT CAC C	CGT GTC ACA AGC CCA CAG ATA T	368
CXCR3	TAG AAG TTG ATG TTG AAG AGG G	CAG CTC TTC CTA TGA CTA TGG A	339

RNA isolation and cDNA preparation by Reverse Transcription (RT)

Total RNA was extracted from culture cells using TRIZOL reagent (Invitrogen Life Technologies), according to manufacturer's instruction. Briefly, after cell lysis, RNA was precipitated with isopropanol, washed with 70% ethanol, and dissolved into diethylpyrocarbonate-treated water. RNA concentration and purity was determined by measuring at A_{260} and A_{280}, and samples were immediately stored at -70°C. cDNA synthesis was performed on 2 µg of RNA in a total volume of 23 µL containing 50 mM of oligo $(dT)_{12-18}$ primers (Promega, Madison, WI), 2.5 mM dNTPs (Invitrogen), 1x first-strand buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$), 20 U of ribonuclease inhibitor, and 200 U/µL of reverse transcriptase (Promega). The reaction mixture was incubated at 42°C for 50 min and stopped at 95°C for 5 min.

Chemokine and chemokine receptors mRNA detection

Expression of mRNA was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) for the following: CXCL10, CCL3, CCL4, CCL5, CCR1, CCR4, CCR5, CXCR3 and β-actin. A 5 µL aliquot of the cDNA obtained was amplified in a 25µL reaction containing 1x PCR buffer (5 nmol KCl, 1 nmol Tris-HCl, pH 8.4, 1.5 nmol $MgCl_2$), 0.2nmol each dNTP, 200 nmol each primer, and 5 U of Taq DNA polymerase (Promega) in a PTC-100 thermal cycler (MJ Research Inc., Waltham, MA). Reaction conditions were 25 cycles of 1min at 94°C, 1min at 55°C, and 2 min at 72°C, with a final extension step of 7min at 72°C. For each set of primers, a negative sample (water) was run in parallel. PCR products were visualized by UV light after electrophoresis through a 6% acrylamide gel and stained with silver nitrate. The sizes of the RT-PCR products were confirmed by comparison with a 100-bp ladder run in parallel on the same gel. The band intensity of the amplified products was analyzed using EagleSight®, version 3.2 software (Stratagene, La Jolla, CA). The results are expressed as a ratio of expression of chemokine or chemokine receptor to β-actin expression.

Statistical analysis

The data are presented as mean±standard error of the mean. Analysis between high and low producers was performed by using the Mann-Whitney- test. For all statistical analysis we used GraphPad Prism, version 5.00 for Windows (GraphPad Software, San Diego, CA) and a p value < 0.05 or < 0.01 was considered significant.

RESULTS

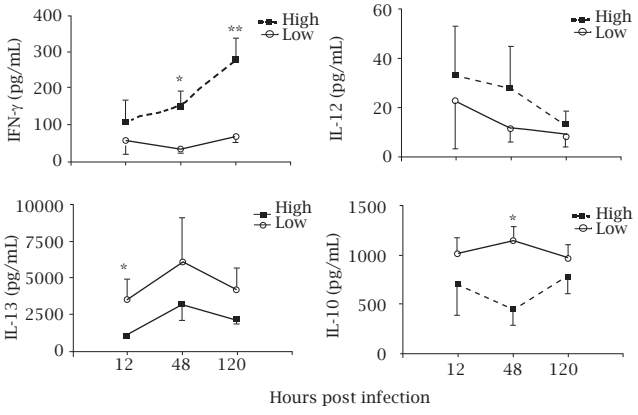
Defining high and low IFN-γ responders

The groups were defined by the concentration of IFN-γ produced after 120h of stimulation with *L. amazonensis* promastigotes. It was adopted as criterion of the division of the groups the same used by Pompeu *et al.*[6] Individuals with IFN-γ greater than 160 pg/mL were classified as high responders (n = 7) and those with lower amount production as low responders (n = 7). In this study, PBMCs from high responders secreted IFN-γ at concentrations ranging from 160 to 584 pg/mL, while in low responders the IFN-γ-secreted ranged from 10 to 135 pg/mL.

