INFECTION AND IMMUNITY, July 1992, p. 2688–2693
0019-9567/92/072688-06$02.00/0

Vol. 60, No. 7

Mapping of a Visceral Leishmaniasis-Specific Immunodominant B-Cell Epitope of *Leishmania donovani* Hsp70

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Received 2 December 1991/Accepted 2 April 1992

We have shown that a member of the 70-kDa heat shock protein (Hsp70) family is a major target of the humoral immune response during *Leishmania donovani* infection. A recombinant fusion protein was recognized by sera from 92% (35 of 38) of patients with visceral leishmaniasis, including representatives from each of the major regions where it is endemic. Serological analysis of recombinant Hsp70, expressed by a series of deletion constructs, identified the carboxy-terminal region as the immunodominant site. This region, which is the most evolutionarily divergent part of the molecule, was recognized by all sera from patients with visceral leishmaniasis which exhibited an anti-Hsp70 response. Purified recombinant *L. donovani* Hsp70 was not recognized by sera from patients with cutaneous leishmaniasis, Chagas' disease, leprosy, malaria, or schistosomiasis. To determine the regions involved in antibody recognition, a series of overlapping peptides were synthesized on polyethylene pins by the Pepscan method, and a hexamer, EADDRA, was identified by the visceral leishmaniasis serum samples as an immunodominant B-cell epitope.

Exposure of cells to a variety of stimuli results in increased synthesis of a family of proteins known as stress or heat shock proteins (29, 37). Although they have important roles in thermotolerance, these proteins also serve vital biochemical functions in the absence of stress. For example, members of the Hsp70 family have been implicated in translocation of proteins across the mitochondrial and endoplasmic reticulum membranes (5, 6), in secretion from cells (15), and in the processing of antigen in macrophages (33). Many of the proposed functions involve the assembly and disassembly of denatured or malformed proteins and the use of the energy of ATP hydrolysis to disrupt protein-protein interactions (7, 23). Functional and structural studies of Hsp70 have suggested that the molecule contains two major domains: a very highly conserved NH_2-terminal region, which contains the ATPase activity, and the more divergent carboxy-terminal region, which is thought to be important in substrate recognition (9).

Stress proteins have been identified as major immunogens in several infectious disease states, such as leprosy and tuberculosis (36), malaria (2), Chagas' disease (8), schistosomiasis (13), and filariasis (28, 31). Both humoral and cell-mediated responses have been detected. The highly conserved nature of the stress proteins has led to the suggestion that cross-reactive immune responses against them may confer a degree of protection against a wide range of pathogens (36). However, it also follows that such cross-reactivity may, under certain conditions or in certain individuals, manifest itself as an autoimmune response. Evidence suggests that this may occur in certain types of arthritis (10, 21, 26, 34).

We have recently described a *Leishmani donovani* antigen belonging to the Hsp70 family which is recognized by sera from the majority of patients with visceral leishmaniasis (VL) (18). VL results from infection with metacyclic promastigotes of the *L. donovani* species complex, which are transmitted by sandfly vectors during a blood meal (4, 24). The parasites are capable of survival within the phagolysosome of macrophages, where they transform to the intracellular amastigote stage. The stress protein against which the immune response was targeted in VL was identified as a 70-kDa heat shock-cognate protein by virtue of its molecular weight, sequence, and constitutive expression at all stages of the parasite life cycle (18).

In order to study the immunogenicity of this protein and to investigate the potential for cross-reactive immune responses, we have sought to define the extent and specificity of the immune recognition of this antigen. The data presented here suggest that in VL, an immunodominant anti-Hsp70 response is raised against a species-specific epitope in the carboxy-terminal region of the molecule.

MATERIALS AND METHODS

Human immune sera. Serum samples from patients with VL (VL sera) from the Belem region of Brazil were a kind gift from R. Lainson and J. J. Shaw, and those from the Piaui region were obtained in collaboration with Adail Fonseca de Castro and colleagues. R. A. Neal supplied VL sera from India and serum samples from patients with cutaneous leishmaniasis (CL sera) from Brazil. Twenty-four VL sera and 20 CL sera were collected by one of us (S.H.E.S.) in the Khartoum district of Sudan. The serum code-named TF and four other serum samples were obtained when patients presented themselves for treatment at the Hospital for Tropical Diseases in London. Serum samples from patients with malaria and leprosy were obtained from P. Graves and M. A. Shaw, respectively. Serum samples from patients with Chagas' disease (Chagasic sera) were obtained from A. O.

