ORIGINAL

Melanoma antigen is a vaccine candidate against Meth A sarcoma

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Abstract : Sarcoma Meth A is widely used in the field of immunology and oncology study. We found that a mutant cell line, Meth A (mMeth A), was rejected in an *in vivo* subcutaneous challenge in BALB/c mice. mMeth A cells were not rejected in athymic BALB/*c-nu*/*nu* mice and $CD8^+$ cell-depleted BALB/c mice, suggesting that $CD8^+$ cells are required for rejection of mMeth A cells. Microarray analysis showed that melanoma antigen (MAg) was one of the most elevated genes in mMeth A cells. Indeed, quantitative gene expression analysis showed that the expression level of MAg in mMeth A cells was one hundred-times higher than that in Meth A cells. We constructed two types of expression vector coding the MAg gene sequence corresponding to 788–1257 and 1611-2043 and immunized mice with these genes by intramuscular injection. Immunization of a plasmid expressing the MAg 788-1257 gene protected the mice from *in vivo* Meth A challenge as evaluated by tumor volume and survival rate. The results reveal that MAg is a potential vaccine candidate antigen against Meth A tumors. J. Med. Invest. 72 : 161-166, February, 2025

Keywords **:** *Meth A, $CD8^+$ cells, melanoma antigen, vaccine*

INTRODUCTION

Immunotherapy is widely used for treatment of cancer in clinical setting. Immunotherapy includes tumor antigen (Ag)-pulsed dendritic cell therapy, cytokine therapy and immune checkpoint therapy (1). Class I major histocompatibility complex (MHC)–restricted cytotoxic T lymphocytes (CTL) recognize peptide Ags (epitopes) has accelerated efforts to identify CTL-defines tumor peptides for the development of peptide-based cancer immunotherapy. There is accumulating evidence that many tumor Ags recognized by autologous CTLs are antigenically normal self-constituents (2). In vaccine-based immunotherapy, identification of an antigenic Ag that is recognized by CTLs would a promising targets for prevention of the development of a tumor.

Melanoma antigen (MAg) has been identified by using a monoclonal (m) antibody (Ab) (M562) that reacts to B16 cell-derived Ag (3). Treatment with the M562 antibody (Ab) has been shown to inhibit anti-melanoma cytotoxic T cell activity and inhibit lung metastasis from a B16 cell challenge *in vivo* (4). Molecular cloning of MAg showed that MAg cDNA encodes the full length of the env gene and long terminal repeat region of endogenous ecotropic murine leukemia provirus of AKV-type (5).

During *in vivo* peritoneal passage of Meth A cells in our laboratory, we obtained mutant cell line Meth A (mMeth A) cells that were rejected from an intradermal syngeneic BALB/c mouse challenge. We tried to characterize the mMeth A cells. Mice that lacked $CD8^+$ cells could not reject mMeth A cells, suggesting that mMeth A cells have a potent antigenic property. Indeed, mMeth A cells highly expressed antigenic MAg. We immunized BALB/c mice with two types of MAg gene segments by intramuscular injection and found that immunization of the MAg 788-1257 gene protected the mice from a Meth A challenge. We showed in this study that MAg is a potential vaccine candidate for Meth A tumors.

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MATERIALS AND METHODS

Mice

Female BALB/c mice and BALB/c *nu*/*nu* mice (Japan SLC, Shizuoka, Japan) were maintained under specific pathogen-free conditions with a 12-h light : dark cycle at 25 ± 2°C and 55 ± 10% relative humidity. All studies were performed in accordance with the ethical guidelines for animal experimentation by the Institute of Biomedical Sciences, Tokushima University, Japan and were approved by the institution review board of the animal ethics committee.

Meth A cell challenge

Meth A cells were obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. Meth A and mMeth A cells were maintained by intraperitoneal passage in BALB/c mice. Meth A cells (1×10^6 cells) were inoculated into the back skin in BALB/c mice. The tumor volume was calculated as $\pi/6 \times [(a \times b)^{1/2}]^3$, where a and b are two perpendicular major diameters.

