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MODIFIED IMMUNOGLOBULIN G GLYCOSYLATION PATTERN DURING TURPENTINE-INDUCED ACUTE INFLAMMATION IN RATS

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Abstract Alterations in the pattern of protein glycosylation have been described during inflammation. In chronic parasitic and tumoral diseases we have reported an increase in the proportion of serum Immunoglobulin G (IgG) molecules possessing an altered Fab glycosylation pattern designated asymmetric antibodies. The alteration results in augmented concanavalin A affinity and functional univalence of the antibody. In addition, Fc agalactosylation has been described as occurring in chronically autoimmune diseases. Therefore, the aim of this paper was to evaluate by analyzing sera whether during an acute inflammatory response in rats produced by subcutaneous inoculation of turpentine oil, there was an alteration in the synthesis and glycosylation of IgG (as revealed by concanavalin A binding). We found that during acute inflammation there was a decrease in the synthesis of IgG which was not affected by prior oral administration of dexamethasone; however, the turpentine-induced increase in IgG binding to concanavalin A was found to be inhibited upon prior administration of the anti-inflammatory agent. As with turpentine, the corticoid used induced an increase in the interleukin-6 levels detected in sera by ELISA. Although we have described an improvement in asymmetric antibody synthesis by low dose of interleukin-6 previously, here we found no correlation between the observed glycosylation pattern of IgG and interleukin-6 concentration assessed in sera of treated rats, probably due to a different dexamethasone mediated pathway.

Key words: rats, acute-phase reaction, interleukin-6, blocking antibodies, IgG, glycosylation

Resumen ***Modificación del patrón de glucosilación de Inmunoglobulina G durante la inflamación aguda inducida por trementina en ratas.*** Entre otras manifestaciones, durante una reacción inflamatoria se producen alteraciones en el patrón de glucosilación proteica. Previamente hemos observado una mayor proporción de moléculas de inmunoglobulina G (IgG) que presentan alterada la glucosilación de un fragmento Fab, denominadas anticuerpos asimétricos, en enfermedades crónicas parasitarias y tumorales. Estas moléculas presentan mayor afinidad por la lectina concanavalina A y se comportan como anticuerpos univalentes. En enfermedades autoimunes crónicas, otros autores han observado una agalactosilación del fragmento Fc de IgG. El objetivo de este trabajo fue evaluar si durante una respuesta inflamatoria aguda en ratas, producida por inoculación subcutánea de aceite de trementina, se produce una alteración en la síntesis y glucosilación de IgG, medida por fijación a lectinas. Se observó una disminución de la concentración de IgG en suero que no se modificó por la administración previa de dexametasona por vía oral y un incremento de la proporción de IgG sérica capaz de fijarse a la concanavalina A que tampoco fue inhibido por el glucocorticoide. Tanto la trementina como la dexametasona indujeron un aumento de la concentracion sérica de interleuquina-6 medida por ELISA. Previamente hemos descripto que la interleuquina-6 a bajas dosis incrementa la síntesis de IgG asimétrica. En este estudio no se detectó, sin embargo, una correlación entre el patrón de glucosilación de IgG observado y la concentración de interleuquina-6 determinada en los sueros de las ratas, debido probablemente a un diferente mecanismo de acción de la dexametasona.

Palabras clave: ratas, reacción de fase aguda, interleuquina-6, anticuerpos bloqueantes, IgG, glucosilación

Trauma is well known as a trigger of inflammatory responses widespread all over the body, involving cascade-activated systems like coagulation, fibrinolysis, complement, and phagocytic cells[1]. Cytokines, such as interleukin-1, interleukin-6, tumor necrosis factor-α, interferon-γ and chemokines are important mediators in the inflammatory processes[2].

It has been observed that during an inflammatory response, there is an increase in the synthesis and an alteration in the pattern of glycosylation of serum proteins produced in the liver and elsewhere, designated acute-phase reactants, and described both in health and disease[3]. Certain cytokines participating in inflammatory

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processes, most notably IL-6[4] as well as corticoid hormones[5], have been involved in protein glycosylation.

