REFERENCE VALUES FOR *LEISHMANIA INFANTUM* PARASITEMIA IN DIFFERENT CLINICAL PRESENTATIONS: QUANTITATIVE POLYMERASE CHAIN REACTION FOR THERAPEUTIC MONITORING AND PATIENT FOLLOW-UP

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Abstract. Quantification of *Leishmania infantum* DNA in blood samples by an ultrasensitive quantitative polymerase chain reaction (QPCR) detected parasitemias in different clinical presentations. We observed a large range of parasitemias, more than 9 log values, and could determine the threshold between asymptomatic carriage and disease in the Mediterranean area (approximately one parasite/mL of blood. Based on kinetoplast DNA amplification, this assay had a sensitivity of 0.001 parasite DNA equivalents/mL and detected asymptomatic carriage of *Leishmania*. It detected parasite DNA in 58% of healthy subjects, while an immunoblot detected specific antibodies in only 16%. For initial diagnosis of disease, this quantitative PCR with blood samples constitutes a non-invasive alternative to bone marrow aspiration. Its main applications are monitoring of drug therapy and follow-up of immunodeficient patients for biologic confirmation of relapses.

INTRODUCTION

The global incidence of visceral leishmaniasis (VL) is estimated by the Word Health Organization to be approximately 500,000 new cases per year. In the Mediterranean basin, coinfections with *Leishmania* and human immunodeficiency virus (HIV) occur in VL patients in at least 30% of the cases.[1] Among these patients, definitive cure is difficult to achieve and relapses are frequent. Diagnosis of relapses relies primarily on clinical criteria and biologic confirmation is performed by detection of the parasite.[2] In patients receiving successive treatments with anti-leishmanial drugs, selection of drug-resistant parasites has been documented. Therefore, clinicians need a reliable and non-invasive biologic test to monitor efficacy of therapy.

Asymptomatic carriage of parasites is common in disease-endemic areas; this concept is defined as persistence of parasites in vertebrate host without clinical manifestations. The ability of *in vitro* assays to detect such silent infections differs among the different disease-endemic areas for VL and the technical means used.[3,4] Increased sensitivity for *Leishmania* detection has been achieved by means of the polymerase chain reaction (PCR), notably by targeting kinetoplast DNA. Analysis of the results obtained with different assays indicates that in the Mediterranean area the parasitic load in asymptomatic carriers is lower than that observed when the disease is diagnosed. This conclusion is based on the results of human and canine studies.[5,6] A quantitative PCR reached such sensitivity that quantification of parasites over an interval of 9 log units, starting at a 1:10,000 single cell equivalent amounts of DNA per reaction tube.[7] The aim of this study was to obtain reference values and thresholds for parasitemias in cases with asymptomatic infections and those with acute-phase infections during and after treatment of Mediterranean VL and to illustrate the usefulness of parasite quantification in blood for therapy monitoring and follow-up of patients at risk of relapse.

PATIENTS AND METHODS

Patients. Blood samples were obtained from four groups. The first group was 17 immunocompetent patients with VL. Diagnosis of the disease relied on clinical signs associated with detection of parasites in blood or bone marrow and/or large amounts of antibodies to *Leishmania* detected by ELISA and immunoblotting. One of these patients was co-infected with hepatitis B virus (HBV). These patients were treated with liposomal amphotericin B (AmBisome®; Gilead, Foster City, CA) as described below. The second group was seven immunosuppressed patients with clinical symptoms of VL (hepatosplenomegaly, fever, anemia). One patient had immunosuppression related to renal graft treatment (patient G), and the others were co-infected with HIV (Table 1). Diagnosis of disease was confirmed by microscopic identification of parasites, culture, and PCR performed on blood and/or bone marrow. Twenty-four blood samples were obtained from these patients at initial diagnosis of the disease and 147 samples were obtained during treatment and/or the survey. Blood was obtained before starting liposomal amphotericin B treatment (day 0) and at days 3, 5, and 10 after beginning therapy, except for patient A, an older patient initially treated with antimony. A biologic survey was performed and blood was obtained periodically (generally at two-month intervals) or when clinical symptoms of VL occurred (Table 1). A quantitative PCR was performed on these samples. The third group was 81 persons living in the leishmaniasis-endemic area of Marseilles who never had any clinical symptoms of VL or immunosuppression. The fourth group was 30 persons from eastern France, an area without *Leishmania* infection, who never traveled outside this area. Anonymity of the 111 healthy subjects (groups 3 and 4) was maintained according to French legislation on biomedical research.

