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Cerium oxide nanoparticles increase the cytotoxicity of TNF-alpha in vitro

O. Shydlovska[1], E. Kharchenko[2], N. Zholobak[1], A. Shcherbakov[1], A. Marynin[2], O. Ivanova[3], A. Baranchikov[3], V. Ivanov[3,4]

[1]Zabolotny Institute of Microbiology and Virology NANU, Kyiv, Ukraine
[2]National University of Food Technologies, Kyiv, Ukraine
[3]Kurnakov Institute of General and Inorganic Chemistry, Moscow, Russia
[4]National Research Tomsk State University, Tomsk, Russia

olgashydlovska@gmail.com, yugin171094@gmail.com, n.zholobak2018@gmail.com, ceroform@gmail.com, andrii_marynin@ukr.net, runetta05@mail.ru, a.baranchikov@yandex.ru, van@igic.ras.ru

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Cerium oxide nanoparticles (CeONP) were used as the modifying agent for the recombinant tumor necrosis factor-α and recombinant tumor necrosis factor-α-thymosin-α1 (rhTNF and rhTNF-T). A notable increase of the biological activity of proteins with antitumor effect was demonstrated. It was established that the cytotoxicity of rTNF-T+CeONP composite increases with the duration of exposure to 7 days. Modification of rTNF-T with cerium oxide nanoparticles provides a stronger and more stable cytotoxic effect in Hep-2, L929, and A-549 tumor cell lines.

Keywords: cerium oxide nanoparticles, tumor necrosis factor, nano-biocomposite, biological activity, antitumor effect.

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1. Introduction

Improving the quality of medication typically relies on the use of totally new (or previously unknown) groups of biologically active substances. One such group includes different cytokines. Therapeutic attempts to use genetically-engineered cytokines in clinical practice have been performed for the last quarter of a century [1]. One of such promising therapeutic cytokines is tumor necrosis factor-alpha (TNF-alpha).

It is well established that the biological effects of TNF depend on its concentration. At low concentrations, it acts as a para- and autocrine regulator of autoimmune reactions against injury or infection [2]. This cytokine is the major stimulator for neutrophils and endothelial cell adhesion and subsequent migration of leukocytes, fibroblasts and endothelial cell proliferation during the healing of wounds [3, 4]. Medium concentrations of TNF-alpha cause a pyrogenic effect, stimulating the formation of phagocytes, increased blood clotting, and reduced appetite. TNF-alpha is an important factor in the development of cachexia in such chronic diseases as tuberculosis and cancer [5, 6].

The use of tumor necrosis factor in the therapy of malignant tumors requires relatively high concentrations, which means that the native form of cytokine cannot be directly used. That is why there is a need to improve the biological properties, namely to increase the activity and to reduce the toxicity of TNF-alpha. Thus, further research aimed at modification and enhancement of the biological activity of TNF and other therapeutic proteins would be the basis for the elaboration of novel highly effective commercial medicines.

Recently, an interesting means of modifying therapeutic proteins was proposed, based on the use of gold or calcium carbonate nanoparticles [7, 8]. Complexes of TNF-alpha with gold nanoparticles (TNF-AuNPs) are formed by nonspecific binding. Interestingly, the formation of the TNF-AuNPs complexes protects TNF-alpha protein from adsorption by specific anti-TNF antibodies [7]. In turn, the layer-by-layer assembled TRAIL/ALG shells have been successfully coated on the surface of highly doxorubicin (DOX)-loaded calcium carbonate ($CaCO_3$) nanoparticles as drug carriers. Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM) have shown that TRAIL/ALG coated DOX-$CaCO_3$ nanocomposites can be readily internalized by cancer cells [8].

In the last few years, cerium oxide nanoparticles (CeONP) have been proposed as a promising carrier for drug delivery and agent for protein modification. CeONP's have been shown to increase immunogenicity of the influenza vaccine [9]. CeONP causes enhanced interferon (rIFNα-2β) response in mice in comparison with the unmodified one [10]. Our preliminary *in vitro* study [11] also showed that CeONP-modified rhTNF-α is more active than the pristine rhTNF-α, that is probably due to adjuvant-mimic properties of CeONP.

