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A Naturally Occurring, Soluble Antagonist of Human IL-23 Inhibits the Development and In Vitro Function of Human Th17 Cells

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Th17 CD4+ cells are critical to inflammation. Their secretion of IL-17 drives inflammation in human diseases, including inflammatory bowel disease. Differentiation of mature Th17 cells depends on stimulation with IL-6, TGF-β, and IL-21 and the induction of RORγt, but IL-23 is essential to Th17 phenotype, stability, and function. Induction of Th17 cells can be antagonized by IL-4 or IFN-γ, but mechanisms through which terminal differentiation can be inhibited have not been identified. Human IL-23Rα (HuIL23Rα)-chain mRNA transcripts exist that lack exon 9 ("Δ9"); these are translated to a truncated receptor containing the entire external domain. This soluble variant of the HuIL23Rα-chain antagonizes Th17 maturation. It is secreted and present at low levels in the blood. It represents 10% of HuIL23Rα-chain mRNA, binds IL-23 in solution, and inhibits the phosphorylation of STAT3 caused by IL-23. In in vitro Th17 cell differentiation experiments, Δ9 inhibits the production of the Th17-associated cytokines IL-17A and IL-17F. Δ9 does not bind IL-12; thus, it is a specific inhibitor of IL-23 and a modulator of Th17 cells. Our results indicate that this soluble form of HuIL23Rα likely functions to regulate Th17 activity. The Journal of Immunology, 2010, 185: 7302–7308.

The recent discovery of the Th17 subset of CD4+ T cells has provided insight into the biology of chronic inflammatory disorders (1, 2). The IL-12 family cytokine IL-23 is critical to the maturation of Th17 cells and maintenance of their function through phosphorylation of STAT3 via its heterodimeric receptor and particularly the IL-23Rα (HuIL23Rα)-chain signaling moiety (3–6).

Th17 cells represent a distinct subset of CD4+ Th cells (7–9) whose functional emphasis is inflammation (10–12). Differentiation of naive human CD4+ T cells to Th17 cells has been the subject of much attention because these cells have been shown to have critical functions in autoimmune disease models in mice (13–15). Their development is driven by IL-1β, IL-6, TGF-β, and IL-21 and may function as an autocrine growth factor, but IL-23 is essential for their final maturation and phenotypic stability (16–22).

IL-23 is a heterodimeric cytokine belonging to the IL-6 helical cytokine family. It is composed of two disulfide-linked subunits, p19 and p40. p40 is also a component (with p35) of IL-12, a cytokine in this same family (3). This alternative pairing allowed the p19 and p40. p40 is also a component (with p35) of IL-12, a cytokine family. It is composed of two disulfide-linked subunits, p19 and p40. p40 is also a component (with p35) of IL-12, a cytokine family. It is composed of two disulfide-linked subunits, p19 and p40. p40 is also a component (with p35) of IL-12, a cytokine family. It is composed of two disulfide-linked subunits, p19 and p40. p40 is also a component (with p35) of IL-12, a cytokine family. It is composed of two disulfide-linked subunits, p19 and p40. p40 is also a component (with p35) of IL-12, a cytokine family. It is composed of two disulfide-linked subunits, p19 and p40. p40 is also a component (with p35) of IL-12, a cytokine family. It is composed of two disulfide-linked subunits, p19 and p40. p40 is also a component (with p35) of IL-12, a cytokine family. It is composed of two disulfide-linked subunits, p19 and p40.
recently, the demonstration of multiple splice forms of HuIL23Ra mRNA (35, 36) led us to ask whether any might encode functionally relevant proteins. In this paper, we describe the function of the peptide encoded by one of these isoforms, HuIL23RaΔ9, “Δ9”, on the generation of human Th17 cells and their in vitro activity.

Materials and Methods

Fragment size analysis of human HuIL23Ra splice variants

Purified RNA from mitogen-stimulated PBMCs was reverse transcribed into cDNA, and PCR was carried out with forward primer (5′-AATGTCGG-GAACTCTACCTACA-3′) and reverse primer (5′-D3-GCTTGGTGTTC-TGGGATGAGATTTCC-3′), which was fluorescent labeled with the “D3” dye. The amplified product was then denatured and analyzed in the Beckman CEQ8000 using their Fragment Analysis Program, calibrated with DNA size standard marker kit 600 (0.5 µL/reaction; Beckman Coulter, Brea, CA), and custom-made D1 labeled 600–1200 size marker (1 µL/reaction; Biowentures, Murfreesboro, CA). Peaks were assigned to corresponding HuIL23Rα-spliced variants based on their size. The fluorescent signal represented the mRNA transcript level.

