Journal of Clinical Laboratory Analysis 23 : 63–69 (2009)

Cellular Immune Response Profile in Patients With American Tegumentary Leishmaniasis Prior and Post Chemotherapy Treatment

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In this study, we have the objective of evaluating the lymphoproliferative response and determining interferon (IFN)-γ and interleukin (IL)-10 cytokine production in the peripheral blood mononuclear cells (PBMC) of patients with American tegumentary leishmaniasis prior and post 12 months of chemotherapy treatment with meglumine antimoniate compared with the PBMC of noninfected donors. Lymphoproliferation, such as cytokine production, was evaluated through in vitro stimulus with the soluble antigenic fraction from *Leishmania* (*Viannia*) *braziliensis* promastigotes (1.25 μg/ml) and Concanavalin A (2.5 μg/ml). Patients showed a significant lymphoproliferative response prior and post treatment compared with the control group. Similar result, prior to chemotherapy treatment, was observed in IFN-γ and IL-10 production when patients were compared with the control group. After chemotherapy treatment, PBMC lymphoproliferative response of the patients revealed an increase, whereas patients have shown a decrease in IFN-γ levels and an increase in IL-10, although without statistical difference. These results may indicate that the patients produced a specific cellular response to the soluble antigenic fraction suggesting that besides Th1 and Th2 dichotomy, immunological regulation mechanisms with the participation of memory T cells and regulatory T cells could be present in the clinical evolution of these patients. This understanding will allow the study and identification of new *L.* (*V.*) *braziliensis* molecules potentially candidates to vaccines. J. Clin. Lab. Anal. 23 : 63–69, 2009.

Key words: American tegumentary leishmaniasis; *Leishmania* (*Viannia*) *braziliensis*; cytokines; chemotherapy treatment; T lymphocytes

INTRODUCTION

American tegumentary leishmaniasis (ATL) is a disease caused by digenetic protozoan that belongs to the *Leishmania* genus of vectorial transmission, which attacks skin, mucous membrane, and cartilages. ATL clinical forms depend on the parasite species, vector, epidemiological characteristics, genetic constitution, and immunological conditions of the host (1). It is known that infections by *Leishmania* induce a specific activation of the immunological response from the host. There is an expansion of various cell types, especially characterized by CD4+T lymphocytes, showing a Th1 and Th2 cytokine profile (2,3). If the response is of Th1 type, cytokines such as interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-12 will be produced, promoting macrophages activation and, consequently, the destruction of the parasites. On the other hand, if the response is of Th2 type, IL-4 and IL-10 will be produced. These cytokines will develop low macrophage activation and clinical manifestation will

Grant sponsors: Molecular and Interfaces Nanotechnology Research Network (RENAMI); Brazilian National Research Council (CNPq); Coordinating Body for Advanced Studies in Higher Education (CAPES); PIBIC/Fiocruz.

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Received 1 October 2007; Accepted 17 October 2008
DOI 10.1002/jcla.20291
Published online in Wiley InterScience (www.interscience.wiley.com).

outcome. *Leishmania* is capable of directing the differentiation of T cells to a Th2-type response characterized by the infection persistence (3,4).

The existing difficulty in the establishment of experimental model for the study of immunity mechanisms to *L.* (*V.*) *braziliensis* has contributed to the performance of studies with patients. It is important to point out that the *L.* (*V.*) *braziliensis* is the major species responsible for ATL in Brazil and it has the capacity to engender from localized cutaneous forms to serious mutilated mucocutaneous lesions, consisting of a severe public health problem in South America (5).

The correct mechanism of the antimonials action is, yet, unknown, even these being the main drugs against *Leishmania* (6,7). Furthermore, the immune mechanism involved in the cure process is probably different for each kind of treatment. Chemotherapy associated with drug resistance and toxicity emphasizes the need of an effective vaccine against leishmaniasis, which may promote a correct immune response such as a Th1 response (8). Owing to this reason, studies of new antigens potentially candidates to vaccines and diagnostic tests are necessary (8,9). Although studies in this context, involving lymphoproliferative techniques and cytokine production, have been performed by other authors, cellular responses to total antigenic fractions from the *L.* (*V.*) *braziliensis* were assessed (10–12).