Thus, the aim of our current study was to establish the possibility of modifying the tumor necrosis factor with cerium oxide nanoparticles and influence on TNF cytotoxicity of tumor cells.

2. Materials and methods

2.1. Cerium oxide nanoparticles and tumor necrosis factor

In this research, we used two different preparations of tumor necrosis factor (TNF) as model objects: Human Recombinant Tumor Necrosis Factor-α (rhTNF-α, Promega Corporation Part# 9PIG524, USA) and Recombinant Tumor Necrosis Factor-α-Thymosin-α1 (rhTNF-T, Refnot-Pharm, LLC, series 020616, Russia).

RhTNF-α is a 17 kDa protein containing 157 amino acid residues; the concentration used was 3.8105 U/ml (10 μg/ml). A rhTNF-α is supplied as a dried powder and contains no additives.

The rhTNF-T is a hybrid of tumor necrosis factor-α and thymosin-α1. It consists of 185 amino acid residues, of which the last 28 at the C-terminus are the thymosin-α1 sequence. The concentration of rhTNF-T was 106 U/ml (55 μg/ml). The rhTNF-α is supplied as a dried powder and contains mannitol, sodium chloride, sodium phosphates (dihydrate and dodecahydrate) additives.

Cerium oxide nanoparticles (CeONP, size ~ 5.8 nm, ζ-potential $\sim +12$ mV) were synthesized as an aqueous 0.01 M sol [12].

2.2. Study of TNF+CeONP complex formation

The interaction of CeONP with TNF and the formation of the corresponding nano-biocomposite should lead to an increase in the peptide's hydrodynamic diameter (HD). HD was monitored by dynamic light scattering (DLS) method using a Zetasizer Nano ZS (Malvern, UK) analyzer. All measurements were performed at a constant temperature (25 °C) and neutral media (pH = 7.2).

2.3. Study of the biological activity of TNF+CeONP nano-biocomposite

The biological activity of tumor necrosis factor was analyzed on murine L929 fibrosarcoma cell line from R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NASU, using Promega Corporation protocol [13]. L929 is commonly used to test the activity of TNF-α [14, 15]. The cells were cultured in 96-wells Sarstedt plates using 199 media (Sigma, USA) supplemented with 10 % fetal bovine serum (FBS, Sigma, USA), 100 units/ml gentamicin (Arterium, Ukraine) and 50 units/ml kanamycin (Arterium, Ukraine). Cells were maintained at 37 °C for 24 hours in TC-80M-2 thermostat with 5 % CO_2 under a humidified atmosphere.

CeONP was added to TNF (rhTNF-α or rhTNF-T) in the weight ratio of 1:3 respectively and the composite was analyzed for biological activity 15 minutes, 24 hours and 7 days after exposition. Samples of non-modified TNF (positive control) and TNF modified with CeONP (test sample) were titrated with two-fold step in 96-wells plates in four repetitions.

According to the standard method, in each well actinomycin-D was added in the concentration of 0.15 mg/ml.

The culture medium was removed after 24 hours of cultivating cells with samples. Cells were dyed and fixed by 0.5 % ethanol solution of crystal violet ("Sigma", USA). Dye absorbed by the cells was dissolved with 70 % ethanol solution. The same procedure was used when testing efficacy for unmodified and modified rhTNF-T.

The optical density was measured by a Thermo Labsystems Multiskan Ascent spectrophotometer at a wavelength of 540 nm. The degree of cytolysis was calculated in comparison with intact cells taken as 100 %. Activities of rhTNF-α and rhTNF-T (both unmodified and modified with CeONP) were analyzed using Area Under Curve (AUC) method [16], which is a simple technique for assessing the activity of biomolecules. The mathematical formula for calculating the AUC is:

$$\mathrm{AUC} = \sum \frac{h(r_n + r_{n+1})}{2},$$

where r_n and r_{n+1} – the percentage of lysis in two nearby dilutions and $h = 1$, because the dilutions are the part of the continuous logarithmic series.

Also, two different cultures of tumor cells were used to test the antitumor activity of TNF+CeONP complexes: A549 (human alveolar adenocarcinoma) and Hep-2 (HeLa contaminant, human carcinoma) from the collection of Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NASU. To determine the biological activity of TNF and TNF+CeONP complexes on cell cultures the above mentioned method was used.

