

Specific post-transcriptional inhibition of mRNA for ligand binding chain of IgE high affinity receptor

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Abstract IgE high affinity receptor (FcεRI) plays an important role in triggering type I allergic reactions. In this study, we have investigated the ability of four synthetic and sequence-specific RNA interfering antisense oligodeoxynucleotides (AS-ODNs) to reduce the expression of FcεRIα gene in granulocytes of allergy sufferers in vitro. Only AS1 out of four AS-ODNs specifically inhibited the FcεRIα gene expression and the dose response assay revealed that AS1 was capable of specific inhibition of target mRNA expression over a linear concentration range without affecting the expression of house keeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Together, these results indicate that sequence-specific RNA interfering ODNs can be effectively used to silence the expression of key genes like IgE high affinity receptor that are involved in chronic inflammatory diseases.

Keywords High affinity receptor of IgE (FcεRI) · Alpha subunit of FcεRI (FcεRIα) · Antisense oligodeoxynucleotides (AS-ODNs)

Introduction

Allergic reactions are responses to antigenic stimuli that result in immunity based mainly on the IgE class of immunoglobulin. Exposure to the antigen (or allergen) triggers an IgE-mediated release of inflammatory mediators that cause symptoms that range from sneezing and dermatitis to inflammation of the airways in an asthmatic attack. Up to 70% of all asthma cases involve IgE-mediated reactions as major pathogenic mechanisms [1]. According to several epidemiological studies, conducted in different parts of the world, the prevalence of allergic diseases has increased dramatically (twofold to fourfold) during recent decades [2, 3]. More than 20% of the general population suffers from allergic diseases in one form or another and the alarming increase in their prevalence over the past decades have placed them among the most common human diseases in industrialized countries. An estimated 40 million Americans (nearly 25% of the population) have allergic diseases [4].

FcεRI plays a central role in triggering the IgE-mediated allergic reaction [5]. FcεRI is expressed in limited types of cells such as mast cells, basophils [6] eosinophils [7], monocytes [8], Langerhans cells [9, 10], platelets [11, 12] and neutrophils [13]. Cross-linking of FcεRI on effector cells by antigen (allergen)–IgE complexes initiates a coordinated sequence of biochemical and morphologic events that result in exocytosis of secretory granules containing histamine and other preformed mediators; synthesis and secretion of newly formed lipid mediators such as prostaglandins and leukotrienes; and synthesis and secretion of cytokines. Together, these mediators are responsible for the majority of the clinical symptoms associated with acute IgE-associated allergic reactions (early-phase reaction), and also contribute to the development of late phase reactions and chronic allergic inflammation [14]. In mast cells and

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basophils, FcεRI has a tetrameric structure composed of a single ligand-binding α chain, a single β chain, and two identical disulfide-linked γ chains [15]. As deduced from transfection experiments, all three subunits of rodent FcεRI must be present for efficient cell surface expression [16]. The tetrameric receptor is the only form found in rodent cells and is also expressed on human basophils and mast cells. Human basophils and mast cells also express a trimeric form of the receptor that is missing the β subunit, and other human cell types express only the trimeric form [17].

Antisense agents are valuable tools to inhibit the expression of a target gene in a sequence-specific manner, and may be used for functional genomics, target validation and therapeutic purposes. Theoretically, antisense molecules could be used to cure any disease that is caused by the expression of a deleterious gene, e.g., viral infections, cancer growth and inflammatory diseases [18]. The concept underlying antisense technology is relatively straightforward: the use of a sequence, complementary by virtue of Watson-Crick base pair hybridization, to a specific mRNA can inhibit its expression and then induce a blockade in the transfer of genetic information from DNA to protein. The difference between antisense approaches and conventional drugs is that the conventional drugs bind to proteins and thereby modulate their function whereas antisense agents act at the mRNA level, preventing its translation into protein [19, 20]. In the current study, IgE high affinity receptor alpha subunit (FcεRI α) was down-regulated at the post transcriptional level. For this purpose, four sequence-specific AS-ODNs targeting different regions of FcεRI α mRNA were utilized during in vitro culturing of granulocytes from allergy sufferers.