Expression and detection of Δ9

Expression constructs for both wild-type (wt)HuIL23Ra and Δ9-spliced variant were prepared. cDNA of each form was subcloned into pcDNA3.3 TOPO vector, using the TA cloning kit from Invitrogen (Carlsbad, CA). Both forms were tagged with the “FLAG” sequence at the C terminus, and 293T cells were transiently transfected using FuGene transfection reagent (Roche, Basel, Switzerland). Cell lysates were prepared using the ProteoJET mammalian cell lysis reagent (Fermentas, Glen Burnie, MD), and culture media were harvested after 48 h and immunoblotted using anti-FLAG M2 Ab. To measure the level of soluble (s)HuIL23RaΔ9 and Δ9, respectively. In some cases, recombinant human His-tagged sIL-23R (R&D Systems) was spiked into the suspension to detect the common p40 subunit of IL-12/IL-23 complexes (20–22), Th17 differentiation was assessed by measuring the mRNA transcript levels of four crucial transcription factors (T-bet, GATA-3, Foxp3, and RORγt). In some cases as shown, IL-17A and IL-17F were quantitated by ELISA (R&D Systems).

Biochemical properties of Δ9

Culture medium from Δ9 transfection experiments was concentrated using Centricon filter (Millipore, Bedford, MA), and then Δ9 was affinity purified using anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO). The immunocomplex was precipitated, washed, and resuspended in PBS. Two hundred nanograms of either IL-12 or IL-23 was added to the suspension and incubated at 4°C for 2 h. FLAG-tagged Δ9 immunocomplex was again precipitated and eluted with excess FLAG peptide. The eluted product was analyzed by immunoblotting using anti-Hup40 or anti-HuIL23R to detect the common p40 subunit of IL-12/IL-23 complexes, respectively. In some cases, recombinant human His-tagged sIL-23RΔβ1 (1 µg/reaction; R&D Systems) was spiked into the suspension containing Δ9 bound to anti-FLAG M2 affinity gel in the presence or absence of 200 ng IL-23. After extensive washing, the eluted product was analyzed using anti-HIS (Invitrogen), anti-Hup40, or anti-HuIL23R (R&D Systems) to detect sIL-12/23RΔβ1, the p40 subunit of IL-23 or Δ9, respectively. To examine the N-glycosylation modification on HuIL23RaΔ9, purified Δ9 was treated with 1000 UPNGase F (New England Biolabs, Beverly, MA) at 37°C for 1 h, followed by immunoblotting using anti-HuIL23R, rIL-23 (200 ng) was added to Δ9 or PNGase F-treated Δ9. Immunoprecipitation was performed and analyzed by immunoblotting, probing with anti-Hup40.

Δ9 and IL-23 signaling in human PBMCs

PBMCs were isolated from heparinized whole venous blood of healthy donors by density gradient centrifugation using Ficoll-Paque (Sigma-Aldrich). Blood was purchased as anonymous buffy coats from a New Jersey blood transfusion service. Isolated PBMCs were rested in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies) and 1 mM glutamine (Invitrogen Life Technologies) at 37°C for 24 h. PBMCs were then stimulated with 10 ng/ml IL-23 for 20 min. Cell lysates were prepared for immunoblots to examine the phosphorylation status of STATs. Membranes were probed with Abs against phospho-(p)-STAT1, p-STAT2, p-STAT3, or p-STAT5 (Cell Signaling Technology, Beverly, MA), then stripped and reprobed for total STAT1, STAT2, STAT3, or STAT5 (Cell Signaling Technology). All Abs were diluted 1:500 in PBS containing 3% (w/v) BSA. In some cases, PBMCs were stimulated in the presence of supernatant containing HuIL23RaΔ9 protein, titrated as shown.

