Clinical effects of actarit in rheumatoid arthritis: Improvement of early disease activity mediated by reduction of serum concentrations of nitric oxide

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Abstract

Objective

We previously reported the presence of high serum concentrations of nitric oxide (NO) in patients with rheumatoid arthritis (RA). In this study we evaluated the effect of actarit on patients with early and advanced stages of RA and the relationship between RA activity and serum NO levels.

Methods

Thirty-seven RA patients who were undergoing care at Sasebo Chuo Hospital were entered into the study. Patients were divided into two groups based on the severity of their disease: group I (stages I and II) and group II (stages III and IV). NO concentrations in serum samples were measured by the chemiluminescence method.

Results

Morning stiffness, the number of tender and swollen joints, grip strength, pain score, modified Health Assessment Questionnaire score (mHAQ), ESR, CRP and the Lansbury index significantly improved during 24 weeks of treatment in group I. Patients in group II did not show improvement in morning stiffness, pain score, ESR or CRP during treatment. The concentrations of NO in group I were significantly reduced at 8 weeks after administration of actarit. Those in group II showed a delayed response; a significant decrease in NO occurred at 20 weeks. The improvement in the number of tender and swollen joints, grip strength, pain score, mHAQ and Lansbury index noted in group I preceded the fall in NO concentrations.

Conclusion

Our results demonstrate that actarit improves disease activity in early phase RA by suppressing serum NO levels. The results suggest that NO is a useful marker for monitoring improvement in the early stages of RA.

Key words

Actarit, NO, rheumatoid arthritis, cytokines.

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Abbreviations

DMARDs: disease modifying anti-rheumatic drugs; ESR: erythrocyte sedimentation rate; mHAQ: modified Health Assessment Questionnaire; NO: nitric oxide; RA: rheumatoid arthritis.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease whose articular and systemic manifestations are thought to be governed by immunological processes within the synovial tissue (1). Recent recommendations for the treatment of RA have focused on early and continued use of disease modifying anti-rheumatic drugs (DMARDs), nonsteroidal anti-inflammatory drugs (NSAIDs), and corticosteroid (2).

Actarit is a newly developed effective DMARD for RA (3). The pharmacological actions of this drug include the inhibition of collagen-induced arthritis via suppression of the allergic reaction (type IV), and inhibition of the production of rheumatoid factor or anti-DNA antibody in MRL/lpr mice (4). Clinical expression of its effect appears 2-3 months after commencement of administration, similar to other DMARDs. Recently, Takahashi *et al.* (5) demonstrated that actarit may be more effective in patients with RA in the early stages of the disease during which inflammation was still not of a severe degree. In contrast, actarit has no effect on acute inflammation such as carrageenin edema in rats. The precise mechanism underlying the improvement in immunological abnormalities seen in RA patients treated with actarit is not well understood.

The small and simple molecule, nitric oxide (NO), has several important physiological and pathological effects including inflammation and immunity. NO exists as an unstable radical under aerobic conditions and is mainly produced by macrophages and endothelial cells (6). Expression of this molecule is regulated by various inflammatory cytokines such as TNF-α, IL-1β and IFN-γ (7), which are abundantly present in the rheumatoid synovium. We have previously reported that NO was an important chemical mediator possibly involved in the pathogenesis of RA (8) and that high concentrations of NO in RA patients correlated with RA disease activity. Despite the circumstantial evidence for NO as a pathogenic mediator in RA, the correlation between *in vivo* NO levels and immunological improvement after treatment has not been examined in patients with RA.

In the present study, we investigated the effect of actarit on endogenous NO production in patients with RA as a mechanism of improvement of immunological abnormalities. We used a highly sensitive chemiluminescence method in this study to measure the concentrations of serum NO.

Materials and methods

Patients

We studied 37 RA patients (4 men and 33 women; mean age; 56.7 ± 1.93 (±SD), range; 26-75 years) who met the RA criteria of the American College of Rheumatology (Table I) (9). Patients were divided into two groups based on the se-

Table I. Characteristics of RA patients entered in this study.

