Glatiramer acetate (Copaxone®) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis

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We examined the effect of glatiramer acetate, a random copolymer of alanine, lysine, glutamic acid, and tyrosine, on antigen-specific T-cell responses in patients with multiple sclerosis (MS). Glatiramer acetate (Copaxone) functioned as a universal antigen, inducing proliferation, independent of any prior exposure to the polymer, in T-cell lines prepared from MS or healthy subjects. However, for most patients, daily injections of glatiramer acetate abolished this T-cell response and promoted the secretion of IL-5 and IL-13, which are characteristic of Th2 cells. The surviving glatiramer acetate–reactive T cells exhibited a greater degree of degeneracy as measured by cross-reactive responses to combinatorial peptide libraries. Thus, it appears that, in some individuals, in vivo administration of glatiramer acetate induces highly cross-reactive T cells that secrete Th2 cytokines. To our knowledge, glatiramer acetate is the first agent that suppresses human autoimmune disease and alters immune function by engaging the T-cell receptor. This compound may be useful in a variety of autoimmune disorders in which immune deviation to a Th2 type of response is desirable.

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Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) white matter. The high frequency of activated, myelin-reactive T cells in the circulation and cerebrospinal fluid of patients with MS is consistent with the hypothesis that an initiating event linked to an antecedent microbial infection in a genetically susceptible host eventually leads to an autoimmune-mediated destruction of myelin followed by the surrounding axons (1). After the initiating event(s), the CNS itself may become a potential depot of antigen and MHC, with expression of critical second signals required for T-cell activation such as B7-1 and CD40 (2, 3) leading to epitope spreading (4). MS is thought to be a Th1-mediated disease based largely on pathological resemblance to a delayed-type hypersensitivity response in the CNS and from observations made in the murine experimental autoimmune encephelomyelitis (EAE) model. However, direct cloning of myelin-reactive T cells from the blood of patients with MS suggests that the majority of T cells can secrete both Th1- and Th2-type cytokines (5).

A major goal in the treatment of autoimmune diseases has been the development of antigen-specific therapies that target autoreactive T cells. The discovery of epitope spreading in the EAE model (4, 6) and observations of diverse T-cell receptor repertoires in response to self-antigens have theoretically made this approach less attractive. Instead, the concept of bystander suppression has emerged in which autoreactive Th2 or Th3 T cells are generated that migrate to the inflamed target organ where they are antigen specifically reactivated, leading to the secretion of cytokines that downregulate inflammation in the local milieu in an antigen nonspecific mechanism (7). Two approaches have emerged for inducing immune deviation of autoreactive T cells: mucosal administration of antigen, which induces Th2 T-cell responses to the antigen (7), and altered peptide ligands (APLs), which, by inducing a weaker strength of signal, lead to Th2 deviation of cytokine secretion (8–11). Both approaches have been used in clinical trials to treat patients with MS, but to date, without success.

An alternative approach to the use of a single self-antigen that has been altered or given mucosally is the administration of peptide mixtures that contain many different antigen specificities. The use of random copolymers that contain amino acids commonly used as

MHC anchors and T-cell receptor (TCR) contact residues are possible "universal APLs." Glatiramer acetate (GA) (Copaxone; Teva Marion Partners, Kansas City, Missouri, USA) (12) is a random sequence polypeptide of the 4 amino acids alanine (A), lysine (K), glutamate (E), and tyrosine (Y) at a molar ratio of A/K/E/Y of 4.5:3.6:1.5:1, respectively, and an average length of 40–100 amino acids. Directly labeled GA efficiently binds to different murine H-2 I-A molecules and to the human counterparts, MHC class II DR, but not to DQ or MHC class I, molecules in vitro (13). Biochemical studies revealed that GA also binds directly and with high affinity to purified HLA-DR1, -DR2, and -DR4 (14), suggesting that GA contains multiple epitopes enabling it to bind promiscuously to MHC class II molecules, where it could potentially be recognized by CD4 T cells.

