Striking Divergence in *Toxoplasma ROP16* Nucleotide Sequences From Human and Meat Samples

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**Background.** ROP16 is a protein kinase of *Toxoplasma gondii* identified in the mouse model as a virulent marker, but it is unknown whether this finding is relevant in human toxoplasmosis.

**Methods.** We obtained the *Toxoplasma ROP16* locus DNA sequence in samples from 12 patients with ocular toxoplasmosis, 1 sample from a patient with congenital toxoplasmosis, 22 samples from soldiers operating in the jungle, 2 samples from urban soldiers, and 10 samples from meat for human consumption. An enzyme-linked immunosorbent assay specific for antibodies against the ROP16 mouse-virulent peptide was performed in 46 serum specimens from patients with ocular toxoplasmosis and in 28 serum specimens from patients with chronic asymptomatic infection, of whom 19 had congenital infection and 11 had toxoplastic lymphadenitis.

**Results.** We found a striking divergence of the ROP16 nucleotide sequences. Ten of 12 sequences (83.3%) from patients with ocular toxoplasmosis clustered with those of mouse-virulent strains, whereas 7 of 7 ROP16 sequences (100%) from meat were clustered with those of mouse-avirulent strains. Only 11 of 104 serum specimens (10.5%) had specific antibodies against the mouse-virulent peptide, and there was no association between clinical forms and positive results of serological assays.

**Conclusions.** The majority of ROP16 nucleotide sequences from Colombian patients with ocular toxoplasmosis belonged to the group of mouse-virulent strains.

**Keywords.** *Toxoplasma*; infectious uveitis; virulence; ROP16 protein; foodborne diseases.

*Toxoplasma gondii* is an obligate intracellular parasite that infects humans and a broad spectrum of vertebrate hosts [1]. In humans, the most important clinical manifestations are the congenital form of retinochoroiditis and, in human immunodeficiency virus-infected patients, the cerebral form of retinochoroiditis [2–4]. There are increasing indications that disease outcomes in human vary with the infecting *Toxoplasma* strain [5–7]. It is also clearly established that there is a geographic divergence in the distribution of *Toxoplasma* strains [8, 9]. The high genetic diversity of *Toxoplasma* strains in the tropical zone of the Americas may partly explain why congenital toxoplasmosis in South America is more symptomatic than in Europe, as was demonstrated when cohorts of congenitally infected children from different continents were compared [10, 11]. Highly divergent strains have been collected in otherwise healthy adults who acquired life-threatening disseminated toxoplasmosis in the Amazon rainforest [12, 13]. Also, the high rate of ocular toxoplasmosis in Colombia [14] is likely attributable to exposure to more-virulent strains of *T. gondii*, even though other factors, such as a high inoculum exposure or the genetic background of the host, may be involved [15].

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A breakthrough in the biology of *Toxoplasma* organisms was the finding of a single major quantitative trait locus on chromosome VIIa that controls virulence [16, 17]. Genes identified as responsible for virulence encode members of a family of polymorphic serine/threonine protein kinases (ROP kinases) [18]. One of the first kinases identified was ROP16, which directly phosphorylates signal transducer and activator of transcription 3 (STAT3) and STAT6, leading to altered cytokine profiles and repression of interleukin 12 signaling [19–21]. Although the discovery of these genetic markers of virulence for the mouse model of infection initiated an explosion of biological work to understand the basis of the virulence in mice and has been the subject of many reviews [22–25], only 1 report has addressed the presence of these mouse-virulent alleles in human infection [26]. The identification of a possible correlation between the severity and type of disease and the genotype of the infecting strain might be very important to determining appropriate treatment and the outcome of the disease in human cases. Here, we report findings from an analysis of 267 clinical samples from patients with congenital, ocular, and asymptomatic toxoplasmosis and from polymerase chain reaction (PCR)–based amplification of *Toxoplasma* DNA from commercial meat.