There is a particular type of antibody molecule, belonging to the IgG subclass, which, due to structural and immunological features has been designated co-precipitating, blocking or asymmetric antibody. Such molecules possess in one of their Fab fragments a mannose-rich oligosaccharide residue preventing them from interacting normally with the antigen. As consequence, these antibodies are incapable of mediating the effector mechanisms of immune response. Asymmetric antibodies have been described in numerous states of chronic infection, in animals repeatedly immunised with particulate antigens and in tumoral processes, as well as in sera and placenta from pregnant women[6]. It has also been reported that during chronic inflammatory responses such as rheumatoid arthritis, there is an alteration of the glycosylation pattern of IgG, showing a lack in terminal galactose residues that is observed in the Fc fragment of the molecule, accordingly designated agalactosyl IgG[7]. Nevertheless, the molecular pathways involved in IgG glycosylation during acute inflammatory responses are poorly understood.

We have already demonstrated that interleukin-6 (IL-6) induces an increase in the ratio of asymmetric antibodies synthesized by a murine hybridoma[8]. Taking into account the mentioned properties of this cytokine, and bearing in mind that dexamethasone acts in part by enhancing the expression of IκBa –an inhibitor of NFκB, an essential transcription factor for the expression of several genes including that of IL-6[9-10]– we speculate that dexamethasone may act on asymmetric antibody synthesis by modulating the IL-6 action during the inflammatory response.

Accordingly, in this study we reproduced an experimental animal model of acute inflammatory response to carry out *in vivo* studies in order to determine whether during its development there is a modification in the IgG glycosylation pattern detected in serum. Furthermore, we investigated whether treatment with dexamethasone, a synthetic anti-inflammatory steroid, is capable of producing an alteration in the type of IgG glycosylation; the possible relationship between this action and the effect of the corticoid in the IL-6 levels detected in serum was also evaluated.

Materials and Methods

Experimental animal model: A model of acute inflammatory response was reproduced in rats[11]. Specific pathogen-free male Wistar WKAH/ HOK F267 rats were distributed in 4 groups of 3 animals each and subjected to the following treatments: Group I: day 0, saline per Os; day 1, subcutaneous (sc.) turpentine oil in a thigh; Group II: day 0, saline per Os; day 1, saline (sc.); Group III: day 0, dexamethasone, 2,5 mg/Kg per Os; day 1, saline (sc.); Group IV: day 0, dexamethasone per Os; day 1, sc. turpentine as in Group I. The experiment was repeated four times using different sets of animals, so the number of animals per group was 12 (4 x 3). The development of the local inflammatory reaction was evaluated by observation of the resultant lesion (oedema, induration). Sera of each animal were collected separately 24 hours before treatments and at days 1, 3, 7 and 15 post-treatment, and run in parallel. The systemic acute inflammatory response was evaluated by assessment of α1-Acid Glycoprotein (α1-AGP) in rat serum. Inhibition Enzyme Linked Immunosorbent Assay (ELISA)[12] was performed, using rat α1-AGP (SIGMA, USA) as standard, a rabbit anti-rat α1-AGP polyclonal antibody produced in our laboratory as first antibody, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins (SIGMA, USA) as second antibody. The specificity of the rabbit anti-rat α1-AGP antibody was assessed by immunoblotting as described by Pos et al.[13].

Measurement of IgG subclasses: Total IgG_1, IgG_{2a}, IgG_{2b} and IgG_{2c} levels in rat serum were determined by ELISA[14]. Microtitre plates were coated with mouse monoclonal antibodies with anti-rat IgG subclass activity. IgG level was determined with a peroxidase-conjugated monoclonal mouse anti-rat immunoglobulins (light chain specific), using 1,2-phenylendiamine plus 0.03% hydrogen peroxide (H_2O_2) as substrate solution.

Assessment of asymmetric IgG: Attending the property of the carbohydrate prosthetic group present in the Fab region of the asymmetric IgG molecule to bind steadily to concanavalin A (Con A), a Sepharose-Con A affinity chromatography was used to measure asymmetric IgG in sera[15].