Materials and methods

For this study, blood samples from patients suffering from IgE-mediated allergy who were either untreated or who had not received treatment in the last 6 months, were collected. For this purpose, services of a number of local hospitals were utilized. Local research ethics committee's permission and individual informed consent were obtained. Healthy non-allergic individuals were included in the study for the comparison of FcεRI α expression in allergic and non-allergic

cases. All blood samples were drawn between 9 and 11 a.m. to minimize the effects of circadian variation on the endpoints assayed. PBMCs and granulocytes were isolated from corresponding blood samples by using Histopaque-1119 (Cat.No.1119-1, Sigma-Aldrich, St. Louis, MO, USA) and Histopaque-1077 (Cat.No.1119-1, Sigma-Aldrich). Granulocytes were utilized for further study. The cells were counted on hemocytometer by using trypan blue.

IgE High Affinity Receptor Alpha subunit (FcεRI α) mRNA was chosen to be targeted by antisense oligonucleotides for post-transcriptional silencing. The sequence of alpha chain mRNA of FcεRI was obtained from GenBank (NM_002001). To determine whether observed biological effects result from an antisense mechanism and not from nonspecific effects; experiments were performed with adequate controls. A total of four specific phosphorothioate internucleotide bond modified antisense oligodeoxynucleotides (AS-ODNs) and one nonspecific ODN which served as control were obtained from Gene Link (Hawthorne, NY, USA). The relevant information has been summarized in Table 1.

The cells were transfected with corresponding AS-ODNs in RPMI medium 1640 (Cat.No.R4130, Sigma-Aldrich) supplemented with sodium bicarbonate (2 mg/ml), FBS (2%), penicillin (100 units/ml) and streptomycin (100 μ g/ml), at a density of 2 million cells/ml in each well of culture plate. The transfection mixture was prepared by mixing corresponding AS-ODN with transfection reagent (TransFectin Lipid Reagent, Cat.No. 170-3351, BioRad, Hercules, CA, USA) according to manufacturer's specifications. After transfection, cells were incubated at 37°C and 5% CO₂ for 4–6 h. After incubation, serum concentration was increased to 10% and the cells were further incubated for 12–14 h. Subsequently, the cells were harvested for total RNA.

Total RNA was extracted by using the Tri-reagent (Cat.No.TR118, Molecular Research Center, Inc., Cincinnati, OH, USA). About 500 μ l of the Tri-reagent was used per 5×10^6 cells. RNA pellet was solubilized in 1% DEPC (diethyl pyrocarbonate)-treated water and cDNA was synthesized by using RevertAidTM First Strand cDNA Synthesis Kit (Cat.No.K1622, Fermentas, St. Leon-Rot/Germany) in a final volume of 20 μ l. The reaction mixture was incubated at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min and then chilled on ice.

Table 1 The sequences and locations of AS-ODNs on target mRNA

AS-ODNs	Sequence	Position on mRNA
AS1	5'- <u>c a g</u> g a g c c a t c t t c t t c <u>a t g</u> -3'	89–109
AS2	5'- <u>a c a</u> c a t c c c a g t t c c t c <u>c a a</u> -3'	501–521
AS3	5'- <u>t t g</u> a g t a a t t a t a t c a g <u>t t g</u> -3'	867–887
AS4	5'- <u>g g c</u> a g g a g c c a t c t t c t <u>t c a</u> -3'	92–112
ASN	5'- <u>a a g</u> c t g g c t c c a g a g a t t <u>t t c</u> -3'	Non-specific control

Bold and underlined nucleotides have phosphorothioate (S-form) linkages, the rest have phosphodiester linkages

Table 2 Primers used for amplification of FcεRIα and GAPDH cDNA by PCR

No.	Primers	Sequences (5'-3')	Melting temperature (T_m) (°C)	% GC content	Length (in nucleotides)
1	FcεRIα-F	5'-gcagcctttcagaagagaca-3'	58.3	50	20
2	FcεRIα-R	5'-ctgaagcctttctgttct-3'	58.5	50	20
3	GAPDH-F	5'-ctgccgtctagaaaaacctg-3'	58.0	50	20
4	GAPDH-R	5'-tcttctcttgctcttgc-3'	58.6	50	20

The first strand cDNA synthesized was used directly for amplification by PCR (polymerase chain reaction). For amplification of cDNA of FcεRIα and GAPDH (a house keeping gene served as internal control) by PCR, specific forward and reverse primers were used. The primer pair for FcεRIα amplified a product of 529 bp and the primer pair for GAPDH amplified a product of 313 bp. The primer sequences, their corresponding melting temperatures and %GC content are summarized in Table 2.