Antibody treatment

Anti-CD4 mAb and anti-CD8 mAb were obtained from ascites fluid from BALB/c *nu*/*nu* mice that had been inoculated with GK1.5 and 53.6.7 hybridoma cells, respectively. The Abs were purified by the ammonium sulfate precipitation method. BALB/c mice were each treated with 0.5 mg of the Ab on days -2, +2, +5 and +8 the challenge of Meth A cells.

In vitro proliferation response

Meth A and mMeth A cells were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoetha-

nol for 24 h in a 96-well plate. For the last 8 h of culture, 37 kBq of [^{3}H]thymidine deoxyribose was added to the wells, and the amount of [^{3}H]thymidine deoxyribose incorporated was measured by a scintillation counter (Aloka, Tokyo, Japan).

Microarray analysis and mRNA expression

Total RNA was isolated from Meth A and mMeth A cells using a RNAiso (Takara Bio, Siga, Japan). Microarray analysis was performed using a 44K Whole Genome mRNA microarray system (Agilent Technologies, Santa Clara, CA, USA). Biological process annotation and pathway analysis with normalized and filtered data was performed with GeneSpring 14 software (Agilent Technologies).

Total RNA was isolated from Meth A and mMeth A cells using an RNAiso (Takara Bio, Siga, Japan). Five thousand ng of the extracted total RNA was transcribed by using a PrimeScript RT Master Mix kit (Takara Bio.). Real-time PCR was performed by using specific primers and SYBR green dye (Applied Biosystems, CA, USA) in ABI StepOnePlus™ (Applied Biosystems) according to the manufacturer's instructions. The primers used in this study were as follows :

Murine MAg forward primer 5'-GGGACTACATCACAGTAAGC-3' ;
Murine MAg reverse primer 5'-CAGGAGGTGGCCTGTTTTCCAA-3' ;
Murine β-actin forward primer 5'-CTGACCCTGAAGTACCCCATTGAACA-3' ;
Murine β-actin reverse primer 5'-CTGGGGTGTTGAAGGTCTCAAACATG-3'.

MAg expression plasmids

One μg of the extracted total RNA from Meth A cells was transcribed with a PrimeScript RT Master Mix kit (Takara Bio.). MAg gene segments (788-1257, 1611-2043) were amplified by PCR targeted to the cDNA of Meth A cells. The products were cloned into a pcDNA3.1 mammalian expression vector with unique Eco RI and Bam H1 restriction enzyme sites. We denoted these plasmids as pcDNA-MAg-788 and pcDNA-MAg-1611. The plasmids were purified by using the Plasmid Giga Kit (Qiagen, MD, USA).

MAg expression in vitro

pcDNA-MAg-788 and pcDNA-MAg-1611 were transfected in COS7 cells using FuGENE HD Transfection Reagent (Roche Diagnostics Gmbh, Germany). Gene-transfected COS7 cells were lysed with lysate buffer. Proteins were loaded on SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. The membranes blocked with 5% nonfat milk were probed with anti-His-Tag Ab (Proteintech, IL, USA) at 4℃ overnight and then incubated with a horseradish peroxidase-conjugated secondary Ab.

Gene immunization

Transfection of the expression plasmid into mouse muscle was done using NAPA21 (NEPA GENE, Chiba, Japan). Immunization of the plasmid (25 μg/mouse) was done 3 times at 2-week intervals.

Statistics

Data were analyzed using Student's t-test for comparison to the control group. Differences in survival *in vivo* studies were determined based on Kaplan-Meier survival analysis. Differences were considered significant at $p < 0.05$.

RESULTS

mMeth A cells are rejected from BALB/c mice in vivo

Meth A fibrosarcoma was maintained by passage of intraperitoneal inoculation in BALB/c mice. In the passage of Meth A cells in BALB/c mice, we found that syngeneic Meth A cells were rejected from BALB/c mice *in vivo* (Fig. 1 A and B). These Meth A cells were named mMeth A cells and the tumor were characterized. First, proliferation response was evaluated by inoculation of ^{3}H-thymidine and it was found that the level of proliferation was same as that of the original Meth A cells (Fig.1 C). *In vivo* proliferation of mMeth A cells was determined in T cell deficient athymic nude mice and it was found that mMeth A cells could grow in those mice (Fig. 2A). Next, the effect of depletion of $CD4^+$ and/or $CD8^+$ cells was examined in mMeth A-inoculated BALB/c mice. Although treatment with an anti-CD4 mAb did not affect the growth of mMeth A cells, treatment with an anti-CD8 mAb or the combination of the anti-CD4 mAb and anti-CD8 mAb promoted the growth of mMeth A cells (Fig. 2 B).