Brito et al. (13) have shown that the soluble antigenic fraction of *L.* (*V.*) *braziliensis* (named L27 and L30 kDa), characterized by proteins from parasite cytoplasm, promoted humoral response in ATL patients. A decrease in the humoral reactivity was observed, comparing serum from patients with active lesions, prior and post specific treatment.

Once immunity mediated by T cell develops a fundamental role in the host response to *Leishmania* and the role of specific antibodies in the immune response is not clear yet, this study has characterized peripheral blood mononuclear cell (PBMC) immune response to the stimulus with the soluble antigenic fraction of *L.* (*V.*) *braziliensis* prior and post treatment.

MATERIALS AND METHODS

Study Population

Fifteen patients from both genders, coming from the municipalities of Amaraji, Camaragibe, Moreno, and Vitória de Santo-Antão, located in Zona da Mata, Pernambuco, Brazil, were selected for this study. The selection of these individuals was based on criteria such as: being more than 12 years old, being active lesion carriers, having confirmed diagnosis, and no previous chemotherapy treatment. Patients were submitted to blood collection prior to chemotherapy treatment with Glucantime® and then 12 months after the end of treatment. Therapeutic scheme was made of doses of 20 mg/kg/day by subcutaneous injections during 20 and 30 days. After treatment, patients were followed up for a period of 12 months to confirm clinical cure and to avoid the appearance of new lesions and relapse. Ten healthy individuals represented the control group from nonendemic areas and without previous ATL infection. All of them signed the "Term of Free and Informed Consent." CPqAM/Fiocruz Research Ethics Committee (Protocol No. 27/04) approved the experimental protocols.

Soluble Antigenic Fraction

Promastigotes forms of *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2903), cultured in vitro, were expanded in Schneider's medium (Sigma, St. Louis, MO) supplemented with 10% of fetal calf serum (Cultilab) and 1% of antibiotics (100 UI/ml penicillin and 100 μg/ml streptomycin; Sigma) until they reached the exponential phase. Afterwards, they were sedimented by centrifugation at $800 \times g$ for 15 min at 4°C and three times washed with phosphate-buffered saline (PBS; pH 7.2). Proteases inhibitors such as 0.1 mM methyl-phenyl-fluoride and 2 mM ethylenediaminotetraacetic acid (Sigma), pepstatine A 0.001 M (Sigma), and, right after, ultrasonicate were added. The parasitic suspension was centrifuged at $10.000 \times g$ for 10 min at 4°C. The supernatant resultant was removed and submitted to a new centrifugation at $100.000 \times g$ for 1 hr at the same temperature. From the supernatant resultant, the soluble antigenic fraction, protein determination was made (13). The antigen was stored at −20°C for further use.

Obtaining the PBMC

Forty milliliters of peripheral blood in heparinized tube was collected and Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) was added to it. After centrifugation at $400 \times g$ for 30 min at 20°C a PBMC ring was obtained. The cells were washed by centrifugation ($400 \times g$ for 30 min at 20°C) in PBS pH 7.2. PBMC was counted in a Neubauer chamber using Trypan blue (Sigma), adjusting the interest concentration according to each performed assay.

Cellular Proliferation Assay

PBMC (2×10^5/well) was cultured (37°C/5% CO_2) in triplicate, in leveled tissue culture plates of 96 wells (TPP, Switzerland), for 5 days. Cultures were stimulated with the soluble antigenic fraction of *L.* (*V.*) *braziliensis* (1.25 μg/ml) by means of previous kinetic assay and Concanavalin mitogen A (ConA) (2.5 μg/ml) (Sigma). The cells, only in the presence of the culture medium,

were used as the negative control. Twelve hours prior to the end of the culture, 0.5 μCi of [^{3}H]-thymidine was added (Amersham Biosciences). At the end of this period, the material was collected via the automatic cells collector (Skatron Instruments, Sterling, VA) and deposited on a glass-fiber paper (Whatman International Ltd., England). The incorporation of [^{3}H]-thymidine was determined through emitted β radiation expressed in rate per minute (RPM). Results were expressed by the stimulation indices (SI), defined as the RPM arithmetic medium of stimulated cultures, divided by the arithmetic medium of nonstimulated cultures, ± standard deviation. The cut-off was determined by the control group medium ± two standard deviations. The representative values of positive proliferation, SI greater than or equal to 3, were considered.