The amplification of FcεRIα and GAPDH cDNA was accomplished via PCR by utilizing the 2× PCR Master Mix (Cat.No.K0171, Fermentas). PCR was performed by initial denaturation at 95°C for 5 min followed by 35 cycles, each of denaturation at 94°C for 2 min, annealing at 55°C for 45 s, and extension at 72°C for 45 s, with final extension at 72°C for 10 min. The amplified DNA samples were run on 1% agarose gel. The DNA bands were visualized directly under the UV and the photographs of the gels were obtained with gel documentation system.

Results

Expression of FcεRIα gene

For comparative expression study of FcεRIα gene, three normal and three IgE-mediated allergic sufferers' blood samples were taken. RT-PCR was performed on the total RNA extracted from granulocytes to amplify the FcεRIα

cDNA by using specific primers (Fig. 1a). The amplified product was resolved on 1% agarose gel (Fig. 1b). The 529 bp FcεRIα specific band intensities in case of allergic sufferers (lane 2–4) were high as compared to band intensities in samples from normal individuals (lane 5–7). Upregulated FcεRIα gene expression in allergic sufferers as compared to its expression in normal non-allergic individuals showed that it could be a potential target for RNA interfering ODNs.

Targeting of FcεRIα mRNA by AS-ODNs

The FcεRIα mRNA was targeted for degradation by using four different sequence-specific synthetic RNA interfering ODNs (Fig. 2a). The potential of RNA interfering activity of all four AS-ODNs is shown in Fig. 2b. The expression of FcεRIα gene remained as such in control without ODN (lane 2) and control with non-specific ODN (lane 7), but was reduced in the presence of AS1 and AS2 (lane 3 and lane 4) indicating their potent RNA interfering activity. AS1 efficiently reduced the expression while AS2 was found to be less effective. In lane 5 and lane 6 appearances of PCR product bands indicated no RNA interfering activity of AS3 and AS4, respectively. For ruling out any nonspecific inhibition by AS-ODNs targeting FcεRIα, expression of GAPDH was also observed. Appearance of almost equal intensity bands for GAPDH in all lanes ruled out the non-specific anti-RNA activity of the four ODNs on other genes' expression (Fig. 2c).

Fig. 1 **a** Positions of primers on FcεRIα cDNA showing a product size of 529 bp. **b** Comparative expression of FcεRIα in granulocytes of allergic and healthy non-allergic individuals with lane 1 showing 100 bp ladder while lanes 2–4 showing FcεRIα gene expression in allergy sufferers and lanes 5–7 in healthy non-allergic individuals