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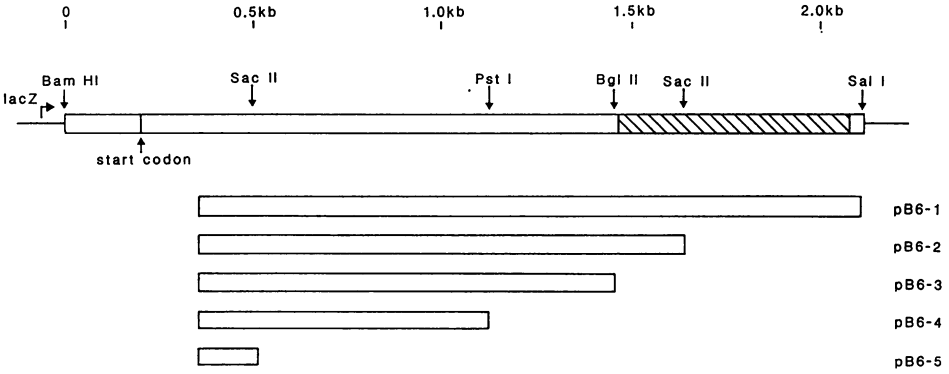

FIG. 1. Deletion constructs prepared from recombinant *L. donovani hsp70* (see text). pB6-1 encodes Asn-62 to Asp-653; pB6-2 encodes Asn-62 to Glu-493; pB6-3 encodes Asn-62 to Ser-427; pB6-4 encodes Asn-62 to Val-322; pB6-5 encodes Asn-62 to Gly-110 (see reference 19 for sequence). The shaded area (Pro-438 to Ala-637) denotes the region corresponding to the cDNA clone which was initially identified by human immune serum (19). The stop codon lies adjacent to the *Sal*I site.

Luquetti. Serum samples from patients with schistosomiasis, provided by K. Schaefer, were from patients infected with *Schistosoma haematobium* in Malawi, and a serum sample from a patient infected with *Schistosoma mansoni* was provided by A. Simpson, National Institute for Medical Research, Mill Hill, London.

Growth of parasites. *L. donovani* promastigotes (MHOM/ET/67/HU3) were cultured at 23°C in Schneider's medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 2 mM pyruvate, 2 mM glutamine, 200 U of penicillin per ml, 200 μg of streptomycin per ml, and 6 μg of hemin per ml. Total cellular protein was prepared for Western immunoblotting by five freeze-thaw lysis cycles.

Production of *L. donovani* Hsp70 fusion proteins. The *Bam*HI-*Sal*I fragment containing the entire *L. donovani hsp70* gene sequence (18) and 164 nucleotides of the upstream region was subcloned by ligation into the Bluescribe SKM13$^-$ plasmid expression vector (Stratagene). IPTG (isopropyl-β-D-thiogalactopyranoside)-induced expression of *L. donovani hsp70* from this recombinant plasmid is prevented by the presence of stop codons between the *Bam*HI site and the *hsp70* start codon (Fig. 1). To overcome this, the DNA was linearized by digestion with *Sac*I and *Bam*HI, and the upstream sequences were removed by the exonuclease III-mung bean nuclease system (Stratagene). The DNA was religated and used to transform *Escherichia coli* JM109 cells. In addition, a 600-bp *Eco*RI insert derived from a λgt11 cDNA clone (18) (corresponding to amino acids Pro-438 to Ala-637) was subcloned into the expression vector pUC8-2. Fusion proteins were induced by adding IPTG (10 mM) to *E. coli* cultures at an optical density at 600 nm (OD_{600}) of 0.45 and incubating at 37°C for 5 to 18 h.

Electrophoresis and Western blotting. Recombinant bacterial lysates containing induced fusion proteins were separated on 10% polyacrylamide–sodium dodecyl sulfate (SDS) gels with sonicated *L. donovani* promastigotes and untransformed *E. coli* lysates as controls. After transfer to nitrocellulose (32), they were incubated with human sera, used in a dilution range from 1:200 to 1:1,000. Positive bands were identified with a peroxidase-labeled second antibody (Jackson Laboratories) and 4-chloronaphthol as the substrate.