mMeth A cells have a high level of MA gene expression

Gene microarray analysis of mRNA expression was performed to characterize mMeth A cells. The microarray analysis showed that melanoma antigen, solute carrier family39, sema domain 3C, glycerol-3-phosphate dehydrogenase 1, prolactin family 2, CD24a antigen I predicted gene were expressed at higher levels in mMeth A cells than in Meth A cells (Table. 1). Quantitative mRNA expression analysis showed that the expression level of MA in mMeth A cells was one handed-times higher than that in Meth A cells (Relative expression of MAg mRNA in mMeth A cells was 100.8 ± 3.5 and that in Meth A cells was 1.0 ± 0.1.) (Fig. 3).

Immunization of MAg gene protects BALB/c mice from Meth A challenge

We hypothesized that melanoma Ag is a target of immune surveillance molecules and we examined the effect of immunization of MAg on protection against a Meth A challenge *in vivo*. We constructed two types of expression plasmid coding MA 788-1257 and 1611-2043, respectively. The expressing protein contains the H-2K^d-binding peptide sequence, *Y******(I or L) (6) (Fig. 4 A). We confirmed MAg 788 and 1611 proteins by Western blot analysis using anti-His-Tag antibody (Fig. 4 B). Mice were immunized with the pcDNA-MAg-788 or pcDNA-MAg-1611 gene three times at 2-week intervals and inoculated with Meth A cells. Fig. 4 C shows the growth of Meth A tumors. Mice immunized with pcDNA-MAg-788 showed smaller tumor volumes than those in control mice (Fig. 4 C). Survival rate of mice immunized with the MAg 788 gene was higher than that of control mice ($p = 0.013$) (Fig. 4 D).

DISCUSSION

MAg cDNA encodes the full length of the *env* gene and long terminal repeat region of endogenous ecotropic murine leukemia provirus of AKV-type. AKV is an endogenous retrovirus causes thymic lymphoma at a high incidence in AKR mice (5). We found that mMeth A cells grew very slowly in the intradermal site and were finally rejected from BALB/c mice (Fig.1 A and B). mMeth A cells proliferated normally in mice deficient in $CD8^+$ cells (Fig. 2). From these results, we hypothesized that mMeth A cells have a potent antigenic property. We immunized MAg that was highly expressed in mMeth A cells and that found that immunization of the pcDNA-MAg-788 gene protected against a Meth A cell challenge *in vivo*. To our knowledge, this is the first study