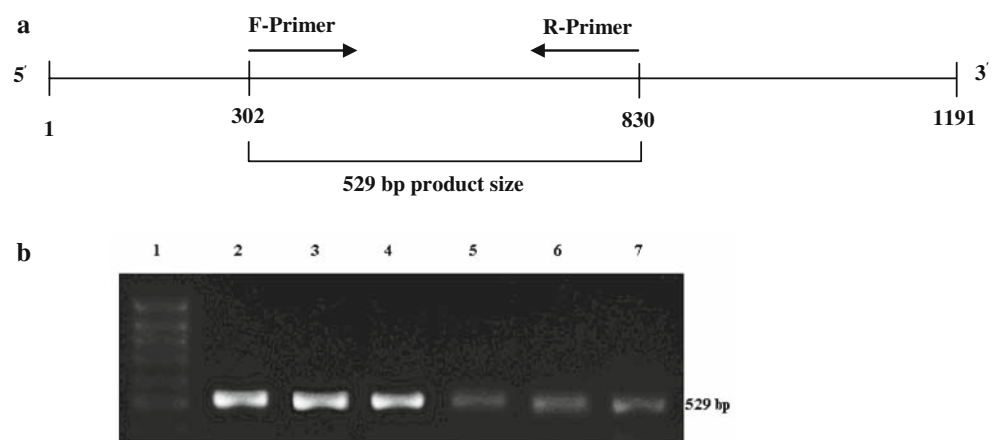
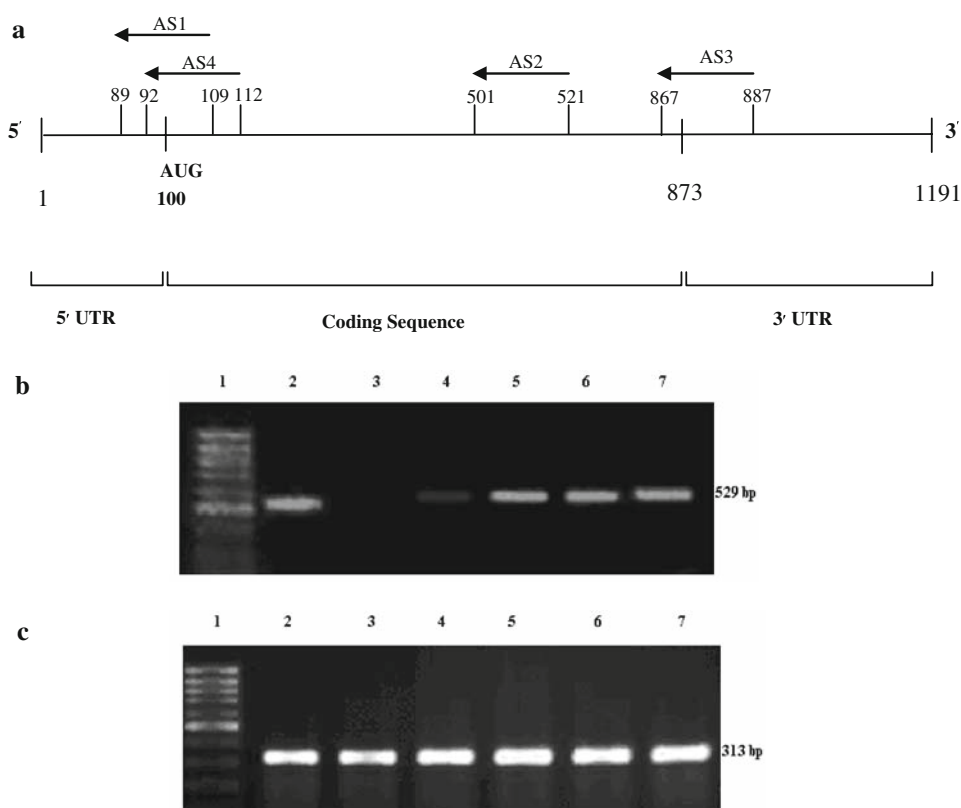


Fig. 2 **a** Positions of AS-ODNs on mRNA of FcεR1α. **b** Effect of synthetic sequence-specific AS-ODNs on FcεR1α mRNA in granulocytes of IgE-mediated allergy sufferers. *Lane 1* shows 100 bp ladder and *lane 2* shows control without AS-ODN. *Lanes 3–6* show effect of four synthetic sequence-specific AS-ODNs AS1, AS2, AS3 and AS4 on FcεR1α mRNA, respectively. *Lane 7* shows effect of non-specific ODN (ASN) on FcεR1α mRNA. **c** Effect of AS-ODNs on GAPDH (served as internal control) with *lane 1* showing 100 bp ladder and *lane 2* showing control without AS-ODN while *lanes 2–6* show effect of AS-ODNs AS1, AS2, AS3 and AS4 on GAPDH mRNA, respectively. *Lane 7* shows effect of non-specific ODN (ASN) on GAPDH



Dose response assay of AS1

For further study of dose-response assay, AS1 oligodeoxynucleotide showing maximum inhibition of target mRNA was selected. The expression of FcεR1α was analyzed by tenfold serial dilutions of AS1 ranging between 1 nM and 1 μM. Controls (without AS-ODNs and non-specific ODNs) were also included. As shown in Fig. 3a, AS1 reduced the expression of FcεR1α gene in cultured granulocytes from allergy sufferers in dose-dependent fashion over a sub-micro molar concentration (lane 3–6), showing maximum reduction at 1 μM (lane 6). Non-specific control at this concentration did not affect FcεR1α expression (lane 7). GAPDH, served as an internal control, showed no effect of AS1 on its expression (Fig. 3b). The dose response assay was performed in three repetitions to confirm the specificity and reproducibility of data. Altogether, these results indicate that AS1 is capable of specific inhibition of FcεR1α mRNA over a linear concentration range.