ATP-agarose chromatograpy. Bacterial lysates containing induced pB6.1 protein (Fig. 1) were dialyzed against 50 mM Tris-HCl (pH 7.5) buffer containing 15 mM 2-mercaptoethanol, 0.1 mM EDTA, 20 mM NaCl, and 5 mM $MgCl_2$ (buffer A). A 5-ml ATP-agarose column (20, 35) was equilibrated with the same buffer, and the sample was loaded at a rate of 20 ml/h. The column was washed with 5 column volumes of buffer A until a steady baseline was achieved. The column was washed in 5 volumes of buffer B (buffer A with 0.5 M NaCl) and then in 5 volumes buffer C (buffer B with 5 mM ATP). Fractions were collected, and the A_{280} was read. Those with the highest readings were pooled, and the sample was dialyzed extensively against water, freeze-dried, and tested for purity by SDS-polyacrylamide gel electrophoresis (PAGE).

DNA sequencing. Sequencing was performed on double-stranded plasmid DNA (1 μg) by the chain termination method with the Sequenase enzyme (United States Biochemical Corp.).

Pepscan synthesis of peptides and pin ELISA. Peptides were synthesized onto polyacrylic acid-coated polyethylene pins, derivatized with hexamethylene diamine, on the Pepscan system (Cambridge Research Biochemicals) (11). Amino acids were added sequentially by standard Fmoc chemistry (3) after deprotection and washing steps. On completion of the synthesis, the peptides on the pins were screened by an enzyme-linked immunosorbent assay (ELISA) with 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) as the substrate, and the A_{410} of the wells was read. The peptides were repeatedly analyzed with different sera after removal of the bound antibody by sonication.

RESULTS

Recombinant *L. donovani* Hsp70 is recognized by sera from most VL patients. A series of plasmid constructs were prepared which allowed expression of *L. donovani hsp70* sequences in transformed *E. coli* cells. In cells transformed with construct pB6-1 (Fig. 1), IPTG-induced recombinant Hsp70 constituted up to 50% of the total cellular protein. DNA sequence analysis of pB6-1 revealed that the expressed fusion protein contained more than 90% of the *L. donovani hsp70*-coded sequence (Asn-62 to Asp-653). On the basis of fusion protein size and level of expression, pB6-1 was used in serological studies. A panel of 38 VL sera, including samples from all the major regions where VL is endemic, were reacted with recombinant Hsp70 on Western blots (data not shown). In this assay, 92% of the samples gave a positive response.

A further series of deletion constructs were produced from pB6-1 by using restriction enzymes to truncate the *hsp70*

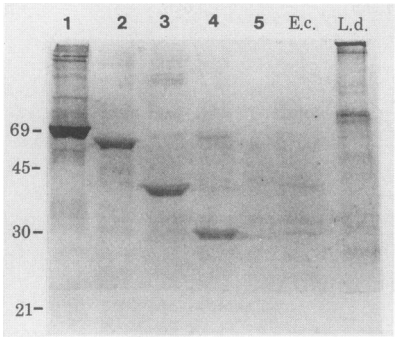

FIG. 2. Western blot of Hsp70 fusion proteins reacted with human immune serum TF (see text). Tracks: 1, pB6-1; 2, pB6-2; 3, pB6-3; 4, pB6-4; 5, pB6-5; E.c., *E. coli* JM109 lysate; L.d., *L. donovani* lysate. Molecular masses are given in kilodaltons.

gene from the 3′ end (Fig. 1). The resultant recombinants were analyzed by SDS-PAGE and Western blotting with TF serum (from an Australian male who acquired VL in the Sudan) (Fig. 2). This serum detected the fusion proteins coded by plasmids pB6-1, pB6-2, pB6-3, and pB6-4 in addition to the fusion protein expressed by a construct containing the 600-bp cDNA sequence (Fig. 1). The serum from this patient therefore recognizes at least two distinct B-cell epitopes on *L. donovani* Hsp70.