Variables		Group I (Stage I, II)	Group II (Stage III, IV)
No.		Stage I (n=0), Stage II (n=20)	Stage III (n=5), Stage IV (n=12)
Sex (M/F)		4/16	1/16
Age (yrs)		53.1 ± 13.4	60.1 ± 10.3
Disease duration	< 3 years	15	1
	3-5 years	1	2
	> 5 years	4	14
	average	3.1 ± 3.0	11.0 ± 6.6
Class	1	5	0
	2	15	14
	3	0	3
	4	0	0

Data represent the mean ± SD of each group. Staging and class were defined by Steinbrocker.

verity of their disease process. Group I consisted of 20 patients classified as stage II RA. This staging was defined by Steinbrocker. Group II consisted of 5 patients with stage III and 12 patients with stage IV RA. The mean duration of RA in these two groups of patients was 3.1 ± 3.0 and 11.0 ± 6.6 years, respectively. Patients were selected at random from those attending the out-patient clinic at the Department of Internal Medicine of Sasebo Chuo Hospital, Sasebo, Japan.

At the time of the study, all patients were being treated with NSAIDs and 18 patients were being treated with DMARDs other than actarit (1 was receiving methotrexate, 3 auranofin, 5 D-penicillamine, 4 bucillamine, 4 sulfasalazopyridine and 1 tiopronin). Nineteen patients with RA had not been previously treated with other DMARDs for six months. Six patients continued to receive DMARDs throughout the study period at the same doses prescribed previously. The administration and dosage of other medications (e.g., NSAIDs and prednisolone) were kept constant throughout the study and for a pre-entry observation period of 3 months. The maximum dose of prednisolone was 5.1 mg in this study.

Actarit was administered at 300 mg/day and was used throughout the study period. The duration of treatment was 24 weeks. During this study there were no significant adverse reactions requiring the withdrawal of treatment. The characteristics of patients are summarized in Table I. Informed consent was obtained from all participating patients and the study was conducted in accordance with the human experimentation guidelines of our institution.

Evaluation of RA disease activity

We used various clinical and laboratory variables to evaluate the effects of actarit on RA activity. These variables consisted of morning stiffness, the number of tender or swollen joints, grip strength, pain score, modified Health Assessment Questionnaire (mHAQ) (10), erythrocyte sedimentation rate (ESR, in mm/h), CRP and the Lansbury index. Grip strength was measured using a mercury barometer. Briefly, its indicator must first be inflated to 20 mmHg. After patients held it firmly, we measured the height of the barometer. The pain score was calculated by the Likert method, in which the score was divided into 5 degrees. To calculate the Lansbury Index we used a simple and easy method in which the duration of fatigability and the dose of aspirin were excluded.

NO assay (Chemiluminescence method).

The concentration of NO in blood samples was determined by chemiluminescence, using a highly sensitive NO measurement system (FES-450, Scholar-Tee Co., Osaka, Japan) (8). In the present study, the plasma concentration of NO represented that of the NO content after the reduction of both nitrite and nitrate to NO using the Vanadium (III) reagent. Nitrites are rapidly reduced to NO at room temperature and nitrates are rapidly reduced at 80-95°C by this reagent. We added 10 µl of plasma samples to a glass syringe that was then sealed with a rubber sleeve-septum. Then 1 ml of vanadium (III) solution in approximately 0.1 M in 1 M HCl was added immediately to the plasma sample using a syringe and the mixture was incubated at 95°C for 10 min. During subsequent incubations, the glass syringe was kept at 95°C in a water bath. NO was stripped from the sample to a gaseous phase in a reaction chamber, and N_2 gas was drawn through one needle into the sample and the headspace gas was then directed via a vacuum through a second needle directly into the NO analyzer and reacted with O_3 gas. The absolute concentration of NO in the sample was determined using a reference standard solution of sodium nitrate. This method is extremely sensitive and can measure picomolar quantities of NO using 10 µ1 of plasma (8).

Statistical analysis

Data were expressed as means ± SD. Differences between groups were examined for statistical significance using the Mann-Whitney test. A P value less than 0.05 denoted the presence of a statistically significant difference.