**MATERIALS AND METHODS**

**Human Clinical Samples and Definition of Clinical Forms**

Peripheral blood samples for PCR assays were obtained from 89 patients with ocular toxoplasmosis and from 15 children with congenital infection. A diagnosis of congenital toxoplasmosis was confirmed using criteria described by the European Network on Congenital Toxoplasmosis [27]. Patients with ocular toxoplasmosis were recruited via the ophthalmological consultation service at the Universidad del Quindio, and diagnosis was based on criteria described previously [28]. We included 123 asymptomatic patients who had serologically confirmed chronic infection (defined as immunoglobulin G [IgG] anti-*Toxoplasma* positive and immunoglobulin M [IgM] anti-*Toxoplasma* negative) and no ocular lesions detected by fundoscopy during previous screening of a young adult population to determine the prevalence of chorioretinal scars in military personnel working in jungle and urban places, as described previously [29]. Additionally, 40 people who were negative for IgG and IgM anti-*Toxoplasma* and visited the ophthalmological consultation service for differential diagnosis of uveitis or toxoplasmic lymphadenitis, tested negative for toxoplasmosis while pregnant, or tested negative for toxoplasmosis during ophthalmological screening were asked to participate as controls for PCR assays in this study.

Serum samples were obtained from 46 patients with ocular toxoplasmosis, 19 children with congenital toxoplasmosis, 11 patients with toxoplasmic lymphadenitis, and 28 patients with chronic asymptomatic infection.

**Meat Samples**

We analyzed 117 meat samples (40 from pork, 40 from beef, and 37 from chicken) obtained from commercial stores from the Quindio department of Colombia. All samples were analyzed by PCR for the B1 gene of *T. gondii*.

**Toxoplasma Clonal Lineages Representative Samples**

We used DNA from the following representative strain types of *T. gondii* for standardization and as controls during PCR assays: TgCtCo1 (type I), TgCtCo8 (type II), and TgCtCo14 (type III). The DNA was a kind gift from Dr Dubey (US Department of Agriculture, Beltsville, Maryland) and Dr Su (Department of Microbiology, University of Tennessee, Knoxville, Tennessee). The RH strain (type I) was cultivated on THP1 cells and originated from Dr Ajzenberg’s laboratory (Limoges, France).

**Isolation of DNA**

DNA extraction from blood was performed using the Wizard DNA purification kit (Promega, Madison, Wisconsin) in accordance with the manufacturer’s instructions. Briefly, DNA from white blood cells was obtained by incubating a blood sample for 10 minutes at room temperature with the cell lysis solution. After centrifugation at 13 000 g for 20 seconds at room temperature, the supernatant was discarded, and the pellet was recovered. The cellular proteins were then removed by a salt precipitation step, and genomic DNA was concentrated and desalted by isopropanol precipitation.

**Detection of *T. gondii* by PCR**

The presence of *T. gondii* DNA in clinical samples was initially confirmed by nested PCR amplification of the repetitive and conserved gene B1 [30]. The positive control was DNA from the RH strain, and the negative control was distilled water in the presence of primers. A control for contamination during DNA extraction was also included and consisted of a tube without template that contained all reagents for DNA extraction and was filled with the same pipette after all clinical samples were prepared. An additional control was a blood sample from a patient who tested negative for IgG against *Toxoplasma*. The sensitivity of PCR was 1 fg of *Toxoplasma* DNA.