2.4. Statistical study

All obtained data are presented as the median and interquartile range Me (LQ–UQ) of AUC, where Me = median (50 % percentiles), LQ = 25 % percentiles, and UQ = 75 % percentiles. Validity check for the null hypothesis was performed using a nonparametric Wilcoxon matched pairs test (WMP-test). The difference between groups was judged to be statistically significant at $p < 0.05$. Statistical calculations were conducted in a Stat Plus Pro 5.9.8. software and STATISTICA data analysis software system, version 10 (StatSoft, Inc. 2011).

2.5. Analysis of cell viability of L929 treated with rhTNF-T and rhTNF-T+CeONP

In this study, murine fibrosarcoma cells (L929) were cultured in 24-wells plates (TPP, Switzerland) on Sarstedt plastic coverslips (Sigma, USA) using 199 media (Sigma, USA) supplemented with 10 % fetal bovine serum, 100 units/ml gentamicin and 50 units/ml kanamycin. L929 cells were maintained at 37 °C for 24 hours in TC-80M-2 thermostat with 5 % CO_2 in a humidified atmosphere.

RhTNF-T and rhTNF-T+CeONP were taken in a concentration of 102 U/ml. The following experimental groups were prepared: CeONP (0.5 μM), rhTNF-T, rhTNF-T+CeONP, actinomycin D (0.15 μg), cells control. The incubation time after the application of the preparations was 24 hours. Following a standard procedure, actinomycin D was added to experimental groups rhTNF-T and rhTNF-T+CeONP.

Propidium iodide (PI, Sigma-Aldrich, USA) and bis-benzimide H 33342 (Hoechst, Sigma-Aldrich, Germany) dyes were used for cell viability analysis. Before dye application, the culture medium was removed from the plate wells. 850 μl of 199 media with 2 % FBS was added to each well. 50 μl of HOE was added to each well and cells were incubated for 30 minutes at room temperature in the dark. After that, 100 μl of PI was added and cells were incubated at room temperature for 15 minutes in the dark. The samples were then analyzed with LOMO MICMED-2 fluorescent microscope equipped with a 480 nm dichroic filter, 450 nm long-pass filter (for Hoechst dye fluorescence measurements), 480 nm dichroic filter, 515 nm long-pass filter (for PI dye fluorescence measurements), and HBO 103 W/2 Mercury lamp (OSRAM, Germany).

3. Results

3.1. The study of TNF+CeONP complex formation

The particle size distributions of the samples are presented in Table 1. DLS data indicate that cerium oxide nanoparticles cause the increase of both rhTNF-α and rhTNF-T hydrodynamic diameters.

3.2. Study of the biological activity of TNF+CeONP preparations

The biological activity of TNF and TNF+CeONP nanocomposites were tested by the standard method on L929 cell culture. The exposure time was varied from 15 minutes to 7 days. The AUC calculation results and the percentage of increased activity are presented in Table 2.

The most effective exposition time for rhTNF-α was 15 minutes – biological activity increased by 23.9 % for rhTNF-α+CeONP in comparison with rhTNF-α. Validity check for the null hypothesis (evidence of the statistically significant difference between two compared groups) was performed using nonparametric Wilcoxon matched pairs test (WMP-test). Since the p-value was equal to 0.0051 upon rhTNF-α and rhTNF-α+CeONP comparison, the null hypothesis was rejected. The difference between unmodified and CeONP-modified rhTNF-α exposition (24 hours or 7 days) was less pronounced – 6.1 % ($p = 0.0077$) and 9.2 % ($p = 0.0051$) of cytotoxicity increase, respectively.

It should be noted, that for rhTNF-T the most effective exposition time was 7 days – biological activity increases by 15 % for rhTNF-T+CeONP in comparison with pristine rhTNF-T ($p = 0.0077$). The increase in cytotoxicity of rhTNF-T+CeONP upon 15 min or 24 h exposition wasn't as high – 1.7 % ($p = 0.8590$) and 2.8 % ($p = 0.3743$), respectively. Different behavior of TNF preparations upon modification with ceria nanoparticles can be related with the differences in the structure of tumor necrosis factor molecules.