Purification of Δ9

293T cell supernatant containing FLAG-tagged HuIL23RaΔ9 protein was collected and concentrated using Amicon ultracentrifugal filter 30K (Millipore), then precipitated using anti-FLAG M2 affinity gel (Sigma-Aldrich). Precipitated Δ9 was eluted with excess FLAG peptide (Sigma-Aldrich). Ten microliters of purified Δ9 was used in the PAGE gels (Bio-Rad, Hercules, CA), followed by Coomassie blue (Bio-Rad) staining.

Differentiation of human Th17 cells

Naive CD4+ human T cells were obtained by negative enrichment (StemCell Technologies, Vancouver, British Columbia, Canada). Their purity was assessed by flow cytometry. Of the 96% CD4+ T cells prepared, 98% were CD45RA-CD45RO-. One million CD4+ naïve T cells were cultured for 5 d in the presence of anti-CD3 plus anti-CD28 (T cell activation expansion kit; Miltenyi Biotec, Auburn, CA) and a “Th17 differentiation” cytokine mixture (10 ng/ml IL-1β, 10 ng/ml IL-6, and 1 ng/ml TGF-β) in the presence of 5 ng/ml IL-23 and 500 ng/ml Δ9 or IL-23/Δ9 complex (20–22). Th17 differentiation was assessed by measuring the mRNA transcript levels of four crucial transcription factors (T-bet, GATA-3, Foxp3, and RORγt). In some cases as shown, IL-17A and IL-17F were quantitated by ELISA (R&D Systems).

Results

One major splice form of HuIL23Ra in human leukocytes

Fragment analysis allowed us to semiquantitate the HuIL23Ra splice forms relative to the full-length (wt) transcript (Fig. 1). Six variants could routinely be detected, and of these, one constituted a single major alternative HuIL23Ra form in the cells tested. The variant lacking exon 9 (HuIL23RaΔ9; “Δ9”) represented between 12 and 20% of the total HuIL23Ra mRNA; we therefore focused our attention on this variant in our subsequent experiments.

The Δ9 variant represents an sHuIL23Ra-chain

The Δ9 variant is generated when the deletion of exon 9 results in a frameshift on splicing of exons 8 and 10 (Fig. 2A). Thus, a potential truncated HuIL23Ra protein whose extracellular domain is left intact, but which lacks the membrane anchor and intracellular signaling domain (Fig. 2B), is generated. Transfection of 293T cells with FLAG-tagged wt or Δ9 constructs clearly demonstrated clearly that Δ9 was equally expressed inside the cells (anti-FLAG Western blotting) as was wt HuIL23Ra. However, only Δ9 was detected in the culture medium, indicating that it was efficiently processed for secretion (Fig. 2B); absence of the wt protein in the culture medium indicated that Δ9 was present via active secretion, rather

![FIGURE 1](http://www.jimmunol.org/)
FIGURE 2. ∆9 represents a naturally occurring, soluble HuIL23R-chain. A, The HuIL23Rα gene contains 11 exons. Protein translation starts at exon 2 with the signal peptide and stops at exon 11 (indicated as #). The TM domain is encoded in exon 9. Deletion of exon 9 results in a frame shift and generation of a premature stop codon, as illustrated (indicated as *). Therefore, the ∆9 protein contains the intact extracellular domain and most likely represents a soluble form of HuIL23Rα. Bi, Expression constructs for both wt HuIL23Rα and the ∆9 splice variant were prepared and FLAG-tagged at the C terminus. Cellular lysates and culture media from transiently transfected 293T cells were prepared 48 h posttransfection, followed by immunoblotting using anti-FLAG M2 Ab. Anti-actin was used to monitor loading of the lysates onto the gels. Both forms were expressed to similar levels in the cell lysates, but only ∆9 was detected in the culture supernatant. Transfections and Westerns were performed three times. Bii, A sandwich ELISA was developed and validated using conditioned media obtained from two independent transfection experiments, successfully detecting soluble HuIL23Rα in the culture medium obtained from 293T cells transfected with ∆9 expression construct. Data from two independent experiments are shown; ELISA wells were tested in duplicate. Biii, The ELISA was also performed on human plasma specimens from six healthy donors calibrated against IL-23R/Fc fusion protein. Each ELISA well was performed in duplicate; five independent human plasma samples are shown.