Production of cytokines by PBMCs during *in vitro* *L. amazonensis* infection

The levels of anti-inflammatory cytokines, IL-10 and IL-13, as well as the levels of inflammatory cytokines, IFN-γ and IL-12 were measured after 12, 48 and 120 hours of sensitization *in vitro*. At 12 hours of infection it was already possible to observe the production of all the cytokines evaluated, coinciding with the peak level of IL-12 in both groups. Secretion of IL-10 and IL-13 reached their peak levels after 48 hours, maintaining a greater response in low than in high responders (Figure 1). Consistent with previous reports,[6,7,8,9] the levels of IFN-γ in low responders were always lower than levels in high responders, while IL-12 was produced in similar amount (Figure 1). However, it is worth to point out that after *L. amazonensis* infection, in both groups, high and low responders, there was a predominance of IL-13 and IL-10 over IFN-γ production (Figure 1).

Figure 1: Cytokine production by PBMCs from high (n = 7) and low (n = 7) IFN-γ responders. PBMCs from different donors were stimulated with or without live *L. amazonensis* promastigotes. After 12, 48 and 120 hours supernatants were assayed for IFN-g, IL-12p70, IL-13, and IL-10 production. Results were obtained by an enzyme-linked immunosorbent assay and are expressed as the mean ± standard error of the mean. Significant difference ($p < 0.05$) between high and low IFN-γ responders are indicated by asterisks. The experiment is representative of at least three independent experiments.



Expression of chemokines and chemokine receptors during *in vitro L. amazonensis* infection

Chemokines ligands for CCR5 (CCL3, CCL4, and CCL5) or CCR1 (CCL3, and CCL5) are involved in differentiation of T-cell subsets, and recruitment of leukocytes to the site of infection whereas CXCL10 is a NK cell-activating chemokine.[16] We next examined whether the expression of these chemokines and their receptors was correlated with Th2 profile presented by low and high responders of IFN-γ after *L. amazonensis* infection. It is interesting that CCL3 and CCL4 showed the same response pattern in both groups. A more striking difference was observed for CCL3 expression; in this case, expression of this chemokine in low responders was significantly lower at 12 and 48h post infection than in high producers (Figure 2).

Regarding expression of chemokine receptors, the principal finding was an almost complete downregulation of CCR5 in low responders at all time points evaluated in this study (Figure 3). Moreover, we found a greater expression of CCR4 (Th2-related) in low responders during the whole period of evaluation, with a statistically significant difference ($p = 0.015$) at 120 hours. CCR1 and CXCR3 did not present a difference between the two groups.

Figure 2: Semi-quantitative RT-PCR analysis of the expression of chemokines from PBMCs from high and low IFN-γ responders. Densitometric data of chemokines expression at 12, 48, and 120 hours post infection are represented as the mean ± standard error of the mean of the ratio of chemokine to β-actin band from PBMCs from high and low IFN-γ responders. The profiles are representative of at least three independent experiments. The asterisks indicate significant differences (*$p < 0.050$) between high and low IFN-γ responders.

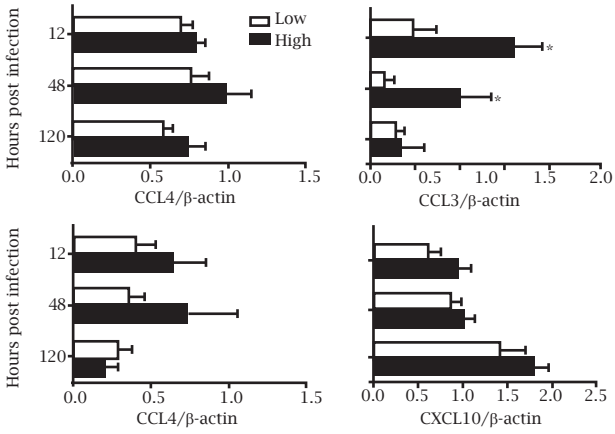
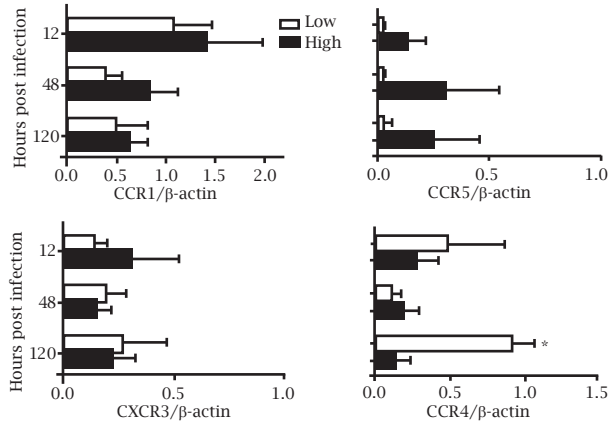