Treatment with liposomal amphotericin B. Patients were treated with liposomal amphotericin B (AmBisome®), 3 mg/kg/day over 5 days, and one perfusion at the same dose on day 10. Treatment of relapses in immunosuppressed patients with liposomal amphotericin B (2–3 mg/kg/day for 15–21 days for coinfected patients) was started when parasites were detected in blood samples.

TABLE 1
Response to treatment of seven immunodepressed subjects with visceral leishmaniasis*

| | | Initial disease | | | | | | Relapse | | | | | | |
| | | Results at diagnosis time | | | Delay of negativity (days) | | | | Results at beginning of treatment | | | Delay of negativity (days) | | |
Patient	Status	P	M	C	P	M	C	Delay from cure	P	M	C	P	M	C
A	HIV	ND	+	+	ND	30	30	1 year	21	−	+	10	−	10
B	HIV	223	+	+	5	3	3	1 year	42	−	−	10	−	−
C	HIV	889	+	+	5	3	3	6 months	321	+	+	Untreated		
D	HIV	305	+	+	3	3	3	2 years	996	+	+	15	5	5
E	HIV	1,000	+	+	5	3	3	No relapse						
F	HIV	1,146	+	+	10	5	5	No relapse						
G	Renal graft	39	+	+	15	3	3	No relapse						

* Delay of negativity for parasitemia is time (days) between start of treatment and aparasitemia > 1 parasite/mL. P = parasitemia/mL; M = microscopy; C = culture; HIV = human immunodeficiency virus; ND = not determined.

Detection of parasites. Nucleated cells were purified from blood on Ficoll gradients (density = 1.119), washed twice in phosphate-buffered saline, and suspended in the same buffer. Microscopic examination was performed after cytocentrifugation and staining with May-Grunwald Giemsa. NNN medium was inoculated with a cell suspension equivalent to 2 mL of blood and incubated at 24°C.

Quantification of parasite DNA was performed according to a previously described technique.[7] DNA was extracted by using the mini Qia-amp kit (Qiagen, Courtaboueuf, France) according to the instructions of the manufacturer. To optimize the extraction yield, proteinase K digestion of the nucleated cells (equivalent to 2 mL of blood) was performed overnight at 56°C. Primers were designed to hybridize to kinetoplast DNA (sequence common to all *Leishmania* species); the hybridization probe was specific for VL parasites. The sensitivity of this assay was 0.0001 parasite DNA equivalents/ reaction tube.

Serologic tests. Sera from healthy individuals were analyzed by enzyme-linked immunosorbent assay (ELISA) using a lysate of *Leishmania infantum* as antigen and immunoblotting as previously described.[3] Immunoblots were scored as positive when antibodies to the 14-kD and/or 16-kD antigens of *Leishmania* were present.

RESULTS

Association of symptomatic carriage of parasites and lower parasitemias. In blood samples from persons living in the Mediterranean area without any history of leishmaniasis, we detected Leishmania DNA in 58% (Table 2). When detectable, the parasitemia ranged from 0.001 parasites/mL to 1 parasite/mL (Figure 1). The mean parasitemia was 0.32 parasites/m: (median = 0.21 parasites/mL). Only 13 of these 81

TABLE 2
Results of qPCR and immunoblot (WB) in 81 subjects living in a Mediterranean disease-endemic area who never presented with clinical symptoms of visceral leishmaniasis*

	qPCR+	qPCR−	Total
WB+	7 (8.6)	6 (7.4)	13 (16)
WB−	40 (49.4)	28 (34.6)	68 (84)
Total	47 (58)	34 (42)	81 (100)

* Values are no. (%) qPCR = quantitative polymerase chain reaction; WB = Western blot.

blood samples contained antibodies to *Leishmania* when tested by immunoblotting. All ELISA results were negative, indicating the low level of antibody response. Among the 47-PCR positive subjects, 7 were positive by immunoblot positive and 40 remaining persons were considered as actually infected with *Leishmania* but did not develop a detectable humoral antibody response. The quantitative PCR and serologic analysis performed on samples from 30 controls living in a non-endemic area showed negative results.