Results

Improvement in various variables of RA activity after treatment with actarit

We initially examined the clinical effects of actarit administered for 24 weeks (Table II). Duration of morning stiffness diminished from 67.5 ± 9.0 and 63.5 ± 8.9 minutes at the beginning of actarit treatment to 38.0 ± 7.7 and 47.6 ± 8.2 minutes after 24 weeks of treatment in groups I and II, respectively. The number of tender joints decreased from 12.7 ± 2.0 and 7.6 ± 1.4 to 6.0 ± 1.4 and 4.2 ± 0.4 in groups I and II, respectively. The number of swollen joints diminished from 0.9 ± 0.3 and 0.9 ± 0.6 to 0.1 ± 0.1 and 0.5 ± 0.2 in groups I and 1I, respectively. Grip strength improved from 167.4 ± 14.6 and 124.4 ± 16.5 mmHg to 201.9 ± 14.5 and 143.0 ± 17.0 mmHg in

Table II. Clinical variables for the RA patients in Group I and Group II at 0 week and 24 weeks later.

Variables	Group I (Stages I and II)		Group II (Stages III and IV)	
	0 week	24 weeks later	0 week	24 weeks later
Morning stiffness (min)	67.5 ± 40.0	38.0 ± 7.7	63.5 ± 36.6	47.7 ± 8.2
Grip strength (mmHg)	167.4 ± 65.3	201.9 ± 14.5	124.4 ± 68.0	143.0 ± 17.0
Pain score (1-5 degrees)	1.6 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	1.2 ± 0.1
mHAQ (points)	10.6 ± 0.9	7.5 ± 0.8	12.4 ± 1.0	10.6 ± 1.1
No. of tender joints (counts)	12.7 ± 2.0	6.0 ± 1.4	7.6 ± 1.4	4.2 ± 0.4
No. of swollen joints (counts)	0.9 ± 0.3	0.1 ± 0.1	0.9 ± 0.6	0.5 ± 0.2
ESR (mm/h)	52.8 ± 6.9	41.7 ± 6.9	75.1 ± 7.3	65.8 ± 5.6
CRP (mg/dl)	2.2 ± 0.6	1.3 ± 0.4	3.6 ± 0.6	2.9 ± 0.6
Lansbury index (%)	43.6 ± 4.0	28.2 ± 3.4	57.9 ± 3.6	50.2 ± 3.7
NO concentration (µM/L)	52.4 ± 3.0	44.2 ± 3.1	63.6 ± 2.8	49.7 ± 4.2

Data represent the mean ± SD for each group. Staging was defined by Steinbrocker. mHAQ: modified Health Assessment Questionnaire, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein.

groups I and 1I, respectively. The pain score diminished from 1.6 ± 0.1 and 1.4 ± 0.1 to 1.1 ± 0.1 and 1.2 ± 0.1 in groups I and II, respectively. mHAQ improved from 10.6 ± 0.9 and 12.4 ± 1.0 to 7.5 ± 0.8 and 10:6 ± 1.1 in groups I and II, respectively. ESR improved from 52.8 ± 6.9 and 75.1 ± 7.3 mm/h to 41.7 ± 6.9 and 65.8 ± 5.6 mm/h in groups I and II, respectively. CRP decreased from 2.2 ± 0.6 and 3.6 ± 0.6 mg/dl to 1.3 ± 0.4 and 2.9 ± 0.6 mg/dl in groups I and II, respectively. The Lansbury index changed from 43.6 ± 4.0 and 57.9 ± 3.6 to 28.2 ± 3.4 and 50.2 ± 3.7 in groups I and II, respectively. All variables improved significantly after 24 weeks of treatment. However, as shown in Figure 1, the patients in group II did not show a significant improvement in morning stiffness, the pain score, ESR or CRP during the 24 weeks of treatment.

Effect of actarit on serum NO levels

Figure 2 demonstrates changes in serum NO concentrations in groups I and II during treatment with actarit. The mean concentration of NO was 52.4 ± 3.0 μM/L in group I and 63.6 ± 2.8 μM/L in group II at the beginning of treatment. In group I, actarit significantly reduced NO levels after 8 weeks of treatment and the effect was persistently seen at 24 weeks (44.2 ± 3.1 μM/L), with the exception of the concentration at 12 weeks (Fig. 2). In contrast, NO concentrations in group II significantly decreased after 20 and 24 weeks of treatment (Fig. 2).