Cytokine Evaluation in Culture Supernatants

Duplicate cell suspensions (10^6 cells/ml) were deposited in culture plates of 24 wells (TPP). Used stimulus was assessed as previously described (cellular proliferation assays) and the plates were kept at 37°C/5% CO_2 for 48 hr by means of previous kinetic assay. After the culture, plates were centrifuged ($1.800 \times g$ for 10 min at room temperature) and culture supernatants collected and stored at −70°C. IFN-γ and IL-10 cytokine levels in culture supernatants were measured through capture ELISA. Used monoclonal antibodies were from Kit OptEIA (BD Biosciences, San Diego, CA), being previous titled. Ninety-six well plates (Nalge Nunc International Corporation, Rochester, NY) were sensitized with specific anti-cytokine antibodies (according to the manufacturer's instructions) and "overnight" incubated at 4°C. Cytokine standards were added after serial dilution from the initial concentration (1.000 pg/ml). After washes, 50 μl of all samples and standards was added in duplicate and the plate incubated for 2 hr at room temperature. Subsequently, the specific antibodies combined with biotin were added (according to the manufacturer's instructions) for 1 hr 30 min at room temperature. The revealer solution containing 2.2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt was added. The reaction was blocked with sulfuric acid 1 M and the reading was carried out in the spectrophotometer (Bio-Rad 3550) at 415 nm. The sample concentrations were calculated in the linear region of titration curve of cytokine standard, and the final concentrations were expressed in pg/ml using the Microplate Manager Version 4.0 software (Bio-Rad Laboratories, Vienna, VA).

Statistical Analysis

The data were analyzed using nonparametric tests. For intragroup comparative analysis, the Wilcoxon test was used and to detect differences between groups, the Mann–Whitney *U*-test was used. All the results were expressed by mean values of groups ± standard deviation and were analyzed considering the value of $P < 0.05$ (statistically significant).

RESULTS

Patients Treatment

In relation to Glucantime® treatment, performed as described in the methodology, all patients have shown complete lesions healing at the end of therapeutic. The therapeutic scheme in the majority of patients (66.7%) was made up of just one series (20–30 days). In 26.7 and 6.6% of patients, treatment was composed of two and three series, respectively. After the end of treatment, none of the patients, followed up for a period of 12 months for the confirmation of clinical cure, has shown anew and relapse lesions.

Lymphoproliferative Responses

PBMC response to ConA, a mitogen used as the test positive control, was strongly positive in the control group and in patients, prior and post treatment, with no significant difference observed between groups. Thus, the result has shown that the immune response of these cells to the unspecific mitogen has not been suppressed (Fig. 1). No one from control group has presented proliferative response to the stimulus with soluble antigenic fraction of *L.* (*V.*) *braziliensis*. As expected, patients PBMC cultures, prior (12.93 ± 22.95) and post treatment (15.60 ± 17.60), have intensely proliferated to

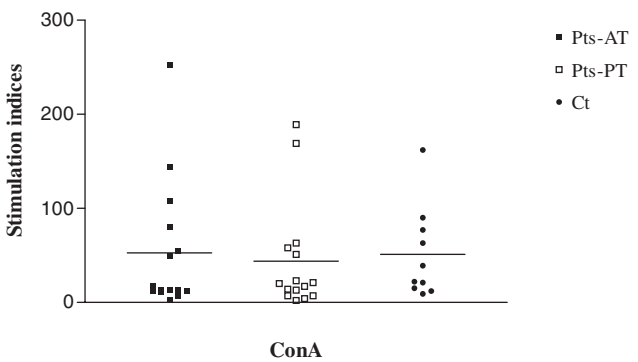

Fig. 1. Comparative graphic of the PBMC lymphoproliferative response of ATL patients prior (Pts-AT), post chemotherapy treatment (Pts-PT), and the control group (Ct) stimulated with 2.5 μg/ml of Concanavalin mitogen A (ConA). SI expressed lymphoproliferative response. Horizontal bars represent the groups' average. PBMC, peripheral blood mononuclear cell; ATL, American tegumentary leishmaniasis; SI, stimulation indices.

antigen. Although a little increase was noticed in SI after treatment, a significant difference among patients was not observed ($P = 0.944$). On the other hand, comparing patient SI prior and post treatment with the control group (1.20 ± 0.42), a significant difference related to proliferation levels was observed ($P < 0.001$) (Fig. 2).