HuIL23Rα ∆9 protein binds IL-23

We next asked whether ∆9 was capable of binding to IL-23. Given the sharing of the p40 chain between IL-23 (p19 + p40) and the related cytokine IL-12 (p40 + p35) (3), it was also important to establish whether ∆9 was specific for IL-23. Culture medium from 293T cells transfected with either empty vector or FLAG-tagged ∆9 expression construct was concentrated and incubated with 200 ng of either IL-23 or IL-12, then immunoprecipitated with anti-FLAG M2 affinity gel; M2 gel alone was incapable of pulling down IL-23 (Fig. 3Ai). The immunoprecipitate was subjected to Western blotting and visualized with either anti-HuIL23R or anti-Hup40 (Fig. 3Aii). Although both experiments confirmed that ∆9 had successfully been precipitated with anti-FLAG, only the incubation of ∆9 with IL-23 allowed visualization of the p40 band, demonstrating that ∆9 binds IL-23 free in solution but will not bind IL-12. We therefore inferred that ∆9 may preferentially bind the p19 chain of IL-23.

The ∆9 variant comprises some 353 aa, predicting a size of 41 kDa. Its observed size, however, was consistently ~65 kDa (Fig. 3B; see also Fig. 5D), suggesting posttranslational modification, most likely by N-glycosylation as is common in cytokine receptors. PNGase treatment of affinity (FLAG)-purified ∆9 reduced its apparent size to <50 kDa, confirming N-glycosylation of the natural form. Interestingly, removing the glycosyl component of ∆9 modestly but clearly reduced its capacity to bind IL-23 (Fig. 3Bii).

HuIL23Rα ∆9 protein forms a complex with IL-23 and IL12Rβ1

On the cell surface, HuIL23Rα combines with HuIL12Rβ1 to form the heterodimeric IL-23R (4). HuIL12Rβ1 also partners with HuIL12Rβ2 to form the cell surface IL-12R (37). We wanted to know whether ∆9 could link to HuIL12Rβ1 via IL-23 or indeed bind it directly. Soluble rHIS-tagged HuIL12Rβ1 (1 µg) was spiked into concentrated FLAG-tagged ∆9-containing 293T supernatant in the presence or absence of IL-23 (200 ng). Immunoprecipitation with anti-FLAG was performed and the Western blots analyzed with anti-HIS, anti-Hup40, or anti-HuIL23R to visualize sHuIL12Rβ1, the IL-23 p40 subunit or ∆9, respectively. ∆9 does not bind directly to HuIL12Rβ1 (Fig. 4). However, when IL-23 was added to the binding assay, immunoprecipitation with anti-FLAG allowed visualization of all three reactants: ∆9, IL-23 (p40), and HuIL12Rβ1, indicating that ∆9 forms a complex with HuIL12Rβ1 through binding to IL-23, thus mimicking the cell surface receptor.

HuIL23Rα ∆9 protein modulates the action of IL-23

Next, we tested the ability of ∆9 to interfere with the natural biological functions of human IL-23, in vitro. Signaling through the IL-23R primarily triggers the phosphorylation of STAT3; this is essential to the formation of Th17 cells. We incubated human PBMCs with 10 ng/ml IL-23 and examined the phosphorylation of multiple STATs. As expected, the greatest influence of IL-23 was on STAT3 (Fig. 5A) although some pSTAT1 and pSTAT5 were also generated. Following titration of IL-23 on STAT3 phosphorylation (Fig. 5B), further experiments were conducted with 5 ng/ml IL-23. In the presence of 5 ng/ml IL-23, pSTAT3 was elevated 7-fold when the PBMCs were cultured in the supernatants from 293T cells transfected with full-length (wt) IL-23R transcript. However, supernatants from 293T cells transfected with the ∆9 expression
construct effected a marked reduction of STAT3 activation, demonstrating a notable inhibition of IL-23’s function on human PBMCs (Fig. 5C). To confirm that this inhibition was indeed because of Δ9, we purified FLAG-Δ9 to apparent homogeneity (Fig. 5D). This purified Δ9 inhibited IL-23-induced STAT3 phosphorylation (Fig. 5E) in a dose-dependent manner (Fig. 5F). Thus, Δ9 negatively regulates IL-23 signaling in human PBMCs.