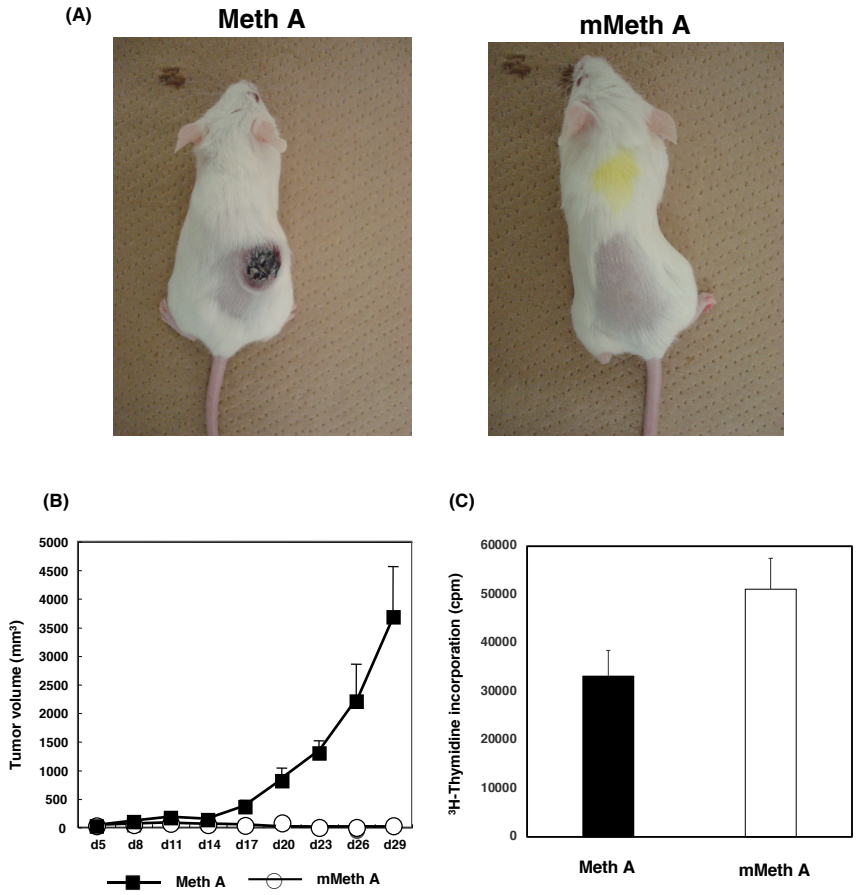

Figure 1. mMeth A cells were rejected from BALB/c mice. (A) Macroscopic observation of tumors in BALB/c mice inoculated with Meth A (left) and mMeth A (right). (B) Meth A and mMeth A were inoculated into the back skin of BALB/c mice and tumor growth was observed (n = 5). (C) *In vitro* proliferation of Meth A and mMeth A was determined as incorporation of [^{3}H]-thymidine. Data are shown as means ± SD.

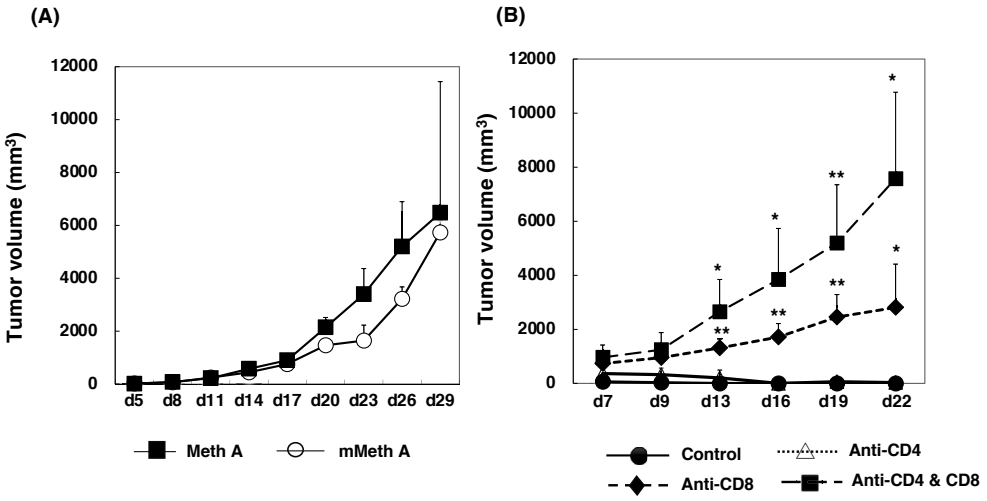

Figure 2. Effects of T cell depletion on mMeth A cell growth *in vivo*. (A) Meth A and mMeth A cells were inoculated in BALB/c *nu*/*nu* mice (n = 5). (B) mMeth A cells were inoculated in BALB/c mice that had been treated with anti-CD4 and/or anti-CD8 mAb as described in the Materials and Methods section (n = 5). Data are shown as means ± SD. * $p < 0.05$ vs control, ** $p < 0.01$ vs control.

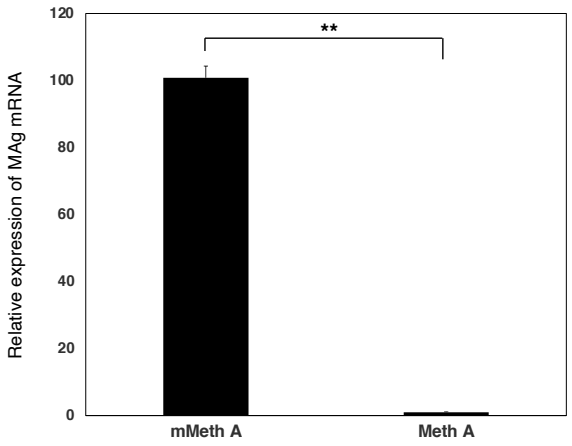

Figure 3. MAg is highly expressed in Meth A cells. Quantitative mRNA expression analysis of MAg in Meth A and mMeth A cells. Data are shown as means ± SD. ** $p < 0.01$.