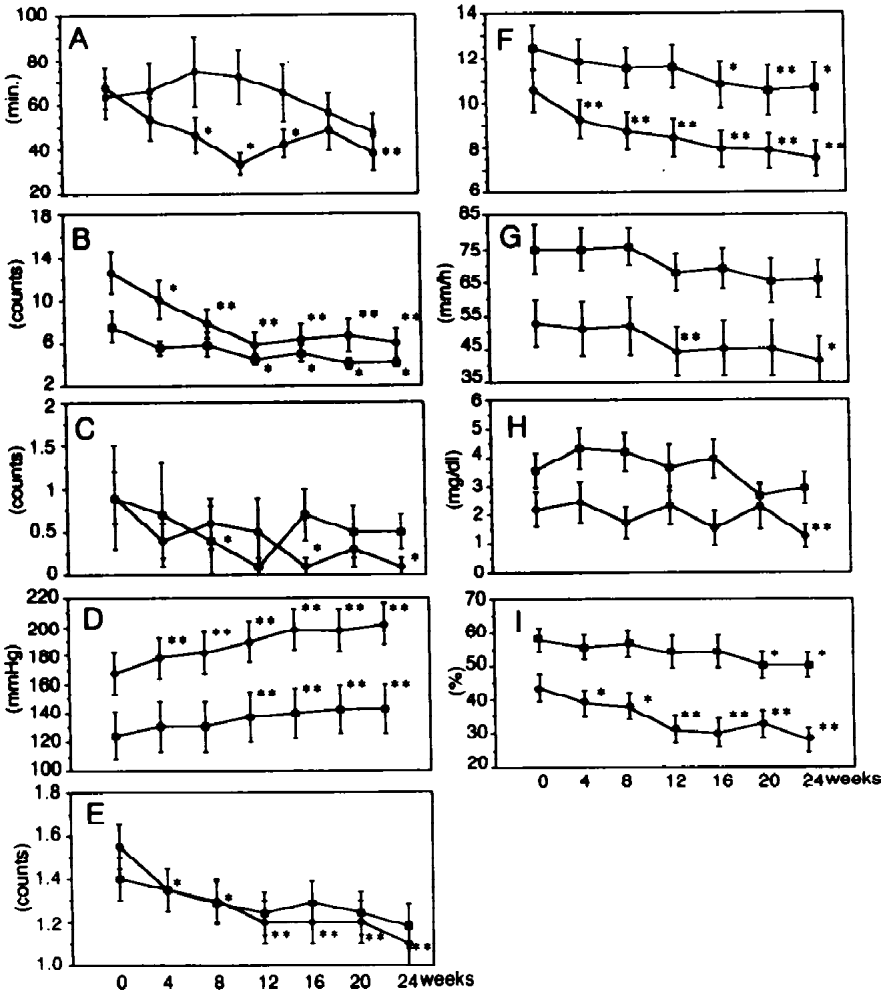

Fig. 1. Improvement of RA activity markers during 24 weeks of actarit therapy. X axis indicates duration of actarit treatment (weeks). Y axis indicates as follows: A: morning stiffness, B: number of tender joints, C: number of swollen joints, D: grip strength, E: pain score, F: mHAQ, G: ESR, H: CRP, I: Lansbury Index. ●: group I;■: group II. Data represent the mean ± SD of 37 RA patients. *p < 0.05, **p < 0.01 versus baseline data.

Relationship between the NO levels in the sera of RA patients and hemoglobin levels

Finally, we examined the relationship between serum NO levels and hemoglobin levels, because NO is trapped by hemoglobin which can therefore affect serum NO concentration (Fig. 3). There was no significant relationship between serum NO levels and hemoglobin although there was a slight tendency toward an inverse relationship between the two variables. In addition, there was positive relationship between NO and CRP levels (data not shown).

Discussion

In the present study, high concentrations of NO were detected in the sera of RA patients; however, the levels were significantly diminished by oral administration of actarit at 8 and 20 weeks, depending on the severity of the disease. Various clinical parameters in the patients with early stage RA, including grip strength, the number of tender or swollen joints, morning stiffness, the modified Lansbury index, and mHAQ, improved following treatment with actarit, although this improvement was more marked in patients with mild RA disease activity.