Cytokine Production by Stimulated PBMC

Under stimulus with ConA, similar results were found between patients and control group, with no significant difference in the detection of IFN-γ. Minimum or undetectable production of IFN-γ was observed in nonstimulus PBMC cultures. On the other hand, with this stimulus, IL-10 production was significantly higher in the patients prior ($1{,}056.53 \pm 497.45$) and post treatment ($1{,}618.13 \pm 866.66$) compared with the control group (526 ± 155.78; $P < 0.01$), standing out the production of this cytokine in patients supernatants post treatment ($P = 0.031$) (Fig. 3). In relation to the production of cytokines in view of antigen, results have shown that patients, prior (465 ± 572.8) and post treatment (262 ± 348.55), have produced a higher quantity of IFN-γ in relation to control group (65.5 ± 25.86). The significant difference was enhanced only among patients before treatment compared with control group ($P = 0.005$). The reduction of this cytokine was observed after the end of treatment. However, no significant difference was verified (Fig. 4). In IL-10 levels, significant difference was also found when comparing prior treatment patients (263.33 ± 253.08) with control group (99 ± 53.35; $P = 0.008$). Despite the higher production of this cytokine after treatment (353.73 ± 324.04), no significant difference was pointed out ($P = 0.307$) (Fig. 5).

DISCUSSION

ATL chemotherapy treatment is primarily based on the administration of pentavalent antimonials, with Glucantime® being used in Brazil (14). Cutaneous lesions caused by *L.* (*V.*) *braziliensis* are usually susceptible to treatment with antimonials, and healing occurs at the end of therapy (15).

Treatment in our patients was 100% efficient and therapy time varied from 20 to 90 days, presenting complete lesion healing. The good therapeutic efficacy, also mentioned by other authors, shows that the efficacy may be related to the parasite characteristics and the evolution time in the majority of patients (16,17). The

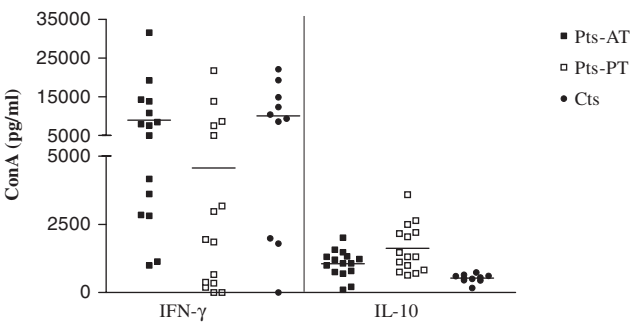

Fig. 3. Graphic of the production quantification of IFN-γ and IL-10 (pg/ml) in PBMC culture supernatants of ATL patients prior (Pts-AT), post chemotherapy treatment (Pts-PT), and the control group (Ct) in the presence of 2.5 μg/ml of Concanavalin mitogen A (ConA). Horizontal bars represent the groups' average. IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell; ATL, American tegumentary leishmaniasis.

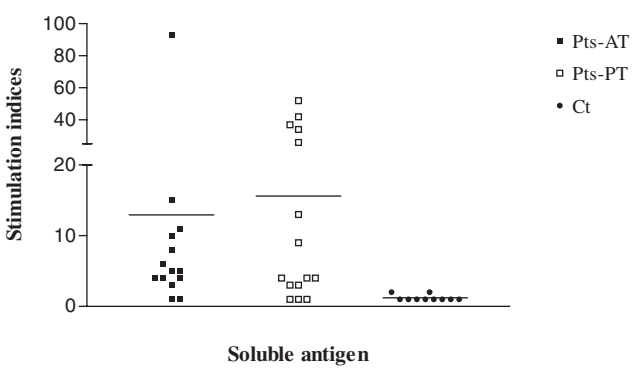

Fig. 2. Comparative graphic of PBMC lymphoproliferative response of ATL patients prior (Pts-AT), post chemotherapy treatment (Pts-PT), and the control group (Ct) with the soluble antigen of *L.* (*V.*) *braziliensis* (1.25 μg/ml). SI expressed lymphoproliferative response. Horizontal bars represent the groups' average. PBMC, peripheral blood mononuclear cell; ATL, American tegumentary leishmaniasis; SI, stimulation indices.