A "universal antigen" containing multiple epitopes would be expected to induce proliferation in vitro, as measured by [^{3}H]thymidine incorporation in naive T cells from the circulation, representing a high degree of cross-reactivity to other peptide antigens. In in vitro cultures of PBMCs from healthy humans, a strong dose-dependent proliferative response to GA has been reported (15). Similarly in our own studies, we found that GA elicits dose-dependent responses in all of more than 50 humans, including healthy subjects and patients with relapsing remitting (RR) and chronic progressive MS (P.W. Duda and D.A. Hafler, manuscript in preparation). The response to GA could be blocked by anti-DR antibodies and the restriction of GA-reactive CD4$^+$ T cells to a particular HLA DR molecule could be shown on a clonal level. The high proliferative and cytokine responses of naive PBMC CD4$^+$ T cells suggest a high frequency of circulating GA-reactive precursor T cells. Our own limiting dilution analysis suggests that the precursor frequency of GA-reactive T cells ranges from 1:5,000 to 1:100,000 PBMCs. Thus, GA appears to constitute a highly cross-reactive antigen preparation.

In animal models of MS, prophylactic subcutaneous administration of GA has been shown to prevent EAE induced by injection of purified myelin basic protein (MBP) (12), proteolipid protein (PLP) (16), or myelin oligodendrocyte glycoprotein (MOG) (17). Of greater importance, in a phase III clinical trial subcutaneous administration of GA has been shown to decrease the rate of exacerbations and to decrease the appearance of new lesions, based on magnetic resonance imaging (MRI), of patients with RR MS (18–20). This represents perhaps the first successful use of an agent that ameliorates autoimmune disease by altering signals presumably through the TCR.

The early observation that cyclophosphamide abrogated the beneficial effect of GA on EAE (21), suggested that the mechanism of action of GA involved the induction of regulatory T cells. Later, adoptive transfer of GA-specific T cells was found to inhibit EAE (22). It was originally thought that GA was structurally cross-reactive with MBP, although this has remained controversial. Recently, TCR antagonism has been suggested to occur in addition to competition for MHC binding (23). Stimulation of murine GA-reactive T-cell lines and clones with MBP was reported to induce the secretion of Th2 and Th3 cytokines to this cross-reactive antigen (24).

Taken together, these data led to the hypothesis that GA acts as an APL in vivo, leading to alterations of responses to myelin antigens by cytokine deviation of myelin-specific T cells and bystander suppression mediated by GA-reactive T cells. Here, we directly tested this hypothesis by investigating changes in antigen-specific responses in patients with MS who were undergoing treatment with daily subcutaneous injections of GA. T-cell reactivity to GA, the immunodominant MBP epitope 84–102 as a model myelin antigen, combinatorial libraries derived from the MBP 84-102 sequence, and a completely random 13mer sequence was examined in vitro before and during a year of treatment. Examination of proliferative responses to combinatorial libraries was deemed potentially informative based on the observation that combinatorial peptide libraries are a powerful tool to examine the degree of T-cell receptor degeneracy. That is, the degree to which a T-cell clone proliferates to a random combinatorial peptide library where all of the 13 amino acids are random, representing a total of 19^{13}independent peptides, to a first approximation provides information regarding the degree of degeneracy for that clone, i.e., the more peptides the clone can recognize, the more degenerate the T-cell receptor. Together, these experiments enabled us to examine whether daily subcutaneous injections of GA induced alterations of the T-cell immune response.

Methods

Patients. Patients with RR MS in early stages of the disease, with MRI findings consistent with the diagnosis, and who decided with their physicians to initiate GA treatment participated in the trial. No clinical exami-

Table 1
Characteristics of the study patients

Patient	Sex	DR	Age at onset of disease	Duration of disease (y)	EDSS[A]	Attacks[B]
1	F	3/13	42	7	1	4
2	M	2/4	48	10	3	0
3	F	2/4	36	2	1	3
4	F	3/4	35	1	2	1
5	M	1/11	28	2	1	2
6	F	2/4	45	1	1	2
7	F	2/8	24	2	0	0

[A]Assessed before initiation of treatment. [B]Number of attacks in the 2 years before initiation of treatment.