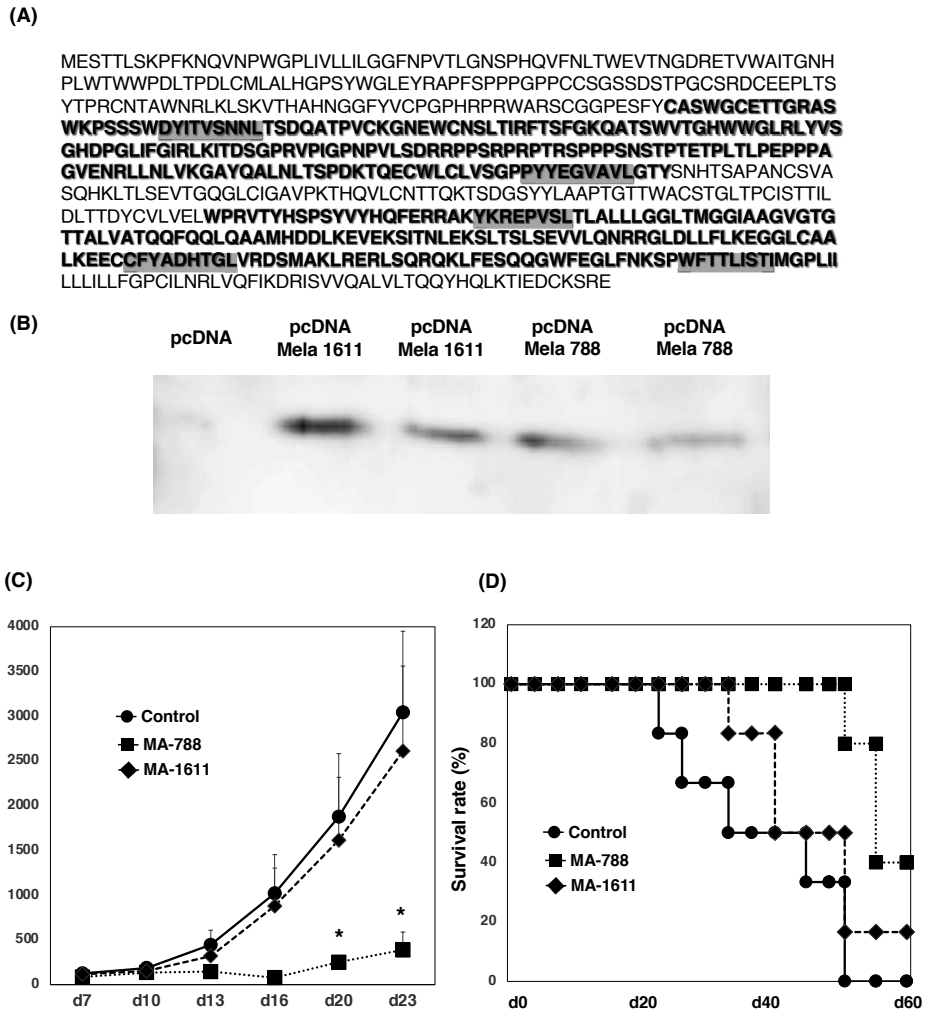

Figure 4. Immunization of the pcDNA-MAg-788 gene protects against Meth A challenge in BALB/c mice. (A) shows the amino acid sequence of MAg. Bold sequences are amino acids corresponding to MAg 788-1257 and 1611-2043 genes. Gray letters indicate the speculated H-2K^d-restrict epitope. (B) Plasmids expressing MAg 788 and 1611 were transfected with COS 7 cells and the protein expression was determined by Western blotting probed with an anti-His-Tag Ab as described in the Materials and Methods section. BALB/c mice that had been immunized with pcDNA (circle), pcDNA-MA-788 (square) and pcDNA-MA-1611 genes (rhombus) three times at 2-week intervals were challenged with Meth A cells. Tumor growth (C) and survival rate (D) are shown. The number of mice in each group was five or six. Data are shown as means ± SEM. * $p < 0.05$ vs control.