Parasitic loads and initial diagnosis of VL. Parasitemia measured at time of diagnosis showed a broad range from 8 parasites/mL to 1,400,000 parasites/mL. The mean parasitemia was 70,560 parasites/mL and the median was 310 parasites/mL (Figure 1).

Kinetics of parasitemia during initial treatment with liposomal amphotericin B. We observed a rapid decrease in parasitemia during treatment with liposomal amphotericin B. In most cases, parasitemia became negative or less than 1 parasite/mL after the fifth perfusion of liposomal amphotericin B. This finding was independent of the initial parasitic load. Control samples taken weeks or months after cure of the disease remained negative or showed parasitemia in the same range as asymptomatic carriers. In our experience, parasitemia represents a good biologic criterion of therapy efficacy
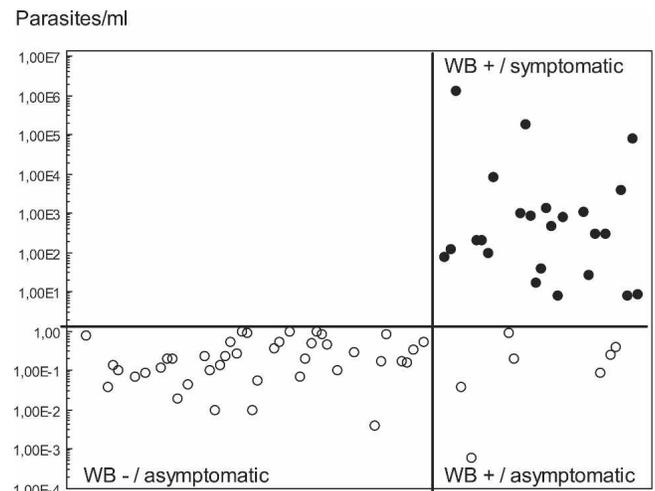

FIGURE 1. Distribution of parasitemias of asymptomatic subjects (○) and visceral leishmaniasis patients at diagnosis (●). WB = Western blot.
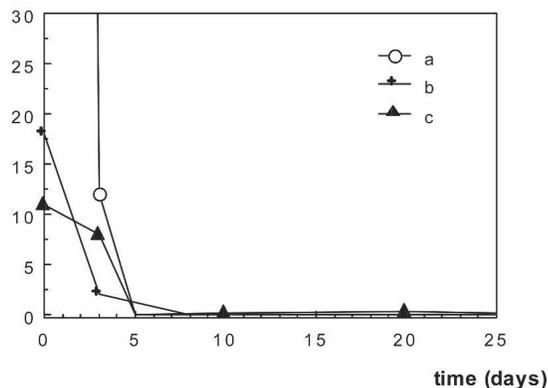
and is available less than 24 hours after blood is sampled. Figure 2A shows the kinetics of three typical cases and Figure 2B shows two patients who required three weeks of treatment to reach a parasitemia less than 1 parasite/mL. One patient was coinfected with HBV and the other patient had undergone a renal graft. Results of a biologic survey during treatment of initial manifestations of VL in patients coinfected with HIV and *Leishmania* were similar to those of immunocompetent persons.

Relationship of parasitemia increase to relapses. In patients coinfected with HIV and *Leishmania*, relapses are frequent and clinical symptoms are often uninformative because fever, hepatomegaly, and splenomegaly are common in these patients. Follow-up of patients at risk of relapse by means of
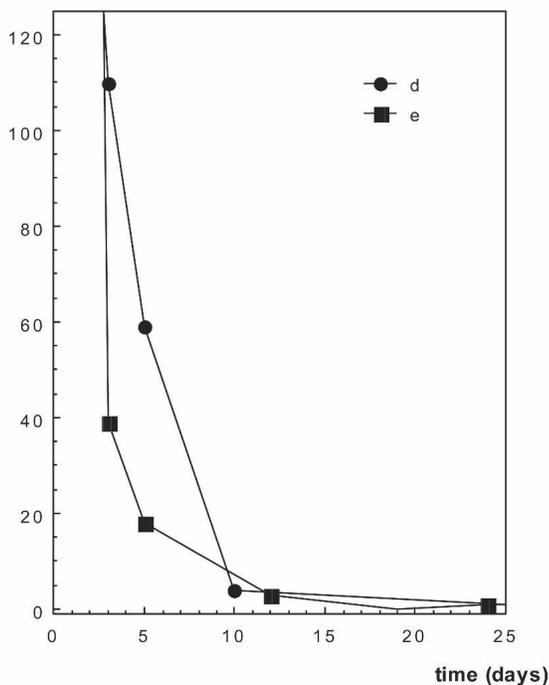
quantitative PCR showed an increase in parasitemia associated with clinical symptoms. Table 1 summarizes the results obtained for the seven immunosuppressed patients and Figure 3 shows the kinetic of parasitemia during relapses.