**ROP16 Locus Amplification**

A nested PCR amplification provided the best strategy for amplification of the gene encoding ROP16 in clinical and meat samples. A first-round PCR targeted the regions between nucleotides 1229 and 1662 (forward primer: 5'–TGTCGGCCGAT GCTGATGCCACGTC-3'; reverse primer: 5'–ATGCCCAAAG GCGTGGAACATCGATC-3'). The second-round amplification targeted nucleotides 1424–1614 (forward primer: 5'–AAG CAACCGGTGTACGTCAGGTTCC-3'; reverse primer: 5'–TCCATGCGCGAATCCAAGTTCGTG-3') obtained from the ROP16 sequence of various strains by using the Gene Runner V.3.01 software (accession numbers: EQ967477, *T. gondii* strain GT1; XM_002365332, strain ME49; E970683, strain VEG; GQ249096, strain TgCgCa1; GQ249095, strain TgCatBr5;
The PCR products were analyzed by 2% agarose gel electrophoresis in a Techne TC-512 thermal cycler (Technelab, United Kingdom). All PCRs were performed in a single cycle consisting of 40 seconds of denaturation at 94°C, 45 seconds at 57°C at the annealing temperature, and 30 seconds at 72°C. The final cycle was followed by an extension step of 10 minutes at 72°C. In the second-round PCR, the first step of amplification was 5 minutes of predenaturation at 94°C. This step was followed by 40 cycles, with 1 cycle consisting of 40 seconds of denaturation at 94°C, 45 seconds at 58°C at the annealing temperature, and 30 seconds at 72°C. The final cycle was followed by an extension step of 10 minutes at 72°C. All PCRs were performed in a Techne TC-512 thermal cycler (Technelab, United Kingdom). The PCR products were analyzed by 2% agarose gel electrophoresis. The expected size of the product of the first round was 434 base pairs, while the second-round amplification yielded a product of 191 base pairs. To avoid possible contamination, several measures, such as performing PCRs in a separate space and using filter tips, were taken, and various negative controls (no DNA, DNA extraction contamination controls, and IgG anti-Toxplasma–negative blood samples) and positive controls from different strains of T. gondii were used to locate any possible contamination.

DNA Sequencing and Phylogenetic Analysis
For sequencing, PCR products were purified from low-melt agarose gels and recovered using the Wizard PCR SV and PCR clean up system kit (Promega, Wisconsin). Sequencing was done under BigDye terminator cycling conditions by using the normal automatic service of Macrogen (Korea) in 3730XL DNA sequencer with the same primers of the PCR amplification. Sequences were aligned with ClustalW, and phylogenetic trees were derived using a maximum likelihood phylogeny test in Molecular Evolutionary Genetics Analysis software, version 5.05 (http://www.megasoftware.net/). Calculations were made with a Kimura 2-parameter model in 2000 bootstrap replicates. Tree inference was made over initial neighbor-joining trees with a strong branch swap filter.

Enzyme-Linked Immunosorbent Assay (ELISA) for Antibodies Against ROP16 Mouse-Virulent Strain Peptide
The ELISA was based on the peptide derived from the sequence described previously [20]. This sequence includes the residue 503L of the ROP16 protein and may act to stabilize the C-terminal surface of the cavity formed by the N- and C-terminal subdomains of the kinase domain. The corresponding cavity has been shown to form the active site of the ROP16 protein from the mouse-virulent Toxoplasma strain; the amino acids that are part of this cavity compose the virulent ROP16 peptide (ALNSLVSQPFP). One control peptide served to establish cross-reaction. This control consisted of a peptide with 1 amino acid substitution (Leu→Ser) at position 503 (ASNSLVSQPFP), to confirm that positive results were not cases of cross-reaction due to recognition of other residues. The peptides were chemically synthesized using the solid-phase multiple peptide technique. MBHA resin (0.49 mg/g), terbutyloxy carbonyl, and low and high molecular weight cleavages were used in the process. Specific enzyme immunoassays were performed by coating 100 µL of each peptide diluted in 0.1 M carbonate buffer at a pH of 9.6 (Na2CO3, 0.159 g/100 mL; NaHCO3, 0.293 g/100 mL) in Maxisorp microtiter plates in 3 incubation periods: 1 hour at 37°C, 48 hours at 4°C, and 1 hour at 37°C. After washing 5 times with phosphate buffer plus 0.05% Tween 20, unspecified ligand sites were then saturated with 300 µL of 3% skimmed milk in phosphate buffer for 1 hour at 37°C. Plates were then washed once with 0.01 M phosphate-buffered saline (pH 7.2) and 100 µL of serum, and 0.05% Tween 20 phosphate buffer diluted 1:100 was added to each well for 1 hour at 40°C. After washing 5 times with phosphate buffer plus 0.05% Tween 20, 100 µL of horseradish peroxidase–conjugated anti-mouse immunoglobulin diluted 1:6000 in 0.05% Tween 20 phosphate buffer was added for 1 hour at 37°C. Finally, after 5 washes with phosphate buffer plus 0.005% Tween 20, horseradish peroxidase activity was detected by using TMB for 15 minutes at 37°C and stopped by adding a 5% H2SO4 solution. Duplicates from each serum were read at 450 nm in an Epoch 2 spectrophotometer (Bio Tek Instruments). A positive cutoff reaction index was determined by calculating the mean average absorbance plus 2 SDs for 6 serum specimens from people for whom sequencing of ROP16 revealed that they were infected with a mouse-avirulent strain. The reactivity index was calculated as the absorbance of each tested sample divided by the cutoff value. Negative reactivity thus yielded a theoretical value of approximately 1.0; values ≥1.0 were considered to indicate significant positive reactivity against the serotyping peptide. All serum specimens that tested positive by ELISA for virulent peptide (503L) were then assayed with the control peptide (503S), and only serum specimens positive for the ROP16 mouse-virulent peptide and negative for the control peptide were defined as true positives.