To examine the mechanism of improvement in these variables, we measured plasma NO levels using a highly sensitive method. Our results showed that actarit significantly reduced the NO level. These changes paralleled the improvement in RA disease activity represented by the above variables. It should be noted, however, that some variables improved earlier than the measured reduction in NO concentration, particularly in those RA patients with early stage disease.

NO is a physiologically important molecule and is also involved in inflammatory and other pathological processes. Articular chondrocytes and synovial fibroblasts synthesize substantial amounts of NO and the inducible form of nitric oxide synthase (iNOS) appears to be responsible for this enhanced synthesis. Furthermore, infiltrating neutrophils, lymphocytes, mast cells and especially macrophages in synovial tissues of inflamed joints are additional potential

sources of NO. We have previously demonstrated that NO levels significantly correlate with various indices of disease activity in patients with RA. To our knowledge, there are no previous studies that have examined changes in NO levels induced by actarit therapy.

The reason for the actarit-induced fall in NO concentrations in RA patients is not clear at present. However, a number of possible mechanisms can be derived from the results of previous studies. For example, the levels of various inflammatory cytokines such as TNF-α, IL-1β and IFN-γ in the serum and synovial fluid of RA patients are up-regulated and these cytokines are considered to be inducers of pannus formation in the RA synovium (11). We have previously reported that serum IL-1β levels reflect disease activity in RA (12) and demonstrated that active RA is associated with increased NO and cytokine concentrations (IL-6, TNF-α), compared to a stable disease process. These inflammatory cytokines induce and up-regulate iNOS (13). Although we did not investigate the effects of actarit on serum levels of inflammatory cytokines, it is likely that the inhibition of these cytokines by actarit explains, at least in part, the suppression of NO production.

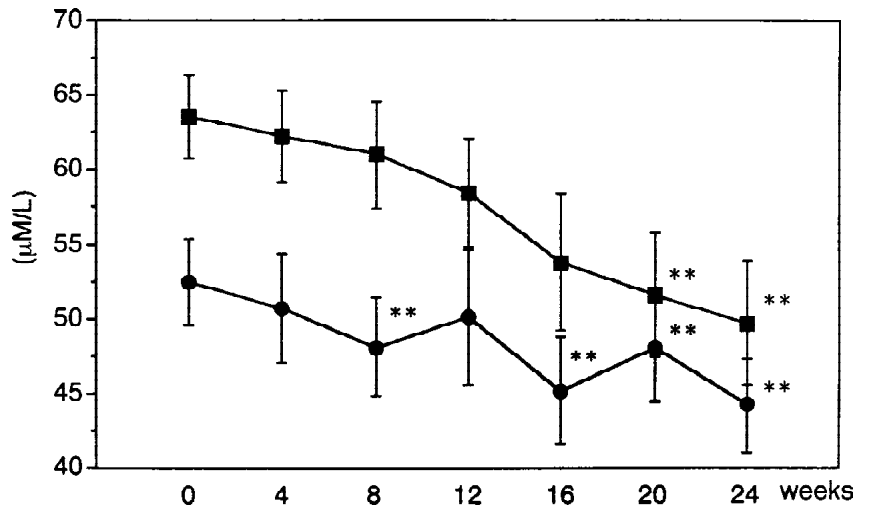

Fig. 2. Effects of 24-weeks actarit treatment on NO concentrations. X axis indicates duration of actarit treatment (weeks). Y axis indicates NO concentration. ●: group I, ■: group II.
Data represent the mean ± SD (μM/L). *p < 0.05, **p < 0.01 versus baseline data. Significant falls in NO concentrations were noted at 8, 16, 20 and 24 weeks in both groups of patients.