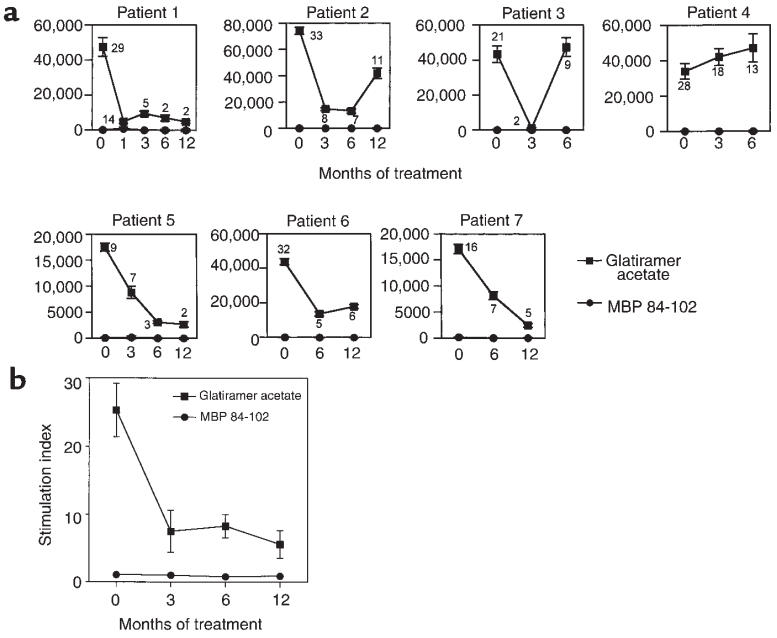

Figure 1
The proliferative response to GA is decreased on average after daily injections of GA. The antigen-specific proliferative response of 20 or 30 primary T-cell lines induced with 40 μg/mL GA as described in Methods was measured by split-well assay for each patient at each time point. Before and at 6 months of treatment, all 7 patients could be tested; at 3 and 12 months, data for 5 patients were obtained. (**a**) Each panel represents data from an individual patient. Squares represent mean ± SEM proliferation in Δ cpm of the GA-specific response compared with the no-antigen control. Background levels of the [^{3}H]thymidine incorporation for all patients of the no-antigen condition were 1,747 ± 111 before treatment, and 3,286 ± 175, 3,785 ± 262, and 3,509 ± 239 at 3, 6, and 12 months of treatment, respectively. The numbers in the figure indicate the SI over the no-antigen control for each time point. (**b**) The mean stimulation index SI ± SEM for all T-cell lines from all patients tested at each time point is shown.

nation other than routine follow-up in the clinic was performed, and no other preselection criteria were applied. Informed consent was obtained before enrollment, and the study was performed in compliance with the rules of the ethical guidelines for human experiments of the Institutional Review Board of the Brigham and Women's Hospital. Table 1 summarizes the patient characteristics.

Antigens. GA (Copaxone; lots 123211 and 123243) was supplied by Teva Marion Partners. MBP 84-102 (DENPVVHFFKNIVTPRTPP) and MBP 93R (ENPVVHFF**R**NIVTPR) peptides were synthesized by standard fmoc technology and HPLC-purified to greater than 99%. Combinatorial peptide library X13 was a 13mer randomized at each position, and combinatorial peptide libraries with random amino acids inserted at position X of the MBP 85-99 peptide (ENPVVHFFKNIVTPR) were: 90X (ENPVV**X**FFKNIVTPR), 91X (ENPVVH**X**FKNIVTPR), 93X (ENPVVHFF**X**NIVTPR), 90X93R (ENPVV**X**FF**R**NIVTPR), and 91X93R (ENPVVH**X**F**R**NIVTPR). All peptide libraries were obtained from Chiron Technologies (Raleigh, North Carolina, USA). Peptides were dissolved at 10 mg/mL in DMSO.