Discussion

Allergic reactions represent, without doubt, an increasing healthcare problem in industrialized countries where an increasing incidence of IgE-mediated allergies such as rhinitis, conjunctivitis, bronchial asthma and atopic

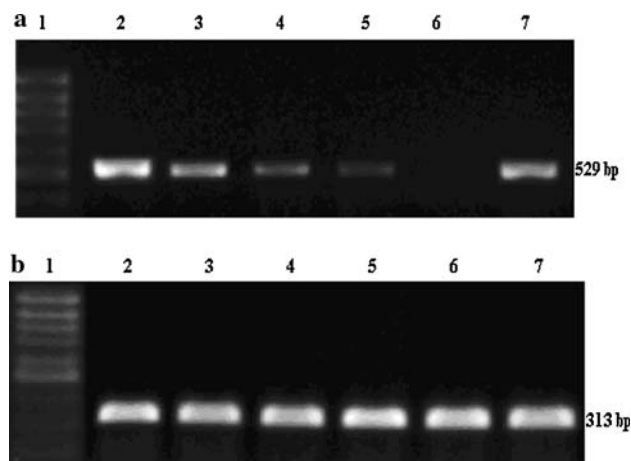


Fig. 3 **a** Dose response assay of AS1 at different concentrations. *Lane 1* shows 100 bp ladder and *lane 2* shows control without AS-ODN. *Lanes 3–6* show the effect of AS1 in concentrations of 1 nM, 10 nM, 100 nM and 1 μM on FcεR1α mRNA, respectively. *Lane 7* shows effect of non-specific ODN (ASN) on FcεR1α. **b** Effect of different concentrations of AS1 on GAPDH (served as internal control). *Lane 1* shows 100 bp ladder and *lane 2* shows control without AS-ODN. *Lanes 3–6* show effect of AS1 in concentrations of 1 nM, 10 nM, 100 nM and 1 μM on GAPDH mRNA, respectively. *Lane 7* shows effect of non-specific ODN (ASN) on GAPDH mRNA

eczema, entails a healthcare burden to society in the order of billions of US dollars [21]. IgE is the major immunoglobulin involved in type I hypersensitivity and IgE high

affinity receptor (FcεRI) plays a central role in triggering the IgE-mediated allergic reaction. The aggregation or bridging of IgE-bound FcεRI is sufficient for initiating downstream signal transduction events that lead to manifestation of an allergic response [22].

In the present study, gene expression of FcεRI alpha subunit, the ligand binding chain of the receptor, is compared in granulocytes from allergic and non-allergic individuals *in vitro*. FcεRIα gene expression is found to be upregulated on granulocytes of allergic individuals as compared to its expression in healthy non-allergic persons. Elevated FcεRI expression on peripheral blood basophils, monocytes, and eosinophils in atopic subjects suggests a role for these receptors in IgE-mediated allergic inflammation [23].

Several strategies for sequence-specific post-transcriptional inhibition of gene expression can be distinguished including small interfering RNA (siRNA) and antisense oligodeoxynucleotides (AS-ODNs). The siRNA molecules bind to a protein complex, termed RNA-induced silencing complex (RISC). It contains a helicase activity that unwinds the two strands of RNA molecules, allowing the antisense strand to bind to the targeted RNA molecule and an endonuclease activity that hydrolyzes the target RNA at the site where the antisense strand is bound [24, 25]. Multiple mechanisms exist by which short synthetic oligonucleotides can be used to modulate gene expression in mammalian cells [26]. A commonly exploited antisense mechanism is RNase H-dependent degradation of the targeted RNA. RNase H is a ubiquitously expressed endonuclease that recognizes a DNA-RNA heteroduplex, hydrolyzing the RNA strand [27, 28]. The studies have suggested that in human cell culture assays, double-stranded oligoribonucleotides that work by siRNA mechanism exhibit similar potency, efficacy, specificity, and duration of action as RNase H oligonucleotides [29].