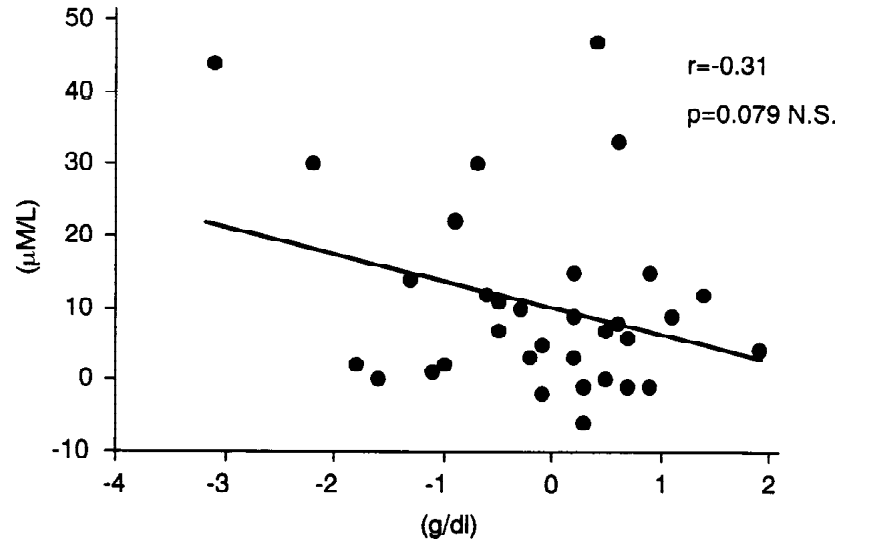

Fig. 3. Relationship between serum NO concentrations and hemoglobin levels. The X axis (hemoglobin) demonstrates the difference in hemoglobin levels between baseline and after 24 weeks of actarit treatment. There was no significant relationship between serum NO levels and hemoglobin, although there was tendency towards an inverse relationship between these variables.

Takeba *et al.* (14) recently reported that actarit acts on RA synovial cells and suppresses cell-to-cell interactions with infiltrating lymphocytes in the synovium and inhibits the production of proinflammatory cytokines and matrix metalloproteinases (MMP). Other investigators showed that NO can directly activate lymphocytes (15) and that natural killer cells and activated killer cells (LAK cells) are activated in the presence of L-arginine. Furthermore, human peripheral blood mononuclear cells stimulated by sodium nitroprusside or S-nitroso-N-acetyl-penicillamine induced NO up-regulated glucose consumption and protein tyrosine kinase ($p56^{lck}$) linked to CD3 or CD8 (15).

Modulation of NO synthesis may represent a new approach to the treatment of inflammatory and autoimmune conditions. Several investigators have reported that a variety of substances presently used in RA therapy interfere with NO activity. For example, auranofin reduces the response of isolated aortas of the rat and rabbit to endothelium-derived relaxing factor (EDRF) (16). In the present study, actarit significantly decreased the level of serum NO in RA patients. However, the relative changes in serum NO levels were quite small (from a mean of 57.5 ± 2.2 to 46.7 ± 2.6 μM, representing about a 10% reduction). Are these changes of any therapeutic benefit? In the present study we examined the relationship between improvement in RA disease activity and the suppression of serum NO, but did not find any direct evidence for an actarit-induced suppression of NO levels. We believe it is important to confirm the direct effect of actarit on endogenous NO formation. Such experiments are currently in progress in our laboratory.

Our study has certain limitations. First, NO diffuses into the vascular lumen, where it is taken up by erythrocytes. In erythrocytes, NO may be inactivated by reacting with venous HbO_2 to nitrate and methemoglobin or with Hb to form HbNO (17). There was no significant relationship between serum NO levels

and Hb levels, although there was a slight tendency toward an inverse relationship between these variables. It is possible that the restoration of Hb may influence the low serum NO in RA patients. Secondly, it is possible that the serial changes in serum NO during actarit therapy may have been influenced directly by the patients' dietary intake of nitrate. The short half-life of NO makes its direct measurement impractical, though in aqueous solutions NO decays to yield equal amounts of nitrite and nitrate (18), which have been used as indices of NO synthesis *in vitro*. It should be noted that the nitrite level is a more sensitive index of endogenous NO production and is less subject to dietary influences than nitrate (19-21). However, in the present study the level of plasma nitrite was very low and we could not detect any change in its level after actarit therapy (data not shown). Therefore, we opted to measure the concentration of serum NO using the chemiluminescence method, which directly measures the gaseous form of NO after converting the nitrate and nitrite in the sample to NO.

In conclusion, we demonstrated in the present study the beneficial therapeutic effects of actarit in patients with early stage RA. These effects were indirectly mediated by inhibition of the production of endogenous NO, probably by suppression of the immune system. Future studies on the direct relationship between NO and the pathogenesis of RA are needed.

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