HuIL23Ra Δ9 protein inhibits the generation of Th17 cells

IL-23 is key to the function of Th17 cells, but is incapable of inducing the differentiation of these cells in vitro, from a starting population of naive human CD4+ T cells. However, the ability of Δ9 to inhibit IL-23 signaling suggested that it would also inhibit the functional maturation of human Th17 cells. We purified human CD3+CD4+CD45RA+ cells (“naive CD4+ T cells”) by negative selection (Fig. 6A) and differentiated them to Th17 cells in vitro, as illustrated. These highly enriched naive CD4+ T cells were stimulated with anti–CD3/CD28-coated microbeads (“beads”) in the presence of a cytokine mixture comprising IL-1β, IL-6, and TGF-β for 2 d, at which point medium, IL-23, Δ9, or IL-23+Δ9 was added. Incubation continued for an additional 2 d, whereupon cells were harvested for RNA, and supernatants were collected for ELISA (Fig. 6B).

The transcription factors T-bet (Th1), GATA3 (Th2), Foxp3 (Treg), and RORγt (Th17) were measured to examine the development of naive CD4+ T cells under the influence of the Th17 differentiation conditions (Fig. 6Ci). In the presence of the “Th17 mixture,” marked skewing in favor of RORγt was observed, indicating the successful differentiation of naive CD4+ T cells to Th17 cells. Terminal maturation of the Th17 phenotype by IL-23 was demonstrated by the enhanced expression of IL-17A and IL-17F mRNA in the presence of IL-23 (Fig. 6Cii, 6Ciii); as expected, RORγt was not elevated by the addition of IL-23 to the Th17 mixture, reaffirming that IL-23 is not a human Th17 cell differentiation factor. Addition of Δ9 alone to the Th17 mixture failed to induce the production of IL-17A or IL-17F, confirming its lack of stimulatory activity. However, when IL-23 was added in the presence of Δ9, maturation of Th17 cells to an active phenotype was greatly reduced; secretion of both IL-17A and IL-17F were significantly diminished (Fig. 6Dii, 6Diii).

Discussion

In our previous reports, we showed that the IL-23Rα mRNA undergoes extensive alternative splicing, which results in 24 different transcripts. Four different classes of putative translation products were deduced from these alternatively spliced mRNA sequences: 1) short premature IL-23Rα extracellular peptides; 2) soluble forms of IL-23Rα lacking TM/intracellular domains; 3) full-length IL-23Rα with a truncated extracellular region; and 4) a membrane-bound receptor isoform of IL-23Rα that lacked likely
intracellular signaling components (35, 36). In this paper, we have presented the results of our studies on the protein translated from the major alternative splice form in human leukocytes, \( \Delta 9 \), detailing its ability to bind p19 and inhibit the generation of functional human Th17 cells in vitro.

We predicted that \( \Delta 9 \) would represent a soluble form of the IL-23R extracellular domain and demonstrated by transient transfection into 293T cells that this was indeed the case. We used commercially available Abs to develop a \( \Delta 9 \)-ELISA and demonstrated that \( \Delta 9 \) is present at low levels in the periphery of healthy individuals. Details of this ELISA will be published elsewhere.

Soluble cytokine receptors may be generated by several mechanisms, including proteolytic cleavage of receptor ectodomains, alternative splicing of mRNA transcripts, or transcription of distinct genes. So, we cannot be completely certain that \( \Delta 9 \) is present in the circulation solely as a result of alternative splicing of the native IL-23R \( \alpha \) mRNA. However, because the human genome project is completed, we believe it is very unlikely that there is a distinct gene encoded for a soluble form of the IL-23R \( \alpha \)-chain.