Eight months after successful treatment of the first relapse, patient A had chronic manifestations with an increase in parasitemia. CD4 cell counts were undetectable in circulating blood and this patient receive continuous therapy over 30 months (Figure 4). Since treatment with liposomal amphotericin B was ineffective, this patient was then treated with pentamidine diisethionate (3 mg/kg every 48 hours, 10 injections, Pentacarinat®; Sanofi-Aventis, Paris, France). This resulted in remission for two months; the last relapse was treated with liposomal amphotericin B because the patient could not tolerate a new course of pentamidine diisethionate.

DISCUSSION

In leishmaniasis studies, determination of susceptibility in animals, vaccination trials, sensitivity analysis of parasite isolates, and surveys of therapy would benefit from an accurate and sensitive quantification of parasites. Flow cytometry has been proposed for use in drug studies, but was it not able to detect rare parasites within human phagocytic cells.[8] Real-time PCR assays targeted to nuclear genome sequences were designed for quantification of parasites in experimental studies in mice, but their sensitivity was only approximately one genome equivalent per reaction tube.[9] Only a newly developed quantitative PCR assay that targeted a consensus sequence of kinetoplast DNA showed a sensitivity of less than one parasite/mL of blood.[7]

This report gives the order of magnitude of parasitemias for different clinical presentations of *Leishmania* infection in the Mediterranean area. In all asymptomatic carriers of *L. infantum*, parasitemia was less than one parasite/mL of blood. Similar results were reported by Lachaud and others, who studied the sensitivity of a PCR that targeted kinetoplast DNA.[10] Our assay provides a better assessment of asymptomatic carriage than immunoblotting because its reflects ac-

parasites / ml

FIGURE 2. Kinetics of parasitemias during liposomal amphotericin B therapy of manifestations of visceral leishmaniasis. **A**, Three patients who responded rapidly to treatment. Patient a was coinfected with human immunodeficiency virus. **B**, Two patients showing a slower decrease of parasitemia after the tenth day. Treatment was given daily until day 21. Patient d was coinfected with hepatitis B virus and patient e underwent a renal graft.
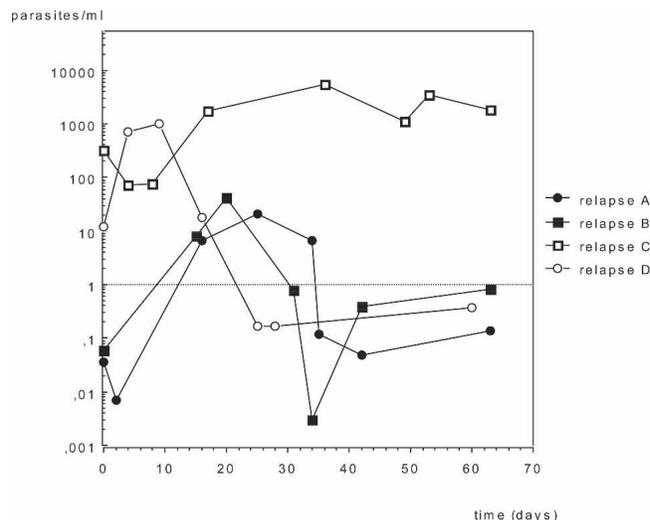