Bioethical Aspects
Informed written consent, according to the regulation 008430 of 1993 of the Ministry of Health of Colombia, was obtained from all people selected to participate in the study. The
University of Quindio Institutional Review Board (act number 14; 23 June 2009) approved the study.

Statistical Analysis
EpiInfo software (Centers for Disease Control and Prevention, Atlanta, Georgia) was used to perform analysis (http://www.cdc.gov/epiinfo/). Differences in proportions were analyzed using the Fisher exact test. Differences in means were compared by the Student t test or a by nonparametric test if values were not normally distributed. One-way analysis of variance was used to evaluate the differences between groups from quantitative clinical or laboratory variables. Odds ratios and their 95% confidence intervals were calculated. P values of <.05 were considered statistically significant.

RESULTS

Toxoplasma B1 PCR Results
Sixty-nine of 89 samples (77.5%) from patients with ocular toxoplasmosis, 7 of 15 (46.6%) from children with congenital toxoplasmosis, and 123 of 625 (19.6%) from infected but asymptomatic people (28 from soldiers operating in Bogota and 95 from soldiers operating in the jungle) were positive for B1 by PCR. Thirty-eight of 117 samples of meat (32.4%) were positive for B1 by PCR. No significant differences in B1 positivity between meat species were found (percentage positivity, 30% [12 samples] for pork, 35% [14] for beef, and 29.7% [11] for chicken; P = .8). All B1 PCR analyses of specimens from 40 people with negative results of serological tests for Toxoplasma were negative.

PCR Detection of the ROP16 Locus
Of B1 PCR-positive samples, those from 12 of 69 patients (17.3%) with ocular toxoplasmosis, 1 of 7 congenitally infected children (14.2%), and 24 of 123 asymptomatic infected soldiers (19.5%; 2 from urban soldiers and 22 from jungle soldiers), as well as 7 of 38 meat samples (18.4%), were positive for the ROP16 locus by nested PCR (Figure 1). No significant differences were observed in positivity for ROP16 between meat species (16.6% [2 samples] for pork, 21.4% [3] for beef, and 18% [2] for chicken; P = .92). The percentage of B1-positive samples positive for ROP16 by PCR was not significantly different between the different types of samples (human or meat; P = 1.0, by the Fisher exact test) or groups of patients (patients with ocular and congenital toxoplasmosis vs asymptomatic patients; P = .71, by the Fisher exact test).

Sequencing and Phylogenetic Analysis
We performed sequencing of 44 ROP16 PCR products (Supplementary Material 1). Sequencing of 2 PCR products from patients with ocular toxoplasmosis was repeated, yielding identical results (Ocular_4 and Ocular_4a and Ocular_2 and Ocular_2a in Figure 2A). Clustal alignment of the new sequences was done with 21 already published sequences between nucleotides 1472 and 1548 of the ROP16 gene. After alignment, phylogenetic analysis of the amplified region (Figure 2A) clustered the ROP16 sequence from the clonal archetype type I Rh strain and the GT1 strain separately from the mouse-avirulent ME49 strain (type II) with bootstrap values of 61 (P = .002). Striking differences were observed in the distribution of the new sequences obtained from PCR products into the 2 major clusters. Thus, 10 of 12 sequences (83.3%) from patients with ocular toxoplasmosis belonged to the group of ROP16 sequences of mouse-virulent strains, whereas 10 of 22 ROP16 sequences (45.4%) from soldiers working in the jungle corresponded to this group. These differences in the proportion of sequences belonging to the mouse-virulent strains cluster between both group of people (83% vs 45%) were significantly different by the 1-tailed Fisher exact test (P = .035). Also, of note, 7 of 7 ROP16 sequences (100%) from meat samples were clustered with type II (mouse avirulent) reference strains.