Figure 3: Semi-quantitative RT-PCR analysis of the expression of chemokine receptors from PBMCs from high and low IFN-γ responders. Densitometric data of chemokine receptors expression at 12, 48, and 120 hours post infection are represented as the mean ± standard error of the mean of the ratio of chemokine receptors to β-actin band from PBMCs from high and low IFN-γ responders. The profiles are representative of at least three independent experiments. The asterisks indicate significant differences (*$p < 0.050$) between high and low IFN-γ responders.



DISCUSSION

The present report shows that few hours following exposure to *L. amazonensis*, PBMCs from naive volunteers presented a predominant Th2 response. Such response is supported by a high production of IL-13 and IL-10 followed by a low IL-12 production. However, despite of this cytokine milieu, IFN-γ was produced.

Th2 cytokine IL-13 is involved in susceptibility to infection with several species of *Leishmania* and the use of IL-13 deficient mice and IL-13 transgenic mice demonstrated that IL-13 is important for the generation of Th2 cells.[17-19] Since IL-13 is a downregulating factor of macrophages function,[20] production of IL-13 after *L. amazonensis* infection might be, in part, a mechanism used by the parasite to evade the immune system.

IL-12 is the key cytokine for induction of Th1 immunity. Although IL-12 was initially detected in macrophages, several reports have shown that dendritic cells (DC) are the primary source of IL-12 in *leishmania*sis.[21-23] Indeed, IL-12 and related cytokines (IL-23, IL-27, and IL-1) are primarily released by infected DC very early on post infection by *Leishmania*, thus efficiently inducing Th1-mediated protection.[24-26] In this current report, although IL-12 was also secreted by low and high responders, it is tempting to hypothesize that the higher levels of IL-13 can render specific T cells unresponsive to IL-12 by inhibiting the expression of the IL-12 receptor β-subunit (IL-12Rβ2) chain, as already demonstrated by others,[27-30] favoring the formation of a microenvironment which promotes early the differentiation of Th2 cells and consequently a lower production of IFN-γ.

IL-10, which was produced in higher levels after *L. amazonensis* infection in both groups, usually exhibits human macrophage-deactivating properties,[31] and also plays a regulatory role in *leishmania*sis.[5,32] High intralesional levels of IL-10 are associated to the persistence of the parasite, even in mice genetically resistant to *L. major* infection.[33] In human localized cutaneous *leishmania*sis, *Leishmania* persistence and high intralesional IL-10 expression is associated with severity and unresponsiveness to treatment of *Leishmania* infection.[34,35] High levels of these cytokines during the first 48 hours of infection with *L. amazonensis* may contribute to parasite survival in macrophages and disease progression.

In this study, IL-4 production was not evaluated. Although it was initially thought that IL-4 was required for the development of Th2 response and the differentiation of naive T cells into Th2 cells, further research has shown that IL-4 knockout mice are capable of mounting Th2 response,[36,37] and DC can induce *in vivo* development of Th2 cells in the absence of IL-4,[38] suggesting that IL-4 is not required for the initiation of Th2 response and other cytokines such as IL-13 and IL-2 influence the development of Th2 response.[39] Indeed, protective roles for IL-4 and IL-13 have been described during *L. major* and *L. donovani* infections but not during *L. mexicana* or *L. amazonensis* infections[40,41] and some workers have shown that, under certain circumstances, IL-4 is capable of directing Th1 response.[42] It suggests that the significance of the type 2 responses to the outcome of infection with *Leishmania* is dependent on the strain or species of parasite studied.[2]