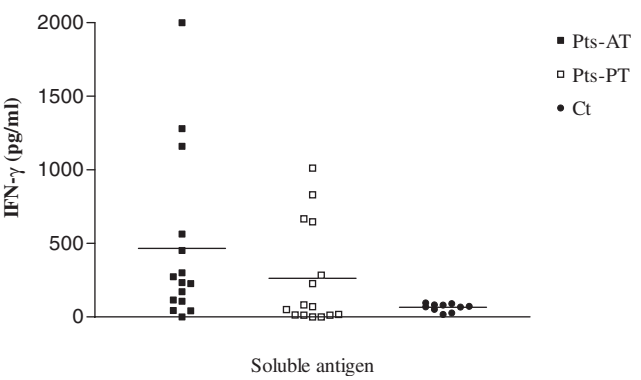

Fig. 4. Graphic of the production quantification of IFN-γ (pg/ml) in PBMC culture supernatants of ATL patients prior (Pts-AT), post chemotherapy treatment (Pts-PT), and the control group (Ct) in the presence of 1.25 μg/ml of *L.* (*V.*) braziliensis soluble antigen. Horizontal bars represent the groups' average. IFN, interferon; PBMC, peripheral blood mononuclear cell; ATL, American tegumentary leishmaniasis.

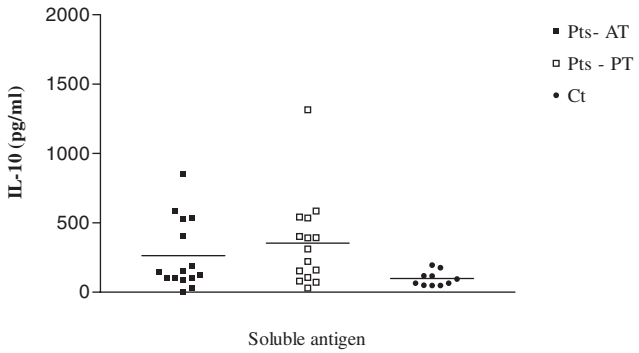

Fig. 5. Graphic of the production quantification of IL-10 (pg/ml) in PBMC culture supernatants of ATL patients prior (Pts-AT), post chemotherapy treatment (Pts-PT), and the control group (Ct) in the presence of 1.25 μg/ml of *L.* (*V.*) *braziliensis* soluble antigen. Horizontal bars represent the groups' average. IL, interleukin; PBMC, peripheral blood mononuclear cell; ATL, American tegumentary leishmaniasis.

cure criterion adopted by many authors is the complete lesion healing. The occurrence of more than 90% of relapses within 1 year in ATL strengthens the suggestion of clinical control for at least 1 year post treatment (5,18–20).

Immunological "status" of the infected host by *Leishmania* may represent an important role in the success of chemotherapy, once infections by this parasite are related to the host immunosuppression. An efficient immune response mediated by cells is necessary for the maximum efficacy of pentavalent antimonial and the relationship between immunosuppression induced by the parasite and the effective chemotherapy is of great importance (21).

All individuals have presented cellular proliferation in response to the mitogen, having no significant difference between the groups, showing that the cellular immune response was not suppressed. This mitogen has also been used by other authors presenting similar results (11,12,22–25).

Analyzing the proliferative response to antigenic stimulus, no individual of the control group has shown lymphoproliferation. Although the medium of SI has increased in patients post treatment, no significant difference was observed when compared with prior treatment. Other studies have shown a decline of the lymphoproliferative response after therapy, even with no significant difference being observed; however, they have used total antigens of *L.* (*V.*) *braziliensis* promastigotes (12,23,26,27). Nevertheless, little is known about antigenicity between soluble and insoluble fractions of *Leishmania* promastigotes.