To confirm the fixation of IgG-$F(ab')_2$ fragment to Con A, a Western blotting assay was performed. Briefly, IgG was purified from sera of normal and inflamed rats respectively by Protein G-Affinity chromatography and the corresponding $F(ab')_2$ fragments were obtained by pepsin-enzymatic digestion as described by Rousseaux et al[16]. The products of the reaction (10 µg) were run on 7.5 % SDS-PAGE[17] and electroblotted to nitro-cellulose. After that, the membranes were blocked and incubated for 30 minutes at 37 ºC with a HRP-conjugated Con A (SIGMA, USA) and developed with 4-Cl-1-naftol plus 0.03% H_2O_2 as substrate solution. Relative protein concentration and Con A reactivity of $F(ab')_2$ in the samples were assessed in gel and membranes respectively, by applying an image processing and analysis software (Scion Image for Windows, Scion Corp, http://www. scioncorp.com).

Quantification of IL-6: The concentration of IL-6 in rat sera during the acute-phase response was determined by a two site-sandwich ELISA[12], by using a commercially available kit specific for rat IL-6 (Quantikyne M, R&D Systems, USA).

Statistical analysis: Repeated Measurement Analysis of Variance (ANOVA) and Student-Newman-Keuls Multiple Comparisons Test or Dunnet test were used to assess the statistical significance of results. Correlation was assessed by Pearson's r-coefficient determination, by using the Prism GraphPad software. P values less of 0.05 were considered significant.

Results

It was observed that as early as 24 h post-treatment, the rats inoculated with turpentine presented a severe inflammatory lesion in their thigh. There was also a significant increase in the serum levels of α1-AGP, which is a major acute-phase protein in rats. The concentration of this protein fell at day 7 post-treatment. The oral administration of one dose of dexamethasone previous to the subcutaneous turpentine inoculation suppressed

the rise in AGP concentration observed at 24 hours post injection. Dexamethasone alone produced no effect on the levels of α1-AGP detected in serum. Results are shown in Figure 1.

Also, it was observed that the inflammatory agent induced a decrease in the IgG level evidenced at 24 and 72 h in all subclasses, the differences reaching statistical significance at 72 h in IgG_{2b} and IgG_{2c} subclasses. These effects were reversed towards the end of the observation period (Table 1). Treatment with dexamethasone alone produced no modifications in IgG values but after 15 days, when an IgG increase in IgG_{2a} and IgG_1 subclasses was observed without reaching significance. As shown in Table 2, at 24 h and 72 h post-treatment there was an increase in the asymmetric ratio of IgG_{2a} and IgG_{2b} from rats treated with the inflammatory agent versus the control (Group II). At 7 and 15 days, asymmetry ratios returned to normal values. Also, it was observed that dexamethasone failed to inhibit the decrease in IgG synthesis when it was administered prior to the inflammatory agent, but it was seen that it inhibited the increase in asymmetry ratio.

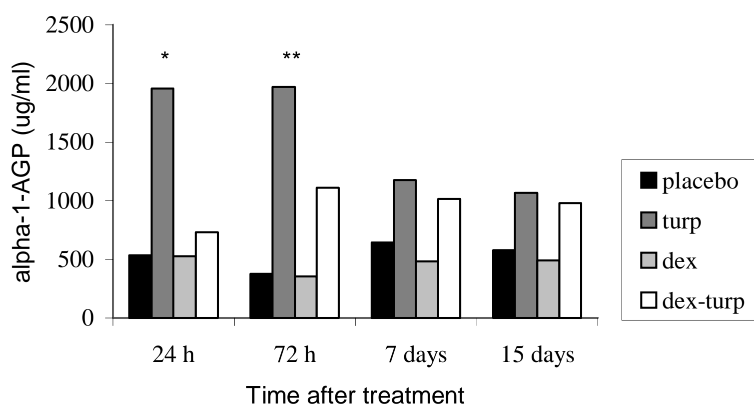

Fig.1.– *α1-AGP levels in rat serum. Effect of turpentine and dexamethasone.* The concentration of α1-AGP was determined by inhibition ELISA assay at days 0, 1, 3, 7, and 15 in serum of inflamed and non-inflamed rats previously treated or not with dexamethasone by oral route. Results are shown as protein concentration in μg/ml (Media of assays performed as triplicates in each individual serum, N:12). *p<0.05; **p<0.01. Turp: turpentine, Dex: dexamethasone.