Potential anti-mRNA drugs in asthma and other allergic disorders may be targeted to cell surface receptors like Adenosine A1 receptor, cytokine receptors, etc. [30]. In order to identify potent antisense agents, many investigators design and test multiple oligonucleotides that target different sites and regions of the target mRNA and often only a few out of many show desired results [31]. We have designed four sequence-specific AS-ODNs targeting different regions of FcεRIα mRNA during *in vitro* culturing of granulocytes from allergy sufferers. Only one (AS1) out of all four AS-ODNs shows the desired RNA interfering activity on target mRNA.

AS1 successfully targets the FcεRIα mRNA for degradation. It is designed to target the 5'-UTR including AUG spanning region of FcεRIα mRNA. The rationale behind the design of effective antisense oligodeoxynucleotides is based on notion that mRNA is single stranded at the AUG

site—to allow ribosomal entry—and thus this site should also be accessible for oligonucleotide hybridization. Oligodeoxynucleotides targeting the start codon have been used successfully to silence the expression of several genes [32]. The reduction in FcεRIα mRNA expression may be mediated by RNase H dependent mechanism as has been shown in case of many other AS-ODNs. The primary mode of action for most antisense oligodeoxynucleotides is the creation of a substrate for endogenous RNase H leading to directed cleavage of the RNA portion of the oligodeoxynucleotide:RNA duplex [33, 34].

The dose response assay of AS1 in our study shows that this synthetic antisense ODN dose-dependently inhibits expression of mRNA of IgE high affinity receptor showing maximum reduction of target gene at a higher dose of 1 μM. The high mRNA levels of alpha subunit of FcεRI in granulocytes from allergic individuals require a high concentration of AS1 to target all the available mRNA. Oligonucleotides in their natural form as phosphodiester are subject to rapid degradation in biological systems by exonucleases and endonucleases. The phosphorothioate oligonucleotides have greater stability in biological systems than normal oligonucleotides [35]. The present study also involved the use of phosphorothioate modified AS-ODNs in which first three and last three nucleotides in each AS-ODN have phosphorothioate linkages (S-form).

Numerous reports have demonstrated that naked oligonucleotides are internalized poorly by cells whether or not they are negatively charged. More specifically, naked oligonucleotides tend to localize in endosomes/lysosomes [35]. As the ability of AS-ODNs which are negatively charged to access specific target site on mRNA of interest are the limiting factors *in vitro*, liposome based transfection reagent for the delivery of all four AS-ODNs to the cells was employed in this investigation. These liposomes, because of their positive charge, have high affinity for cell membranes, under physiological conditions [36].

Involvement of the α-chain in FcεRI-mediated allergic reaction was definitively proved by the absence of the allergic reaction in α-chain-deficient mice [5]. The binding itself of monomeric IgE to FcεRI was shown to promote the survival of mast cells without cross-linking of the receptor [37, 38], also suggesting that an increase in FcεRI α-chain on the cell surface accelerates the IgE-mediated allergic reaction. Recently, three single nucleotide polymorphisms were detected in the 5' flanking region of the FcεRI alpha chain gene, although no variants were detected in the gene itself. The most common was a T/C translocation at −335 bp before the translational start site [39–41]. In the current study, none of the designed ODNs included these suspected regions of polymorphism. Moreover, if the α-chain of the IgE high affinity receptor is silenced at post-transcriptional level, there would be no

chance of IgE–FcεRIα interaction even in the presence of high levels of IgE which could lead to allergic cascade.

In conclusion, our results demonstrate that AS1 can specifically inhibit mRNA of FcεRIα during in vitro culturing of granulocytes. Further studies can be carried out to determine the functional efficacy of AS1 in the inhibition of effector cells degranulation and release of inflammatory mediators such as histamine, leukotrienes and prostaglandins, which are of pharmaceutical importance in the treatment of human allergic disorders.

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This erratum has been initiated as discrepancies with authors were observed after publishing.

Mr. Muhammad Imran was wrongly included in the author group, and is now to be read in the acknowledgement section.

“Centre of Excellence in Molecular Biology, University of the Punjab, LHR” has been added and should be read in the acknowledgement section.

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