Since the most notable effect of rhTNF-T modification with CeONP was registered upon 7-day exposure, these conditions were also chosen for confirmation experiment with other cultures of tumor cells – A549 and Hep-2. Results of these studies are presented in Table 3.

Determination of biological activity of nano-biocomposites on A-549 and Hep-2-cells has confirmed the efficiency of rhTNF-T modification with ceria nanoparticles. The increase in the biological activity of rhTNF-T+CeONP composite compared to the unmodified rhTNF-T is more pronounced in A-549 cells (29.3 %) than in Hep-2 cells (12.0 %). The p-value for both cultures according to WMP-test was 0.0277, thus the null hypothesis was rejected.

TABLE 1. The study of TNF+CeONP composites formation by DLS

	Size distribution by intensity, nm (median [interquartile range])		
	Peak 1	**Peak 2**	**Peak 3**
rhTNF-α	499.4 [441.2–572.3]	75.4 [64.4–79.9]	5352.5 [0.0–5560.0]
Area.%	66.6 [56.7–70.3]	31.4 [24.3–40.3]	2.5 [0.0–3.8]
Z-Average	508.3 [452.2–676.1]		
PdI*	0.6 [0.5–0.7]		
rhTNF-α+CeONP	898.3 [817.4–990.8]	0.0 [0.0–0.0]	0.0 [0.0–0.0]
Area.%	100.0[100.0–100.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]
Z-Average	1510.0 [1356.0–1604.5]		
PdI*	0.7 [0.3–0.8]		
rhTNF-T	125.3 [112.8–142.0]	20.2 [10.1–29.1]	0.0 [0.0–0.0]
Area.%	94.9 [93.2–97.5]	5.1 [2.6–6.8]	0.0 [0.0–0.0]
Z-Average	315.1 [269.8–397.8]		
PdI*	0.4 [0.3–0.5]		
rhTNF-T+CeONP	2832.5 [2168.8–3212.5]	220.3 [165.9–536.8]	0.0 [0.0–215.9]
Area.%	91.8 [88.1–93.9]	7.0 [5.5–8.5]	0.0 [0.0–3.7]
Z-Average	2320.5 [2110.3–2506.3]		
PdI*	0.5 [0.4–0.5]		

*PdI – Polidispersity Index

TABLE 2. Biological activity (AUC) of TNF and TNF+CeONP composites depending on the exposure time

Preparation name	**Exposure time of pure TNF and TNF+CeONP composites**		
	15 min	**24 h**	**7 days**
rhTNF-α	646.7* [641.4–651.9]	1778.0* [1752.2–1815.4]	1244.2* [1238.0–1250.4]
rhTNF-α+CDN	800.9* [782.1–819.9]	1886.6* [1856.7–1905.7]	1358.0* [1348.7–1367.4]
Increase in activity, %	23.9 [20.1–27.8]	6.1 [2.3–8.8]	9.2 [7.9–10.5]
rhTNF-T	1479.1* [1475.6–1496.3]	1027.6* [1018.0–1087.0]	996.4* [996.1–998.7]
rhTNF-T+CeONP	1504.0* [1479.2–1504.9]	1056.7* [996.3–1097.0]	1146.3* [1135.2–1161.4]
Increase in activity, %	1.7 [0.2–2.0]	2.8 [7.7–7.9]	15.0 [13.7–16.6]

*The table shows the values of the area under the curve (AUC) for the medians of the experimentally obtained values

TABLE 3. Cytotoxicity (AUC) of TNF and TNF+CeONP composites to A-549 and Hep-2 cell cultures

	A-549	**Hep-2**
rhTNF-T	290.0* [285.8–291.9]	1317.4*[1315.3–1388.0]
rhTNF-T+CeONP	375.1* [370.4–378.1]	1475.6* [1473.4–1532.1]
increase in activity, %	29.3 [26.9–32.3]	12.0 [6.2–16.5]

*The table shows the values of the area under the curve (AUC) for the medians of the experimentally obtained values

3.3. Analysis of cell viability of L929 treated with rhTNF-T and rhTNF-T+CeONP

Analysis of viability of L929 cells treated with rhTNF-T and rhTNF-T+CeONP was performed using fluorescent dyes. After staining with fluorescent dyes, the cells were studied with a fluorescent microscope using relevant filters (480 nm dichroic filter, 450 nm long-pass filter for Hoechst dye fluorescence; 480 nm dichroic filter, 515 nm long-pass filter for PI dye fluorescence).