TABLE 1.– *Total IgG levels in rat serum*

Time	Group	IgG_{2c}	IgG_{2b}	IgG_{2a}	IgG_1
	II (control)	1.003±0.054	0.186±0.012	1.159±0.055	1.454±0.138
24 h	I	0.668±0.097	0.160±0.030	1.014±0.103	0.981±0.257
	III	1.151±0.093	0.198±0.028	1.048±0.085	1.117±0.207
	IV	0.663±0.099	0.148±0.019	0.972±0.111	1.207±0.221
72 h	I	0.501±0.067 * #	0.098±0.010 * #	0.901±0.062	0.931±0.354
	III	1.059±0.126	0.170±0.013	1.217±0.015	1.324±0.187
	IV	0.515±0.092 * #	0.101±0.011 * #	0.876±0.096	0.900±0.154
7 days	I	0.886±0.177	0.197±0.008	1.075±0.056	1.693±0.302
	III	1.156±0.352	0.224±0.035	1.151±0.059	1.628±0.117
	IV	0.871±0.0671	0.189±0.034	1.070±0.082	1.322±0.230
15 days	I	0.824±0.018	0.188±0.008	1.347±0.008	1.970±0.070
	III	1.602±0.242 *	0.273±0.018 *	1.449±0.116	1.866±0.088
	IV	0.880±0.219	0.172±0.032	1.295±0.097	1.163±0.002

*Serum IgG concentration was determined by ELISA. Rats were bled at 24 h, 72 h, 7 days and 15 days post-inoculation of either turpentine or saline. Values are expressed as OD at 490 nm (Means ± SEM, N: 9 corresponding to one representative of four assays performed as triplicates in each individual rat serum). Group I: Turpentine; Group II: Saline; Group III: Dexamethasone; Group IV: Dexamethasone plus Turpentine. * p<0.05 vs control, ** p<0.01 vs control, # p<0.05 vs Group III.*

TABLE 2.– *Asymmetric IgG molecules in rat serum*

	Con A-Fixation of Rat IgG_{2a} (%)			
Sera	24 h	72 h	7 days	15 days
Group II	29.15±2.30	31.67±1.44	30.8±1.20	35.4±4.30
Group I	49.20±8.10*	53.75±1.60 ***	23.80±1.30	25.20±1.10
Group III	36.25±5.45	29.65±8.25	37.95±1.05	36.90±1.25
Group IV	40.80±10.20	29.20±5.10	38.25±0.95	38.35±0.95
	Con A-Fixation of Rat IgG_{2b} (%)			
Sera	24 h	72 h	7 days	15 days
Group II	29.6±2.44	32.55±1.90	29.8±3.00	24.4±2.50
Group I	64.90±5.00***	49.00±13.00 *	41.40±8.90	45.03±8.82
Group III	27.05±0.45	25.65±2.35	38.65±5.35	24.75±1.35
Group IV	27.15±6.75	30.40±0.80	28.80±9.40	35.60±0.90

*Serum asymmetric IgG_{2a} and IgG_{2b} were determined by Con A fixation. Rats were bled at 24 h, 72 h, 7 days, and 15 days post-inoculation of either turpentine or saline (control). Values are expressed as percentage of asymmetric IgG as indicated in Materials and Methods (Means ± SEM, N:12). Group I: Turpentine; Group II: Saline; Group III: Dexamethasone; Group IV: Dexamethasone plus Turpentine. * $p<0.05$, *** $p<0.001$ vs control (group II).*

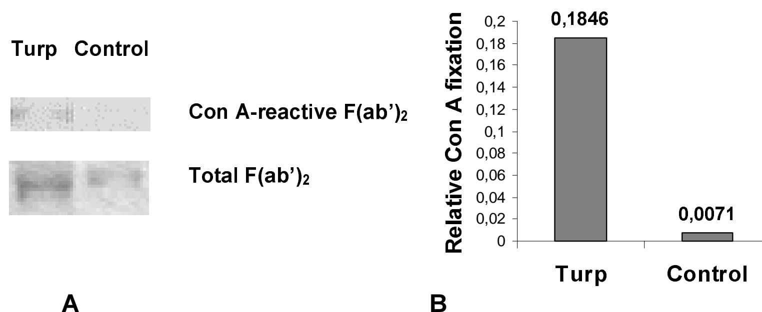

Fig. 2.- *Binding of Rat IgG-F(ab')$_2$ fragment to Con A. Effect of turpentine.* **A.** Serum IgG from inflamed (Turp) and non-inflamed (control) rats were pepsin-digested and separated on 7.5% SDS-PAGE. Western blotting assay was performed using HRP conjugated-Con A. One representative assay of two performed is shown. **B.** Histogram plot corresponding to Con A binding relative to F(ab')$_2$ protein concentration (OD units) for inflamed and normal rats, as determined by image analysis.