Generation of antigen-specific T-cell lines. PBMCs were isolated from fresh drawn heparinized blood by Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation according to manufacturer's protocol. Antigen-specific T-cell lines were generated by culturing 150,000 PBMCs per well in the presence of 40 μg/mL GA or MBP 84-102 peptide. Unless otherwise indicated, all cell cultures were done in 96-well U-bottom microtiter plates in 200 μL complete medium (RPMI 1640; BioWhittaker Inc., Walkersville, Maryland, USA) containing 2.5% heat-inactivated pooled human AB serum (PelFreeze, Brown Deer, Wisconsin, USA), sodium pyruvate, HEPES, nonessential amino acids, and glutamine) in a humidified 8% CO_2 incubator at 37°C. For patient 5, whole human MBP was used instead of the MBP 84-102 peptide throughout. On day 5 of culture, 120 μL of culture supernatants was removed and replaced by 140 μL complete medium containing 10% phytohemagglutinin-free (PHA-free) T-stim (Collaborative Biomedical Laboratories, Bedford, Massachusetts, USA). On day 7, each GA-induced T-cell line was transferred into 1 mL of complete medium containing 10% T-stim for further expansion. On day 12, split-well assays were performed to test for antigen-specific and cross-reactive proliferation and cytokine secretion. For patients 1, 2, 3, and 4, 40 T-cells lines to MBP 84-102 and 20 T-cell lines to GA were generated at each time point. For patients 5, 6, and 7, 30 T-cell lines were generated for each antigen.

Cross-reactivity assays. Equal aliquots of primary T-cell lines were stimulated with irradiated (33 Gy) autologous PBMCs that had been preincubated with antigen in 96-well U-bottom microtiter and ELISPOT plates at 37°C for 1 hour. The conditions tested with primary GA-reactive lines were 20 μg/mL GA, 100 μg/mL X13, 40 μg/mL MBP 84-102, 20 μg/mL 90X, 20 μg/mL 91X, 20 μg/mL 93X, 20 μg/mL 93R, 20 μg/mL 90X93R, 20 μg/mL 91X93R, and the no-antigen control. Primary MBP 84-102–induced lines were tested with 20 μg/mL MBP 84-102, 40 μg/mL GA, and no antigen.

Proliferation assay and cytokine measurement by ELISA. Equal aliquots of primary T-cell lines were stimulated with antigen-pulsed autologous PBMCs (100,000 per well). After 48 hours, 160 μL of supernatant was removed and frozen at –80°C for future cytokine analysis. The cells were pulsed with 1 μCi/well of [^{3}H]thymidine in 100 μL of complete medium. After a further 24 hours, cells were harvested onto filter paper, and incorporation of [^{3}H]thymidine was measured in a scintillation counter (Wallace, Gaithersburg, Maryland, USA). Supernatants were tested for cytokines in duplicate by performing standard sandwich ELISA using matched antibody pairs according to the manufacturer's protocol (Endogen Inc., Woburn, Massachusetts, USA).

ELISPOT assay. ELISPOT plates (Millipore Corp., Bedford, Massachusetts, USA) were coated with an optimal concentration of 100 μL of primary antibody diluted in 0.1 mM $NaHCO_3$ (pH 8.3) and incubated overnight at 4°C. Antibody pairs were the same as those used in the sandwich ELISA assay described earlier here. Plates were washed 3 times with PBS and blocked with 1% BSA in HBSS at 37°C for 1 hour. Plates were again washed with PBS 3 times, and antigen-presenting cells were added together with antigen and placed in a 37°C, 8% CO_2 incubator for 60 minutes. Responder T cells were added, and plates were placed in a 37°C incubator for 24 hours. The plates were then washed 3 times with TP buffer (0.05% Tween in PBS) and incubated with 100 μL of biotinylated secondary antibody in TP buffer overnight at 4°C. Plates were washed again 3 times with TP buffer and incubated at room temperature with 100 μL of a 1:1,000 dilution of streptavidin alkaline phosphatase conjugate Extravidin (Sigma Chemical Co., St. Louis, Missouri, USA) for 2 hours. Plates were washed, and viewing of spots was carried out with 100 μL BCIP/NBT substrate (Sigma Chemical Co.) prepared according to manufacturer's instructions and by developing in dark for up to 20 minutes. The reaction was stopped by washing the plates with distilled water.

Measurement of T-cell proliferation in primary in vitro culture. PBMCs, isolated from heparinized blood as already described here, were incubated at 50,000 PBMCs per well in the presence of PHA or at 150,000 PBMCs per well in the presence of tetanus toxoid (Massachusetts Biological Laboratories, Jamaica Plain, Massachusetts, USA). On day 6, 160 μL of culture supernatant was removed from each well and replaced by 100 μL of complete medium containing 1 μCi of [^{3}H]thymidine. After further incubation at 37°C for 18 hours, cells were harvested onto filter paper, and thymidine incorporation was measured by scintillation counting.