FIGURE 3. Kinetics of parasitemias in three patients during relapses (A, B, and D) and in a patient who refused any specific therapy (C).
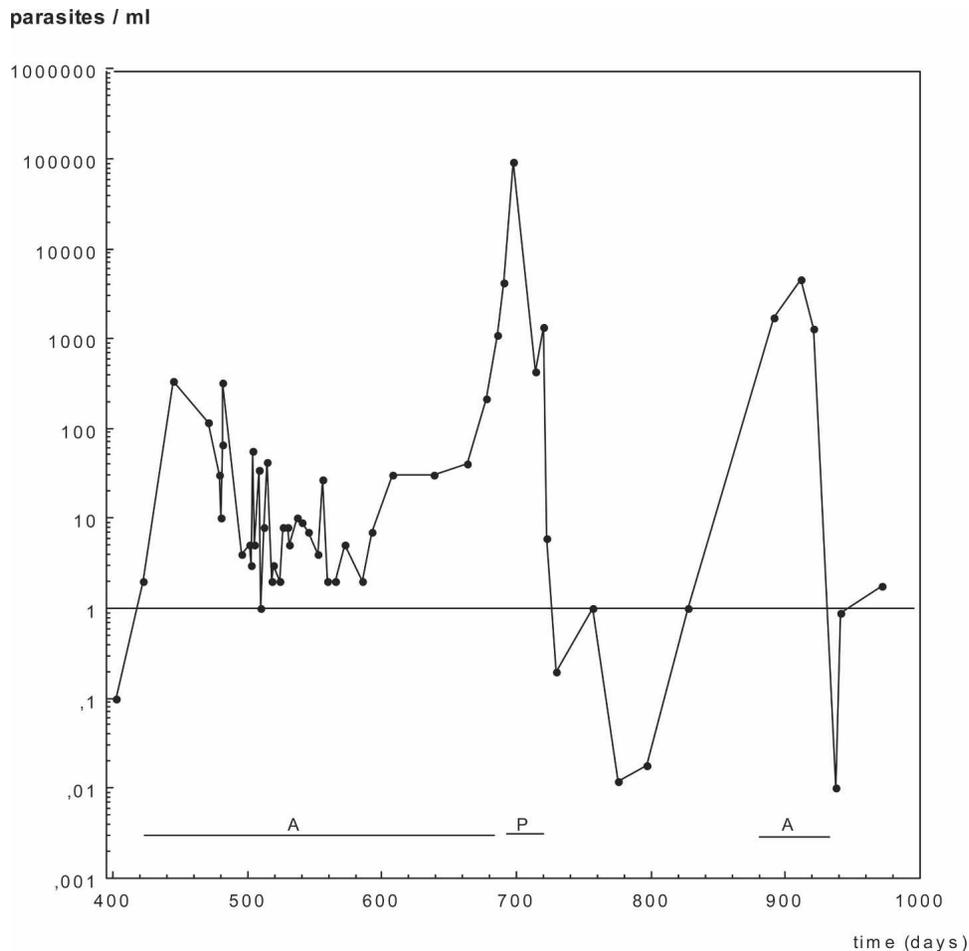
FIGURE 4. Evolution of parasitemia in a patient infected with human immunodeficiency virus. Lines at the bottom indicate treatment with liposomal amphotericin B (A) or pentamidine diisethionate (P).

tual parasitism rather than a previous infection by *Leishmania* with development of a detectable immune response. Among 81 healthy subjects, 58% had positive results and 85% were negative for specific antibodies by immunoblot, the most sensitve antibody assay. Thus, immunoblot positivity is an insufficient criterion for characterization of asymptomatic carriage of *L. infantum* in the Mediterranean area.

Forty of the 68 immunoblot-negative subjects harbour parasites without detectable humoral immunity. The insensitivity of serologic assays to detect subclinical infection was previously described by Costa and others in a South American focus.[11] Our results show that frequency of human asymptomatic carriage in the Mediterranean area is comparable to canine carriage in the same area and to healthy humans in a Sudanese population.[5,12] Le Fichoux and others underestimated the frequency of *Leishmania* infection in healthy humans in southern France because they performed the PCR after selection of seropositive samples.[13] The low level of parasitemia we observed in asymptomatic subjects reflects the host-parasite relationships in the Mediterranean area. However, higher levels could be found in other disease-endemic areas for VL. For example, India, Sharma and others found *Leishmania* in blood of asymptomatic subjects by microscopic examination, which suggested a higher parasitic load.[14]