Immune recognition of *L. donovani* Hsp70 is disease specific. Hsp70 molecules are among the most highly conserved in nature. Since these proteins are also known to be highly immunogenic in a wide spectrum of pathogenic infections, we sought to investigate the extent of the cross-reactivity between *L. donovani* Hsp70 and sera from patients infected with agents which also elicit an anti-Hsp70 response. Sera from patients with VL which had previously given a positive response (31 samples: 21 from the Sudan, 7 from Brazil, and 3 from the Hospital for Tropical Diseases in London) were tested by Western blot analysis against recombinant *L. donovani* Hsp70 (encoded by pB6-1) purified by ATP-agarose chromatography (20) in parallel with sera from patients with CL (21 samples), lepromatous leprosy (8 samples), malaria (8 samples), schistosomiasis (8 samples), and Chagas' disease (8 samples). While all of the VL sera tested positive, none of the above 53 sera cross-reacted with *L. donovani* recombinant Hsp70. Therefore, immune recognition of this protein appears to be specific to patients with VL.

Mapping the immunodominant region of Hsp70 by Western blotting. The fusion proteins expressed by the pB6 family of constructs were analyzed by Western blotting with a panel of 27 VL sera, each of which had previously been shown to react with the pB6-1-encoded fusion protein. There was a range of reactivities (Table 1). Nine sera (group 1) gave the same recognition pattern as TF serum (Fig. 2), implying recognition of at least two distinct epitopes, one or more in the region of the molecule corresponding to the cDNA clone (Pro-438 to Ala-637) and at least one in the *Sac*II-*Pst*I fragment (Gly-110 to Val-332) (Fig. 1). Group 2 (three sera) also recognized at least two epitopes: one or more in the carboxy-terminal region, as described for group 1, and at least one in the region corresponding to the *Bgl*II-*Sac*II fragment (Ser-427 to Glu-493) (Fig. 1). The largest group (group 3), comprising 15 serum samples, identified an epitope(s) only in the portion of the molecule encompassed by amino acids Glu-493 to Asp-653, encoded by nucleotides between the 3′ *Sac*II and *Sal*I sites (Fig. 1). All the sera that tested positive recognized an epitope(s) in the carboxy-terminal region of Hsp70.

Identification of an immunodominant epitope by Pepscan. To determine the species-specific epitope(s) involved in antibody recognition of the carboxy-terminal region of Hsp70, a series of peptides, decamers overlapping by 5 amino acids, were synthesized on polyethylene pins (see Materials and Methods). Based on the Western blot data, peptides spanning the region from Glu-493 to Ala-620 were synthesized. This region constitutes the portion of the molecule identified by sera from all VL patients who produced an anti-Hsp70 response. When these pins were screened with TF serum, two predominant responsive sequences were identified, Glu-529 to Arg-538 and Tyr-614 to Gly-623. On the basis of the intensity of the response, it was decided to investigate the region Glu-529 to Arg-538 more thoroughly. A second series of decamer peptides were prepared, from Arg-520 to Ala-541, overlapping by 9 amino acids. When screened with TF serum, the hexapeptide 529-EADDRA-534 was identified as the minimal peptide sequence required for recognition by this serum (Fig. 3). Removal of the NH_2-terminal glutamic acid or the carboxy-terminal alanine greatly reduced the response. This sequence is specific to *L. donovani* Hsp70 and has not been found in Hsp70s from any other species (18). It immediately follows a region of 200 amino acids which are identical to the sequence of *L. major* Hsp70.

To confirm the hexamer as an epitope, a further series of peptides were prepared on which the hexamer sequence was

TABLE 1. Recognition of recombinant *L. donovani* Hsp70 fusion proteins with sera from VL patients

Origin of serum samples	No. of samples	Proven or probable agent	Fusion proteins detected[a]					
			pB6-1	pB6-2	pB6-3	pB6-4	pB6-5	cDNA
Group 1								
Sudan	6	*L. donovani* sensu lato	+	+	+	+	−	+
India	2	*L. donovani*						
Portugal	1	*L. donovani infantum*						
Group 2								
Sudan	2	*L. donovani* sensu lato	+	+	−	−	−	+
Brazil	1	*L. donovani chagasi*						
Group 3								
Sudan	14	*L. donovani* sensu lato	+	−	−	−	−	+
India	1	*L. donovani*						

[a] Fusion proteins were expressed from the plasmid constructs shown in Fig. 1.