Low IFN-γ responders displayed lower expression of CCL3 and CCL4 than high responders. Additionally, a stressed suppression of CCR5 seems to have an important role in the induction of Th2 response in the first hours of *in vitro* lymphocytes sensitization by *L. amazonensis*. CCL3 and CCL4 are known as being involved in the development of Th1 cells.[43] Linkage of CCL3 or CCL5 to CCR5 and CCR1 leads mainly to early IFN-γ production by NK cells.[16] It is worth to point out that although CCR1 expression occurred in low responders, its chemokine ligand (CCL3) was not expressed in those individuals.

Interestingly, low IFN-γ responders presented upregulation for CCR4 that had a higher expression in low than in high responders at practically all time points evaluated in this study. CCR4 is a chemokine receptor found on DC, macrophages, NK cells, and basophiles, but it is predominantly known for its expression on T cells, especially of the Th2 phenotype.[44]

CXCL10 and its receptor (CXCR3) were expressed in low responders, and since this chemokine is capable of recruiting and activating NK cells,[45] which are IFN-γ producers,[46] we speculate that IFN-γ levels observed in these individuals is being produced by NK cells. However, this production is not maintained in low responders probably due to a decrease in IL-12 production in these individuals.

It had been demonstrated that *L. amazonensis*-infected mice presented significantly delayed and depressed expression of inflammatory cytokines (IL-12, IFN-γ, IL-1α, IL-1β), chemokines (CCL3, CCL4, CCL5, and MIP-2), and chemokine receptors (CCR1, CCR2, CCR5) in foot tissues and draining lymph nodes compared to the expression in *L. major*-infected controls.[47] The authors suggest that there is impairment in multiple immune functions at early stages of infection with *L. amazonensis* parasites.

Collectively, the findings presented here suggest that the pattern of immune response to human cutaneous *leishmania*sis due to *L. amazonensis* is mounted in the first hours after parasite stimulation with a strong Th2 response, supported by a predominance of IL-13 and IL-10 production, and a decreased secretion of IFN-γ and IL-12, the signature cytokines for Th1 responses. These date suggest that in the early phase of *L. amazonensis* infection macrophages can be being activated by the "alternative" via,[48] which leads to upregulation of genes for TGF-β and the enzyme arginase-1, and downregulation of TNF-α, thus contrasting the effects of IFN-γ, which induces upregulation of iNOS2 enzyme and TNF-α, and downregulation of the immunosuppressive TGF-β.[48] Furthermore, IL-4 and IL-13 have also been

reported to regulate the iron metabolism of activated macrophages by increasing transferrin mRNA expression and ferritin translation,[49] thus leading to iron depletion and consequently downregulation of macrophage effector functions. Besides, recently it has been shown that high concentrations of IL-10 can enhance IFN-γ-induced NO production, and even low concentrations of Th2 cytokines are capable of exerting a strong inhibitory effect on both NO production and microbicidal activity of macrophages.[50] This means that the levels of Th2 cytokines *in vivo* are critical even when in the presence of a strong IFN-γ response.

Interestingly, despite of this early Th2 environment, IFN-γ was produced, mainly in high IFN-γ responders. These data correlate with what it is known in murine and human *L. braziliensis* infection, since after a short period of a down modulated immune response, a strong type 1 immune response is predominate later.[9,51] The correlation between the *in vitro* difference and the response *in vivo* in terms of susceptibility to infection remains to be determined. Further studies are required to establish the mechanisms of protection in *leishmania*sis and their surrogate markers. This complex interplay of cytokine activities reflects the overall complexity of the immune response to *leishmania*sis and ultimately determines the outcome of the infection.

ACKNOWLEDGMENTS

This study received financial support from the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES - PROCAD 0018/01-5), and *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq). We thank Cristiane M. Millanezi for technical assistance, and Clarissa R. Teixeira for the critical review of this manuscript.

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