In patients treated with Glucantime®, it is expected that in vivo T-cell stimulation decreases during the progress of treatment for the cure. This will happen provided that the reduction in the parasite charge may probably cause the reduction of the number of responsive T cells (12). An indirect beneficial effect of the treatment with antimonial would be an increase of T-cell immune response after treatment (25).

Furthermore, after clinical cure, an important characteristic of infection by *Leishmania* is the occurrence of parasite persistence leading to the production of a protective immune response and re-infection resistance (28,29).

Cellular immune response to *Leishmania* parasite happens with the production of cytokines that are important for the development and control of the immune response. In addition, the major event for the induction of this response is the efficient activation of cells capable of producing these protective cytokines (30).

Statistical differences found in our study, regarding cytokine production when stimulated with ConA, may be explained by the fact that this mitogen induces mitoses by a mechanism that is TCR dependent and not all T cells are affected by this process, that is, ConA has not equally stimulated mitoses in groups (31). IFN-γ and IL-10 production in view of the soluble antigen fraction of *L.* (*V.*) *braziliensis* has shown a significant difference when comparing patients group with control group. After chemotherapy treatment, although without statistical difference, patients have shown a decrease in IFN-γ levels and an increase in IL-10.

The existence of immunoregulation mechanisms necessary to promote a Th1 effective response in ATL may explain our results. These mechanisms are important to maintain the host tissue integrity against a subsequent inflammatory response. Study has demonstrated that Th1 response, formed after *L.* (*V.*) *braziliensis* infection, is followed by T-cell responses that produce IL-10 (30).

Cytokines such as IFN-γ, TNF-α, and IL-12 may be toxic when produced in high quantities and IL-10 blocks Th1 cell activation. Consequently, it prevents these cytokine superproduction, avoiding tissue damage (32). Pompeu et al. (33) have identified that IFN-γ production is proportional to TNF-α and IL-10 production. IL-10 high levels may represent a necessary counterbalance to an exaggerated response.

In our study, the increased production of IL-10 post treatment may suggest a regulatory T-cell action that uses this cytokine to limit the action of the immune system, favoring the parasite persistence after clinical cure (34).

Previous studies evaluating IFN-γ production in patients with localized cutaneous lesions, stimulated with total antigens of promastigotes of *L.* (*V.*) *braziliensis*, have shown an increase in these cytokine levels right after the end of therapy with Glucantime®.

Despite this increase, no significant difference prior and post treatment was observed. This increase is probably related to the increase of expression of CD8+ cells that produce IFN-γ (11,12,23,26,27).

Therefore, differences in the results we obtained could also be associated with the antigen used, showing differences in the antigenicity when stimulating the memory T cells. Studies with T-cell response to total antigens are as important as the study of T-cell response to peptides and/or epitopes derived from *Leishmania* antigens (27). Moreover, these results may also be influenced by the time used as a criterion for the clinical cure. In our study, the evaluation was performed 1 year after the end of treatment. In other studies, evaluations were performed right after the end of therapy, although all studied individuals showed healing lesions.

It is important to enhance the fact that the same Th1-type cytokines involved in the infection control can be related to the pathogenesis of the disease. Thus, the determination in Th1 and Th2 response can also be influenced by the strain of the *Leishmania* and the parasite-inoculated dose, by the inoculation site and vector sand fly saliva, besides the immunological aspects and genetic predisposition of the host (1). Therefore, the existence of a counterbalance with cytokine cell producers of the type Th2 seems necessary, that is, a Th1 × Th2 dichotomy (3).

These results suggest that besides Th1 and Th2 dichotomy, immunomodulatory mechanisms with the participation of memory T cells and regulatory T cells are present in the clinical evolution of these patients. This understanding will allow, altogether with the development of models for the study of *L.* (*V.*) *braziliensis*, the study and identification of news molecules from this parasite potentially candidates to vaccines.

ACKNOWLEDGMENTS

We are grateful to Mineo Nakazawa, Wlademir Melo, and Simone Reis for their technical assistance. Luiza de Campos Reis received a CAPES MSc scholarship for the duration of this research project. Marina de Assis Souza received a scholarship from PIBIC/Fiocruz.

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