Serotyping for ROP16 Virulent Peptide
The cutoff index was calculated with mean serum absorbance from patients in whom the ROP16 sequence of the infecting Toxoplasma strain was shown to be from the mouse-avirulent group, as determined by the phylogeny reconstruction analysis. In the first ELISA, which involved the mouse-virulent peptide,
37 of 104 serum specimens were positive. The 37 serum specimens were then tested with the control peptide to determine cross-reaction with the mouse-avirulent peptide, and only 11 were negative in this second assay. Therefore, only 11 of 104 serum specimens (10.5%) had specific antibodies against the mouse-virulent peptide. The percentage of true-positive ELISA results for IgG antibodies against the ROP16 virulent peptide (Table 1) was similar between people with and people without ocular lesions \((P = .235)\). There was no association between clinical forms and the positivity of this assay \((\chi^2 = 1.64; P = .65)\). Complete data for serum specimens and results of both ELISAs are showed in Supplementary Figure 2. To understand the low positivity for this peptide among people with toxoplasmosis, we analyzed the predicted location of B epitopes within the amino acid sequence of ROP16. We found that the mouse-virulent peptide was located in a region with low immunogenicity (Figure 3). This could explain the low level of antibodies against this peptide.

**DISCUSSION**

Strain-related factors can explain differences in clinical severity \([26]\) and immune response \([31]\) in ocular toxoplasmosis. We

<table>
<thead>
<tr>
<th>Group</th>
<th>Negative, No. (%)</th>
<th>Positive, No. (%)</th>
<th>Total, No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic asymptomatic</td>
<td>24 (85.8)</td>
<td>4 (14.2)</td>
<td>28</td>
</tr>
<tr>
<td>Congenital</td>
<td>17 (89.5)</td>
<td>2 (10.5)</td>
<td>19</td>
</tr>
<tr>
<td>Lymphadenitis</td>
<td>10 (91.0)</td>
<td>1 (9)</td>
<td>11</td>
</tr>
<tr>
<td>Ocular</td>
<td>42 (91.3)</td>
<td>4 (8.7)</td>
<td>46</td>
</tr>
<tr>
<td>Overall</td>
<td>93 (89.5)</td>
<td>11 (10.5)</td>
<td>104</td>
</tr>
</tbody>
</table>

Mouse-virulent ROP16 peptide was derived from the sequence of the mouse-virulent *Toxoplasma* GT1 strain with the polymorphism 503 S → L.
reported that Colombian patients with ocular toxoplasmosis have a different cytokine pattern if they are infected by type II or non-type II strains, as determined by a serotyping test that uses peptides derived from GRA6 and GRA7 proteins [31]. The problem with current available serotyping assays is that they discriminate very well if the patient is infected by strains from different geographical origins but do not discriminate between asymptomatic or symptomatic infection in patients originating from the same country or between type I and type III infecting strains [6, 9, 32]. To determine the proportion of patients with ocular toxoplasmosis infected by ROP16 mouse-virulent strains, we tested whether serotyping with a peptide derived from the mouse-virulent strain can help with this purpose, and we included an additional assay with a control peptide to identify cross-reactions. Theoretically, serotyping would have an advantage over genotyping because it would not be biased by the amount of parasite DNA circulating in clinical samples. The frequency of a particular strain, if determined on the basis of genotyping results, potentially can result in a biased high frequency of strains that induce a higher parasite load, and information is lost for the vast majority of cases in which parasite DNA amplification or strain isolation is not accomplished. For example, Brazilian researchers have reported reaching only 20 genotypes (32%) from 62 clinical DNA samples [33] and obtaining 20 isolates from 178 clinical samples (15%) from congenitally infected children [34]. In our case, only 18% of clinical samples with *Toxoplasma* B1–positive results were also positive for ROP16 by PCR. Hence, only a small portion of clinical samples can be genotyped. Our attempts to perform serotyping on the basis of the ROP16 mouse-virulent peptide were disappointing, and this could be explained by a low humoral immunogenicity of this peptide. Use of relevant peptides for clinical prognosis would be hazardous because they would be both indicative of virulence and immunogenic. Therefore, one alternative is to improve DNA extraction methods to ameliorate sensitivity of genotyping assays.