Levels of parasite DNA found in samples from most asymptomatic subjects are less than the DNA content of one *Leishmania* cell analyzed in 2 mL of blood. We assume that specificity is 100% on the basis of numerous reports on the minicircles targeted by PCR[15–19] and controls performed in non-exposed individuals. We previously verified that the DNA extraction yield is approximately 100% for one parasite/mL of blood and DNA present in plasma is eliminated by Ficoll purification and washing of the nucleated cells. Thus, the only remaining hypothesis is that phagocytic cells that recently engulfed *Leishmania* and destroyed these parasites contain minicircles that are not yet hydrolyzed. Epidemiologic studies based on a quantitative PCR need optimal conditions to detect these minute amounts of DNA. The discrepancy between our actual results and previous results (21% positive samples among the healthy subjects[7]) indicates that efficiency is needed at each step: e.g., DNA extraction, quality of the reagents, and performance of the thermal cycler. In this particular application, results would only provide a minimal frequency of the asymptomatic carriage of the parasite.

Our results indicate that parasitemia threshold between asymptomatic subjects and VL patients is in the range 1–8 parasite/mL: the parsitemia of asymptomatic subjects was less than one parasite/mL and that of symptomatic patients at diagnosis was 8 parasites/mL or more. A comparable finding was reported by Bossolasco and others, who estimated that

relapses of coinfected patients occur when parasitemia was greater than 10 parasites/mL, but the sensitivity of their assay was insufficient to detect a lower level.[20] In symptomatic cases, parasitemia at time of diagnosis of the disease showed individual variations that had no relationship to the immunologic status of the host.

Most available data on variations of parasitemia in patients being treated for VL were obtained with qualitative PCRs with medium sensitivity to avoid positive results related to asymptomatic carriage of the parasite.[21–23] These assays gave negative results after treatment and were unable to analyze the decrease in parasitic load during treatment. In typical cases of VL, the quantitative PCR showed that parasitemia decreases to undetectable levels between the third and the fourth administration of liposomal amphotericin B independently of the initial parasitemia. In some cases, the decrease in parasitemia is slower and the level observed at day 10 (after the fifth administration of liposomal amphotericin B) could be an indication to stop or continue therapy. Two of the patients we studied need prolongation of treatment: one was coinfected with HBV and the other had a renal graft. This must be taken into account when shortened treatment protocols based upon one or two perfusions of liposomal amphotericin B are used; parasitemia levels should be verified at the end of treatment.

The ability of the quantitative PCR to quantify low parasitic load with good accuracy constitutes a major advantage for early biologic diagnosis of relapses in immunocompromised patients. Appearance of clinical symptoms is correlated with an increase in parasitemia above the threshold fixed for asymptomatic carriage. In two cases, parasitemias were 21 and 42 parasites/mL; culture was positive in one case and microscopy was negative in both cases. These results agree with the sensitivity limits of classic techniques previously described.[7]

Since the interval of time between the first episode of VL and the relapse is variable, the frequency of biologic tests should be adapted to the clinical presentation of the patients. In a patient coinfected with HIV and *Leishmania* whose leishmaniasis was previously treated with antimony, a long-term survey showed efficacy of liposomal amphotericin B treatment of the first relapses. Eight months later, CD4 T cells were undetectable in the blood of this patient and parasite development was controlled with iterative liposomal amphotericin B until refractoriness appeared. Treatment with pentamidine diisethionate drastically, but only temporarily, reduced the parasitemia.

These cases illustrate the usefulness of the quantitative PCR for survey of parasitic loads in immunocompromised patients. Other biologic assays have been proposed for diagnosis of relapses; however, qualitative analysis of specific antibodies is dependent on the sensitivity of immunoblotting and the immune status of the host, and antigen detection in urine during patient follow-up is not correlated with clinical symptoms.[24,25]

In conclusion, quantification of *L. infantum* DNA in blood samples showed a dispersion of the results over 9 log values and determined a threshold between asymptomatic carriage and the acute phase of the disease. Sampling blood rather than bone marrow for initial diagnosis of the disease constitutes an additional advantage and a better approach for analyzing asymptomatic carriage of *Leishmania* in epidemiologic and genetic studies. The main application of this assay is the monitoring of therapy and the survey of immunodeficient patients at risk of relapse. This same work needs to be performed in the other areas endemic for VL because host-parasite relationships could differ from those in the Mediterranean focus.

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