Table 1. Top ten genes that highly expressed in mMeth A by microarray analysis.

Fold change	genedescription	genebank	genesymbol
181	melanoma antigen	D10049|BC113756|AK145028	Mela
127	solute carrier family 39 (zinc transporter), member 4	BC023498	Slc39a4
121	melanoma antigen	U63133	Mela
96	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	BC066852	Sema3c
53	glycerol-3-phosphate dehydrogenase 1 (soluble)	BC019391	Gpd1
46	prolactin family 2, subfamily c, member 5	AF128884	Prl2c5
44	CD24a antigen | predicted gene, EG621324	BC075622	Cd24a|EG621324
40	prolactin family 2, subfamily c, member 3	BC100299|K03235|BC100300	Prl2c3|Prl2c4|Prl2c2
38	tensin 4	BC055820	Tns4
35	predicted gene, EG432466 | argininosuccinate synthetase 1	M31690	EG432466|Ass1

providing evidence that MAg is a potential vaccine candidate Ag.

We constructed two types of expression plasmid that code MAg genes corresponding to 788-1257 and 1611-2043. pcDNA-MAg-788 and pcDNA-MAg-1611 contain two and three H-2K^d-restricted Ag epitopes, respectively. The results of the gene immunization study showed that the vaccine efficacy of pcDNA-MAg-788 is superior to that of pcDNA-MAg-1611 (Fig. 4 D). Speculated H-2K^d-restricted Ag epitopes in pcDNA-MAg-788 are DYITVSNNL and PYYEGVAVL. Further study is needed to identify which epitope is important for the protection or whether both are needed. Moreover, it should be identifying that contribution of CD8$^+$ cells by pcDNA-MAg-788-mediated protected immunity in Meth A challenge.

It has been shown that MAg is highly expressed in B16 melanoma cells but is not expressed in other types of tumor cells including EL-4 cells, P815 cells, L cells, BW5147 cells and P3U1 cells [5]. We compared the levels of MAg mRNA expression in fibrosarcoma Meth A, thymoma EL-4 and B lymphoma A20 cells. Among those tumor cells, the expression levels of MAg in A20 cells and EL-4 cells were sixty-times and twenty-times higher, respectively, than expression level in Meth A (data not shown). It would be interesting to examine the role of MAg in the antigenic property of A20 and EL-4 cells.

BALB/c murine sarcoma Meth A is known to have three missense point mutations in p53. It has been shown that a nonamer peptide containing the codon 234 mutational product (234CM) elicited mutation epitope-specific CTLs and that immunization with 234CM in an adjuvant inhibited Meth A growth *in vivo* (7, 8). In Meth A p53 mutation, vaccine application have been tried in both dendritic cell- and gene-based approaches (9, 10). In addition to the p56 protein, Meth A-derived heat shock protein 70 and 17b-hydroxysteroid dehydrogenase type 12 have been shown to be candidates for a vaccine for Meth A (11).

In the course of investigating the characteristics of mMeth A cells, we found a new tumor vaccine candidate Ag, MAg, in this study. We hope that this new tumor vaccine candidate will have clinical application in the future.

CONCLUSIONS

We obtained an mMeth A tumor that was rejected from syngeneic BALB/c mice. Interestingly, mMeth A cells could grow normally in mice that lacked CTLs, suggesting that CTL recognized antigenic Ag expressing mMeth A cells. Microarray analysis showed that expression levels of specific genes in mMeth A cells were elevated compared to those in Meth A cells. We focused on MAg that is highly expressed in mMeth A cells. Immunization of pcDNA-MAg-788 protected against a Meth A challenge *in vivo*. This study provided the evidence that MAg is a potential vaccine Ag for Meth A sarcoma.

CONFLICT OF INTEREST

The authors declare no conflict of interest

ACKNOWLEDGEMTS

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