Figure 1 shows that under the action of rhTNF-T (B1) and rhTNF-T+CeONP (A1) complexes L929 cells lose their natural morphology (as compared to the control cells – E1), with those for rhTNF-T were observed the single cells, which kept natural morphology. Analysis of groups A and B and their comparison with the control (E), allows to conclude that the intensity of cells coloration with Hoechst dye in these groups is rather small, which may indicate the inhibition of active transport across cell membranes. It should be noted that the intensity of coloration in Group A is the lowest. In comparison with E3 image, A3 and B3 images demonstrate an intense staining of the cells with fluorescent PI dye, which may indicate an integrity violation of cell membranes and the development of necrosis.

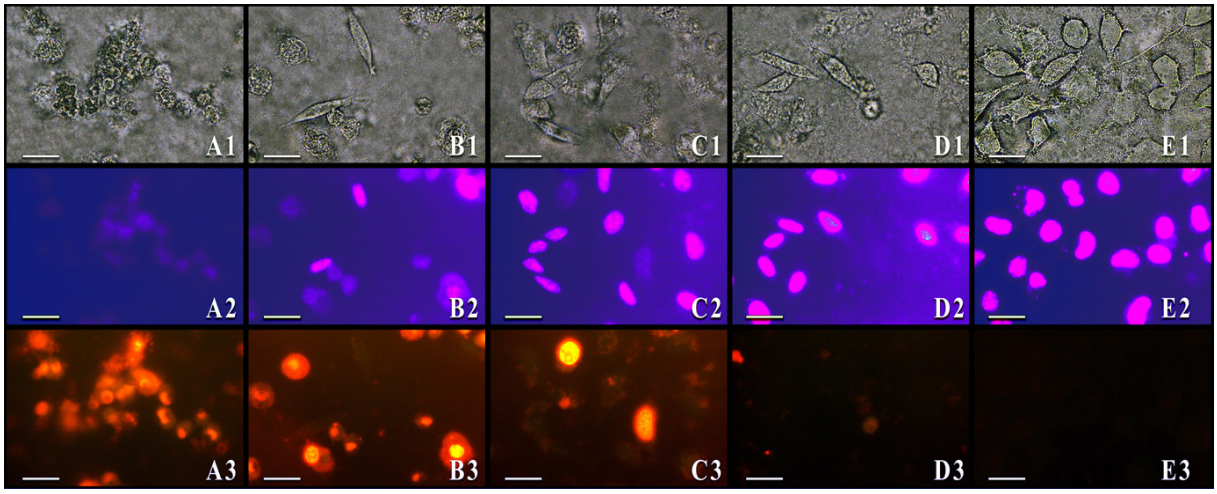

FIG. 1. Bright field (1) and fluorescent (2 – Hoechst dye, 3 – PI dye) micrographs of L929 cells treated with rhTNF-T and rhTNF-T+CeONP. L929 cells treated with rhTNF-T+CeONP – A1-A3; rhTNF-T – B1-B3; CeONP – C1-C3; actinomycin D – D1-D3; cell control – E1-E3. Scale Bar = 30 μm

It should be mentioned that the treatment of cells with bare CeONP (Group C) results in quite intense coloration of Hoechst dye, which indicates good cell viability and active membrane transport. Nevertheless, a few cells intensely colored with PI dye were found, indicating the presence of necrotic cells in this experimental group. Cell groups treated with actinomycin D (Group D) shows virtually no PI coloration, which indicates that the experimental groups treated with antibiotic show no signs of cell death, and their intense coloration with Hoechst dye indicates cell viability.

4. Discussion

Several proteins belonging to a tumor necrosis factor superfamily were investigated in this study: rhTNF-α (a natural form of TNF-α) and rhTNF-α (a hybrid form of TNF and thymosin-α1). Based on the results of preclinical and clinical studies of *Refnot*, a commercial drug, whose major active substance is rhTNF-T, it can be stated that rhTNF-T has up to 100 times less toxicity to normal cells in comparison with the natural form – rhTNF-α [17, 18].