We focused on the ROP16 protein of *Toxoplasma* because it has been shown to influence the immune response in human cell lines and because strains with mouse-virulent *ROP16* gene have been shown to produce higher mortality in the mouse model of infection [16, 19]. For *ROP16* genotyping in clinical samples, we performed PCR amplification of this gene in clinical samples, and then the amplified DNA was sequenced. Phylogenetic analysis showed that *ROP16* sequences amplified by PCR were assigned to one of 2 clusters that separated reference mouse-virulent strains from reference mouse-avirulent strains. The distribution of sequences by group of samples was not homogenous, and it is striking that the great majority of sequences from patients with ocular toxoplasmosis were grouped with the mouse-virulent strains. Soldiers were selected as controls because they had negative fundoscopy results and were IgM negative. Although they worked in a wild environment, were heavily infected, and had greater parasitemia [29], their *ROP16* DNA sequences were nearly equally distributed into 2 clusters. In contrast, the *ROP16* DNA sequences from meat samples were all grouped with the cluster of mouse-avirulent reference strains. This indicates that strains infecting meat are different from those producing ocular toxoplasmosis, because meat

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**Figure 3.** Prediction of linear B epitopes by the Bepipred server (http://www.cbs.dtu.dk/services/BepiPred/) for the ROP16 protein of mouse-virulent *Toxoplasma* strains. The location of residue 503L is shown by the black circle.
samples were obtained in the same region where patients with ocular toxoplasmosis live and purchase meat for consumption. There were no differences between sequences for meat of different species (beef, pork, or chicken), and consequently it will be very important to examine oocysts recovered in water samples from the same region, to verify whether these are the source of strains responsible for ocular forms. The oocysts acquired from water were the source of important outbreaks of ocular toxoplasmosis in Canada [35], India [36], and Brazil [37].

Our recent reports about the immune response in Colombian patients with ocular toxoplasmosis showed that they have a T-helper type 2 (Th2)–deviated response [5, 31, 38], and it is well demonstrated that ROP16 from virulent strains induces activation of STAT6, which impedes the Th1 immune response [19, 21]. The present findings of the predominance of ROP16 sequences that belong to the cluster of mouse-virulent strains in patients with ocular toxoplasmosis are congruent with our hypothesis that mouse-virulent strains are responsible for the preferential Th2 immune response seen in Colombian patients with ocular toxoplasmosis. Definitive conclusions can be drawn if we are able to develop more-sensitive genotyping (or serotyping) methods that can inform us about the infecting strain in all of the patients. Accurate serotyping or genotyping methods are necessary for future studies. For instance, we need to establish the percentage of infections with virulent ROP16 parasites among cases of congenital or cerebral toxoplasmosis and their relation with disease severity. In addition, longitudinal studies can be done by following up on recent acquired toxoplasmosis, to determine whether positive results of this genotyping or serotyping test is predictive of a higher probability of ocular involvement. This information would be useful to determine whether longer or intermittent therapy is needed for people infected with the virulent strain. In addition, this information will support therapeutic immunomodulatory strategies to improve prognosis and resolution of this significant cause of visual damage.

In conclusion, our results indicate that ROP16 DNA sequences in samples from humans and meat in Colombia were distributed into 2 highly divergent clusters, in which >80% of sequences from patients with ocular forms were grouped with the cluster of reference mouse-virulent strains.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. J. E. G.-M. has been a speaker for Roche, Abbott, and Biomerieux. A. d.-l.-T. has been a speaker for Abbvie and Euroetika. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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