HLA typing. Determination of the HLA DR and DQ phenotypes of each patient was determined by standard PCR and hybridization methods.

Statistical analysis. Statistical analysis was performed using the STATISTICA for Macintosh package (StatSoft, Tulsa, Oklahoma, USA) as indicated. Unless otherwise indicated, results are given as mean ± SEM.

Results

The in vitro proliferative response of PBMCs to GA decreases upon in vivo administration of GA. PBMCs were isolated from 7 patients with RR MS before and at various times after subcutaneous administration of GA. At each time point tested, primary and secondary in vitro proliferation and cytokine assays in the presence and absence of GA were performed. We found that before treatment, there was a significant proliferative

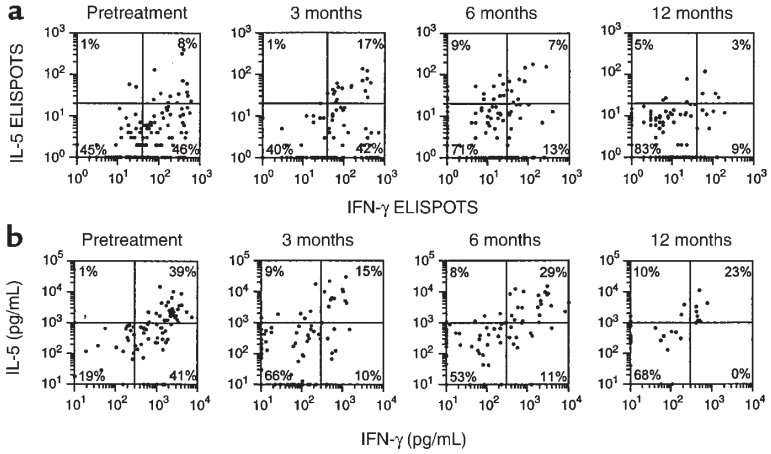

Figure 2
GA-specific secretion of cytokines is polarized toward a Th2 response after daily injections of GA. The GA-specific secretion of the cytokines IL-5 and IFN-γ was measured in T-cell lines by 2 methods: ELISPOT (**a**) and ELISA (**b**) assays. Each symbol represents the difference of spots counted or Δ pg/mL measured in split-well assays between the GA (20 μg/mL) condition and the no-antigen control. The limits of detection were 1 spot and 10 pg/mL, respectively. Numbers represent the percentage of T-cell lines in each quadrant with a minimum difference in spots of twice the SD of the negative controls for IL-5 and IFN-γ, respectively.