In this study, the formation of nano-biocomposites of TNF and CeONP was revealed. It should be noted that according to previously published results hydrodynamic diameter of TNF measured by DLS is ~ 4.5 nm [19]. In our experiments, the hydrodynamic diameter for pure TNF samples was in 130 – 500 nm range. This can be explained by the ability of nanoparticles and macromolecules to form large agglomerates in buffer solutions like serum or PBS [20]. Previously published results for the formation of TNF complexes with its antagonists were based on DLS data collected also using PBS solution, but the samples were filtered through 0.2 μm syringe filter prior to the measurements that could be the reason for different results obtained by us [21].

Investigation of the dependence of the biological activity of TNF and TNF+CeONP composites on the exposure time showed that the modification of therapeutic proteins with cerium oxide nanoparticles efficiently increase the biological activity of TNF-α at various exposure times, with the most pronounced effect registered at 15 min exposure. The rhTNF-T+CeONP nano-biocomposite demonstrate the inverse trend in the biological activity changes: with exposure time increasing the rhTNF-T+CeONP complex activity increases. At 7-days exposure, the biological activity increased by 15 %. This result suggests that CeONP has a positive effect on the biological activity of rhTNF-T and proves that CeONP is not only a stabilizer and a substance that prevents the loss of biological activity of TNF, but it actually forms a promising therapeutic rhTNF-T+CeONP nano-biocomposite with enhanced activity. One important thing is that the natural form of rhTNF, even modified with the CeONP, eventually loses its biological activity, whereas the presence of thymosin fragment in rhTNF-T causes increased biological activity of rhTNF-T+CeONP composite and preserves it over time.

To confirm the positive effect of ceria nanoparticles on rhTNF-T protein, the study of cytotoxicity was carried out on two tumor cell cultures, A549 and Hep-2. The results obtained showed that the biological activity of the rhTNF-T+CeONP nano-biocomposite is higher by 29.3 % compared to the pristine rhTNF-T on the A-549 cell culture. An increase of 12.0 % in biological activity was noted for the Hep-2 cell culture.

The results obtained using different cell cultures allowed us to state that the rhTNF-T+CeONP composite definitely has a higher biological activity in comparison with the native form of the rhTNF-T therapeutic protein. It should be noted that the cytotoxicity of the rhTNF-T+CeONP composite depends strongly on the cell culture. This allows us to suppose that human lung carcinoma can be regarded as a good target for rhTNF-T+CeONP nano-biocomposite anticancer therapy.

Data obtained indicate that rhTNF-T preparation causes necrosis of tumor cells. When CeONP is used to modify rhTNF-T, the necrotic effect of the therapeutic protein increased.

In recent years, information about the mechanisms for the formation of nanoparticle-therapeutic protein complexes appeared in the scientific literature. It has been established that nanoparticles, entering the protein-containing medium (a solution of therapeutic cytokines), are covered with a kind of a “crown” – a layer of proteins, which are adsorbed on the surface of the particles [22]. As a result of a mutual influence of the components, the properties of the nanoparticles change under the influence of the “crown”, and the proteins that contact the particles are prone to modification. We believe that the presence of the thymosin domain in the structure of the rhTNF-T molecule is the factor facilitating the formation of such a “crown” structure, which leads to a greater biological activity of the rhTNF-T+CeONP composite in comparison with rhTNF+CeONP.

5. Conclusions

In this study, we demonstrated that cerium oxide nanoparticles can be used as an effective modifying agent for therapeutic proteins such as rhTNF and rhTNF-T. The obtained rhTNF+CeONP and rhTNF-T+CeONP nano-biocomposites have a stronger cytotoxic effect to malignant tumor cells than pristine proteins. Modified rhTNF-T+CeONP demonstrated greater efficacy and stability over time compared to other samples. The enhanced cytotoxic effect of this composite was confirmed by fluorescent microscopy study of stained cells.

Our study allows considerion of nanocomposites of rhTNF+T with CeONP as a promising therapeutic agents with direct antitumor action.

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