As shown in Figure 2, it can be observed that inflamed rat-IgG- F(ab')$_2$ fragment behaved more reactive to Con A than IgG- F(ab')$_2$ fragment from normal control rats. Blots were then assessed by image analysis. Results expressed as Con A binding relative to F(ab')$_2$ protein concentration (OD units) are shown as histogram plot.

Levels of IL-6 in sera of rats treated or not with the corticosteroid previous to the turpentine inoculation were determined. Results in Table 3 show that during the acute-phase response there was an increase in IL-6 levels and that the oral dexamethasone administration induces *per-se* an increase in the IL-6 concentration. Moreover, the effect of dexamethasone on IL-6 levels was synergistic to the effect observed when turpentine was inoculated to the rats. Analysis of correlation between IL-6 levels and IgG_{2a} or IgG_{2b} asymmetric ratio in rat sera showed no significant correlation (r= 0.2326 and 0.0649 respectively). No dexamethasone-treated rats showed an increase in asymmetric IgG ratio at 24- 72 hours after turpentine injection coincident with the IL-6 peak.

TABLE 3.– *IL-6 levels in rat serum. Effect of Turpentine and Dexamethasone*

Sera	IL-6 Concentration (pg/ml) 24 h	72 h
Group II	1.10±0.03	1.10±0.03
Group I	41.20±4.00 ***	18.10±1.00 *
Group III	4.75±1.05	14.10±1.00 *
Group IV	46.10±5.63 ***	56.45±0.05 ***

*Serum IL-6 concentration was assessed by sandwich ELISA at 24 h and 72 h after administration of Turpentine or saline respectively, as described in Materials and Methods. Results are shown as picograms per millilitre (pg/ml) of IL-6 (Media±SEM of assays performed as duplicates in each individual serum, N:9). Group I: Turpentine; Group II: Saline; Group III: Dexamethasone; Group IV: Dexamethasone plus Turpentine. *p<0.05; ***p<0.001 with respect to the control (group II).*

Discussion

In order to assess whether turpentine inoculation had provoked a systemic acute-phase reaction, levels of α1-acid glycoprotein were measured in the serum of treated rats. As expected, we observed an increase in serum α1-AGP concentration at 24 and 72 hours after turpentine injection. Although a stimulatory effect of dexamethasone on the *in vivo* synthesis of α1-AGP was described elsewhere[13, 18, 19] , this effect was not observed by us. Furthermore, here we found that one dose of dexamethasone, administered by oral route 24 h before turpentine, inhibited the turpentine-induced increase in serum α1-AGP. Previously, also Bauer et al[20] and Princen et al[21], showed no effect of dexamethasone on the *in vivo* synthesis of this and other acute-phase glyco-proteins. Bauer et al[20] showed that turpentine-induced α1-AGP synthesis could not be observed in hypophysectomized rats and that dexame-thasone-induced increase in α1-AGP concentrations is higher in those rats than in normal ones. Thus, we speculate that our results, corresponding to exogenous dexamethasone administration, could be due to an acute inhibitory effect exerted by dexa-methasone on the endogenous corticoid-induced synthesis of α1-AGP, probably by down-regulation of steroid receptors.