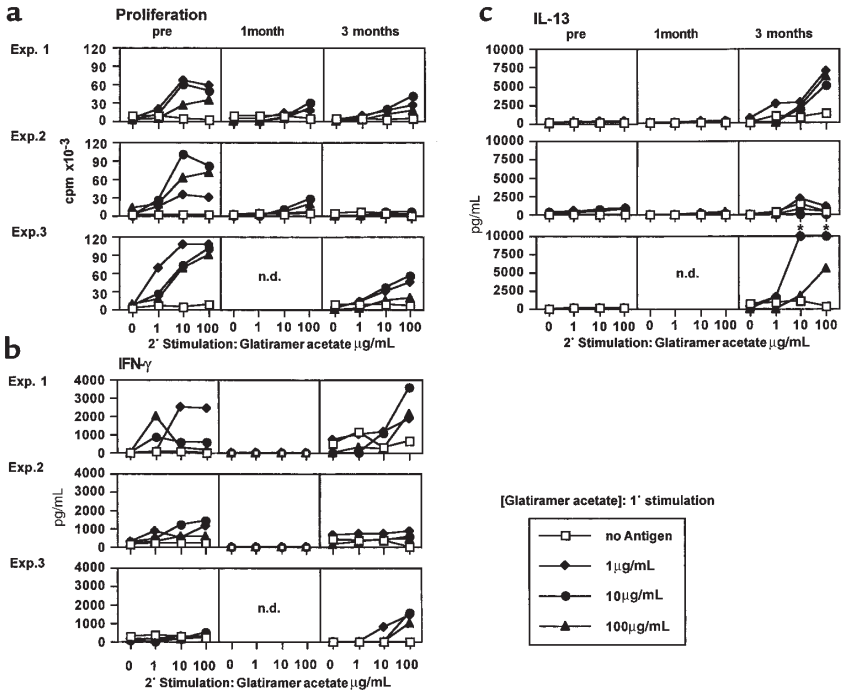

Figure 3
IL-13 secretion is increased after daily injections of GA. Primary T-cell lines were set up in 10 identical wells each in the presence of no antigen or with 1.0, 10, and 100 μg/mL GA and cultured as described in Methods. Identical wells were pooled on day 11, and 30,000 T cells each were restimulated with no antigen or 1.0, 10, and 100 μg/mL GA pulsed on 100,000 autologous APCs. Cytokines were measured in supernatants by ELISA after 48 hours as described, and proliferation was measured by [^{3}H]thymidine incorporation. Asterisks point at values that were above the upper limit of detection of the IL-13 assay of 10,000 pg/mL.

response as measured by [^{3}H]thymidine incorporation to GA in all 7 patients, with an average stimulation index (SI) in vitro of 24.8 ± 1.1; the average Δ cpm was 37,241 ± 1,766 cpm. Additionally, all of a total of 170 independently derived T-cell lines stimulated in primary in vitro culture with GA proliferated in response to the antigen (data not shown). After treatment with GA 20 mg subcutaneously daily for 3, 6 and 12 months, the proliferative response as measured by SI and Δ cpm significantly decreased (Figure 1a) (P < 0.001), although, as expected, individual patients varied in their response to GA (Figure 1b).

In vitro–generated GA-reactive T-cell lines deviate toward a Th2-cytokine profile upon treatment with GA. Having demonstrated that the proliferative response to GA changed after in vivo subcutaneous administration of GA, we next examined whether the cytokine profile also changed. The cytokine response was measured for the prototypic Th1 and Th2 cytokines IFN-γ and IL-5 by ELISPOT and sandwich ELISA in a total of 590 T-cell lines generated before and at various times after GA injection in all 7 patients. As shown in Figure 2, a and b, compared with the values detected before treatment, the average IFN-γ secretion to GA measured by either ELISA or ELISPOT was significantly decreased (P < 0.001 by Tukey's honest statistical difference test) after treatment for 3, 6, and 12 months, except for the measurement by ELISPOT at 3 months, which did not reach significance, and the ELISA measurement at 6 months, which only reached a significance level of P < 0.01. The GA-dependent IFN-γ secretion as determined by ELISPOT as Δ spots between the cells tested with antigen and the no-antigen control was 104 ± 10 before treatment, 132 ± 18 at 3 months, 28 ± 4 at 6 months, and 18 ± 3 at 12 months. IFN-γ secretion measured by sandwich ELISA in Δ pg/mL was 1,405 ± 150 before treatment, 222 ± 38 at 3 months, 797 ± 185 at 6 months, and 5.9 ± 46 at 12 months. When patients were analyzed individually, 5 of the 7 patients had statistically significant decreases in IFN-γ secretion (P < 0.001) by ELISPOT or sandwich ELISA (data not shown). The levels of IFN-γ secretion were correlated with the decreased proliferative capacity in these patients (r^2 of > 0.8 for ELISA values in all patients tested, measurement by ELISPOT correlated less well with r^2 > 0.5 in 4 patients). This is in accordance with previous observations that the proliferative and IFN-γ responses are correlated (25).

GA-specific IL-5 secretion was, on average, not statistically significantly altered during treatment with GA. By ELISPOT assay, the average IL-5 secretion in Δ spots was 10 ± 3 before, 10 ± 2 at 3 months, 11 ± 2 at 6 months, and 6 ± 1 at 12 months after the initiation of treatment. When measured by sandwich ELISA as Δ pg/mL, IL-5 secretion was 1,484 ± 266 before treatment; 1,940 ± 554 at 3 months; 1,738 ± 343 at 6 months; and 1,146 ± 303 at 12 months after the initiation of treatment. One patient had a sustained statistically significant decrease in IL-5 (P < 0.001), and 1 had a sustained statistically significant increase (P < 0.001) in IL-5 secretion as measured by ELISPOT during treatment.

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