In their original report, Parham et al. (4) described the human and mouse IL-23 receptor and, despite showing binding by their external domain Fc and V5-HIS6 constructs, commented that neither could “act as effective antagonists” of IL-23. In this paper, we demonstrate that the natural \( \Delta 9 \) form inhibits STAT3 phosphorylation and Th17 cell differentiation in a human in vitro assay, starting from naive CD4\(^+\) T cells. The discrepancy between the two reports may be due to several factors, including differences in the IL-23 assay used or their use of fusion proteins versus our naturally occurring variant, for example. Similarly, however, our study also showed binding of IL-23, but not its sister cytokine IL-12, to the IL-23 external domain (\( \Delta 9 \)) and further demonstrated that this was via the p19, not the p40, component. Our data also agreed that STAT3 phosphorylation is the primary consequence of IL-23/IL-23R interaction (Fig. 5A); in our experiments using primary naive CD4\(^+\) human T cells responding to IL-23, this was dose-responsively inhibited by \( \Delta 9 \).

IL-23 is the key element in the final stages of Th17 phenotypic maturation. In our Th17 maturation assays, naive CD4\(^+\) T cells
were successfully differentiated to Th17 cells, based on their upregulation of RORγt (the signature transcription factor for this cell subtype), and secretion of IL-17A and IL-17F. The induction of IL-17A&F expression was abolished when Δ9 was present but, consistent with others’ reports of Th17 development, induction and levels of RORγt expression were not affected. Notwithstanding our data on the ability of Δ9 to dose-dependently inhibit STAT3 phosphorylation in primary naive CD4+ T cells, we cannot formally exclude the possibility that blocking IL-23 with Δ9, and so reducing Th17 induction, might interfere with a signaling pathway other than that involving STAT3. Although a full analysis of IL-23 signaling is outwith the scope of the present report, two complementary studies lead us to believe that this is in fact the most likely scenario—experimental deletion of STAT3 impaired RORγt expression whereas hyperactive STAT3 promoted Th17 development (6), and human patients suffering dominant-negative mutations in STAT3 had much diminished Th17 cell development (38); furthermore, the STAT3 locus is itself associated with inflammatory bowel disease (39). These results reinforce our belief that Δ9 functions as a naturally occurring specific inhibitor of IL-23 that has the ability to regulate Th17 cell development, most likely by causing a failure in STAT3 signaling.

In our in vitro cell culture experiments, we required a relatively high concentration of Δ9 protein to inhibit IL-23R signaling and the expression of IL-17A and IL-17F. However, the circulating level of Δ9 in a small random selection of healthy individuals was much lower. We believe that the generation of Δ9 through alternative splicing of the IL-23Rα transcript belongs to a negative feedback mechanism that dampens the effect of IL-23 in vivo and prevents excessive inflammatory damage to the body. We speculate that exon 9 skipping and generation of Δ9 likely increases during an inflammatory response, or under inflammatory conditions. This posttranscriptional regulation, exon 9 skipping, not only increases the expression of soluble Δ9 protein but also diminishes the cell surface expression of IL-23Rα (data not shown). Therefore, the amount of Δ9 required to be produced locally may well be less than that needed in vitro, but still considerably higher than that seen in the circulation of a healthy person.

It is of interest to consider the question of Δ9 in the mouse. The intron/exon structure is conserved between mouse and human; in
both species, the IL-23R gene comprises 11 exons. Translation begins in exon 2 and ends in exon 11. In each case, exon 9 encodes the TM region of the mature protein. As we show in this paper, skipping of exon 9 during mRNA splicing results in the introduction of an early translation termination signal, thus generating the Δ9 protein, which contains the extracellular region of IL-23R. In this paper, we have shown that human Δ9 protein regulates human IL-23 signaling. It is very likely that this same regulatory mechanism is also conserved in mice.

In summary, therefore, our results demonstrate the presence of a hitherto unsuspected soluble form of the human IL-23R, Δ9, whose mRNA is spliced from that encoding the native protein and represents up to 20% of human leukocyte IL-23 transcript. This Δ9 is secreted and binds IL-23 in solution, blocking IL-23–induced STAT3 phosphorylation. Furthermore, naturally-secreted Δ9 suppresses the secretory phenotype of human Th17 cells, demonstrating the existence of a regulatory mechanism for human Th17 cells. We anticipate that understanding the function of Δ9 will provide insight to the mechanisms of human Th17-dependent conditions such as Crohn’s disease, asthma, and psoriasis. IL-23 is a target for anti-inflammatory therapies, particularly in the intestine; Δ9 may represent a tool in this process.

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Disclosures

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References