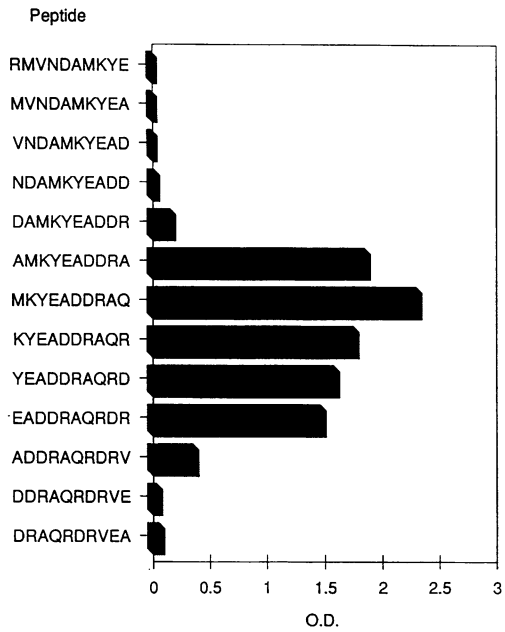

FIG. 3. Overlapping peptides, 10-mers overlapping by 9 residues, covering the region from Arg-520 to Ala-541, synthesized on polyethylene pins, and screened in a standard ELISA with TF serum (see text) at 1:200. The OD_{410} values are the mean of duplicate pins. This is a representative example of three experiments.

synthesized on six pairs of pins. The corresponding region of *L. major* Hsp70 (EEDDKA) was synthesized on one pair of pins as a control. TF serum recognized the hexamer peptide, as did five other VL sera tested, three from the Sudan and two from Brazil (Fig. 4). Sera from patients with Chagas' disease, schistosomiasis, or CL did not recognize this *L. donovani* peptide. Conversely, the corresponding *L. major* hexamer was not recognized by VL serum TF.

DISCUSSION

The data presented here demonstrate that in VL infections, the carboxy-terminal region of *L. donovani* Hsp70 is a major target of the humoral immune response. Furthermore, we show that, despite the highly conserved nature of stress proteins, recognition of *L. donovani* Hsp70 is disease specific, as sera from patients with CL, malaria, schistosomiasis, Chagas' disease, or leprosy did not recognize a recombinant *L. donovani* Hsp70 on Western blots. Consistent with this, an immunodominant epitope identified in this study (EADDRA) was localized to a divergent region of the molecule, as was a second immunoresponsive peptide (Tyr-614 to Gly-623). Therefore, one explanation for the disease-specific immune response to the Hsp70 may be that immunodominant epitopes are confined to the regions of the sequence that are not highly conserved. The findings in this article extend observations made for schistosome infections, in which the immunodominant region of Hsp70 is also located in the carboxy-terminal region and the responses against *S. mansoni* and *S. japonicum* were found to be immunologically distinct and non-cross-reactive (14), although the epitopes involved have not been identified.

The species specificity of these epitopes is, however, insufficient to account for the differential anti-Hsp70 responses which we have observed in the visceral and cutaneous forms of leishmaniasis. The Hsp70s from *L. donovani* and *L. major* have greater than 95% identity (16, 18), and at least some of the *L. donovani* Hsp70 epitopes recognized by sera from patients with VL are also present in the *L. major* protein. This can be inferred from the observation that anti-*L. donovani* Hsp70 antibodies affinity purified from VL sera recognize a 70-kDa band on Western blots of *L. major* protein (18). In the cases investigated in this study, the *L. major* Hsp70 epitopes did not trigger a detectable immune response during CL. A possible explanation for this differential response may be the different sites of infection in the two clinical forms of the disease. *L. donovani* and *L. major* may encounter different populations of antigen-presenting cells in the viscera and the skin, respectively. The lower temperatures (30 to 31°C) encountered by parasites during cutaneous infection (27) might be associated with reduced