Then we studied the synthesis and glycosylation pattern of IgG and we observed a decrease in IgG levels in the serum of turpentine-treated rats. Previous admi-nistration of dexamethasone by oral route produced no modification in this effect. Such results are in agreement with previous publications[22-24], showing that IgG could be a negative acute-phase reactant. A drop of IgG concentration in sera as soon as 48 h after injury has been described[24]. Our results also show that the diminished levels of rat IgG during inflammation are observed mainly at IgG_{2c} and IgG_{2b}, which are the IgG subclasses in rat with the lowest half-life[25]. In normal rats, blood clearance rates of radiolabelled antibodies injected intravenously were found to be dependent on immuno-globulin subclass (IgG_{2b} >IgG_{2a} >IgG_1)[26]. As IgG_{2a} and IgG_{2b} are the two major IgG subclasses in rat sera[27], therefore we evaluated the IgG glycosylation pattern by assessing the IgG_{2a} and IgG_{2b} binding to Con A during the acute-phase reaction. We observed that after 24 and 72 hours upon turpentine inoculation there was a significant increase in Con A fixation; this effect was inhibited by previously administered dexamethasone by oral route. Dexamethasone alone had no effect on the IgG glycosylation pattern. These findings directly correlate with the observed effect on α1-AGP concen-tration in sera (Pearson's r: 0.5758, p<0.05 and r= 0.8936, p<0.0001 for IgG_{2a} and IgG_{2b} respectively). Although most oligossacharide residues are described as present in the Fc region of the antibody molecule, Coloma et al[28], by working with monoclonal antibodies, described that additional glycosylation sites at the variable region of the antibody molecule promote variable changes in affinity. Thus, by performing a Western blotting assay we demonstrated an increase in Con A reactivity of the $F(ab')_2$ fragment of IgG, when it was obtained from inflamed rat sera. Nevertheless, the blocking activity of these unusually glycosylated IgG molecules was not investigated in this study.

It has already been described that IL-6 concentration increases during acute as well as chronic inflammatory responses and that this cytokine is capable of modifying protein glycosylation such as IgG galactosylation, and it can modulate glycosyltranferase activity[29]. Therefore, we intended to determine if a modulation of IL-6 synthesis during an acute-phase reaction could be involved in the modifications observed in the IgG glycosylation pattern. Surprisingly, we found increased IL-6 serum levels in rats treated with dexamethasone alone. Also, dexamethasone enhanced the turpentine-induced IL-6 increase. Although glucocorticoids generally turn down the production of pro-inflammatory cytokines, it has been described that intravenous infusion of corticosterone at nonstressed and stressed levels increased TNF and/or IL-6 release. In addition, when LPS was combined with corticosterone, the lower dose of corticosterone facilitated the release of cytokines[30]. Consequently, although we observed that in turpentine-treated rats there was an increase in asymmetric IgG ratio at 24-72 hours after turpentine injection coincident with an IL-6 peak, in this study we could not find a correlation between IL-6 synthesis and asymmetric IgG ratio. Dexamethasone action on IgG glycosylation might occur by a different molecular pathway, possibly involving direct glycosyltransferase activity modulation.

We conclude that during an acute inflammatory response there is an alteration in the synthesis and glycosylation pattern of IgG antibody molecules. Anti-inflammatory corticoids such as dexamethasone could be involved in these processes in a way that seems independent of the effect of the corticoid on IL-6 synthesis. An increase in the natural immune response has been described as taking place during an acute-phase response, characterised by a rise in acute-phase protein and natural-autoantibody synthesis[31]. Results recently obtained in our laboratory showed that 78% of asymmetric IgG molecules isolated from rat-non immune sera were able to react with host antigens (Canellada et al, unpublished results). A rise in glycosylation of variable regions of IgG has been described to render these molecules natural polyreactive antibodies[32]. Although generally beneficial to the host, inflammatory processes are intrinsically destructive to the surrounding tissues and can result in major tissue injury. Thus, further investigation should be necessary to assess the biological significance of an asymmetric IgG antibody increase, during an acute inflammatory reaction, in order to improve host protection. It will also be necessary to evaluate whether this increase could be involved in an early non-specific immune defence mechanism.

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Croire ou ne pas croire. Telle est la question.

C'est comme le dit François Jacob, une question "de gout", de choix individuel. Croire ou non en Dieu est et reste un choix individuel qui doit être respectable et respecté. C'est un fondement essentiel de la liberté.

Creer o no creer. Esta es la cuestión.

Es como dice François Jacob, una cuestión "de gusto", de elección individual. Creer o no creer en Dios es y sigue siendo una elección individual que debe ser respetable y respetada. Es un fundamento esencial de la libertad.

Claude Allègre

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