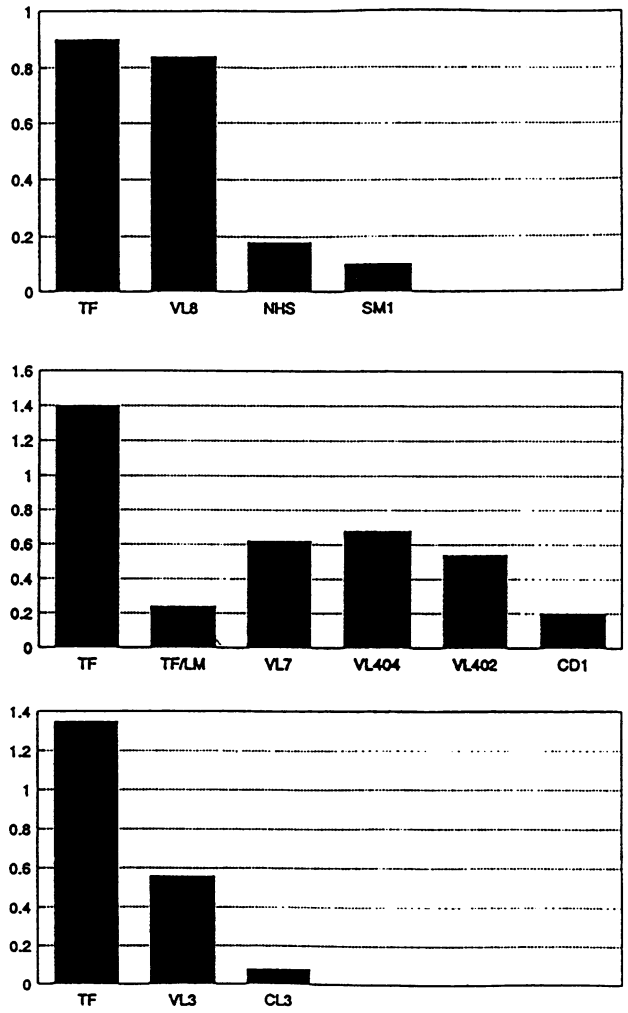

FIG. 4. EADDRA hexamer synthesized on a series of duplicate pins and screened in a standard ELISA with a variety of sera. Sera VL3, VL7, and VL8 were from VL patients in the Sudan. Sera VL402 and VL404 were from patients in Brazil. Serum CL3 was from a CL patient in the Sudan, serum CD1 was from a patient with Chagas' disease in Brazil, and serum SM1 was from a patient with *S. mansoni* infection in Kenya. TF/LM, TF serum tested on the corresponding hexapeptide from *L. major*. NHS, normal human serum. All sera were used at a 1:200 dilution. The ordinate shows the OD_{410}. Each panel represents a separate experiment.

levels of expression of Hsp70-related molecules. In this context, it would be of interest to examine antibody recognition of Hsp70 in patients with cutaneous disease due to *L. donovani infantum* infection (12) and visceral disease due to *L. mexicana amazonensis* infection (1).

Definition of both T- and B-cell epitopes in Hsp70 will be important if synthetic peptides derived from the sequence are to be used in protection studies. One prerequisite for such peptides is that they not possess the potential for stimulating autoimmune reactions against the host homolog. In malarial infections, for example, monoclonal antibodies raised against a *Plasmodium falciparum* 72-kDa antigen, a homolog of Hsp70, cross-react with the corresponding host protein (19). In addition, T cells from patients with rheumatoid or reactive arthritis recognize the mycobacterial 65-kDa heat shock protein (11, 21). An important finding of this study is that species-specific epitopes can be identified from a highly conserved protein that presumably will not induce an autoimmune response, although the importance of B-cell epitopes may be limited in immunity to *L. donovani* infection. Resolution of VL infection is thought to depend on the production of gamma interferon by parasite-specific $CD4^+$ T cells and its subsequent role as a macrophage-activating factor (17, 22, 25). It will therefore be of interest to determine whether Hsp70 is a target for T-cell-mediated responses during VL infection, to identify immunodominant T-cell epitopes on the molecule, and to define the T-cell subsets which they stimulate.

ACKNOWLEDGMENTS

This work was supported by a grant from the Wellcome Trust. Juan MacFarlane was in receipt of a Medical Research Council Studentship, and Sayda El Safi was in receipt of a W.H.O. fellowship.

We thank Keith Howard for his critical reading of the manuscript.

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