Collagens Serve as an Extracellular Store of Bioactive Interleukin 2*

The binding of certain growth factors and cytokines to components of the extracellular matrix can regulate their local availability and modulate their biological activities. We show that interleukin 2 (IL-2), an important stimulator of T cell growth, preferentially binds to collagen types I, III, and VI and to a lesser degree to collagen types IV, V, and V, immobilized on polystyrene or nitrocellulose. These interactions are inhibited by denatured, single collagen chains or a subset of their cyanogen bromide peptides in a dose-dependent manner. Cross-inhibition experiments and ligand blotting of collagen-derived peptides point to a limited set of collagenous consensus sequences mediating the binding of IL-2. This interaction is saturable, with dissociation constants of $10^{-11}$ M, and estimated molar ratios of 4–6 molecules of IL-2 bound to one molecule of triple helical collagen. Furthermore, collagen-bound IL-2 stimulates proliferation of mouse lymphocytes. We conclude that its specific binding to the abundant interstitial collagens leads to a spatial pattern of bioavailable IL-2 which is dictated by the local organization of the collagenous extracellular matrix. This interaction may contribute to the particular phenotype of stromal lymphocytes and could be exploited for devising collagenous peptide analogues that modulate IL-2 bioactivity.

The interaction of growth factors and cytokines with components of the extracellular matrix (ECM) has received increasing attention. Binding to ECM can influence the availability and modulate the biological activity of these factors (1–5) which by themselves influence matrix remodeling, e.g. by stimulating matrix metalloproteinase expression (6). Glycosaminoglycans and proteoglycans were initially found to be the major growth factor binding ECM components. Examples are the interaction of basic fibroblast growth factor, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), granulocyte-macrophage colony-stimulating factor, and interferon-γ with heparin and heparan sulfate chains of proteoglycans (3, 7–13). In addition, cytokines can bind to glycoproteins of the ECM. Thus transforming growth factor-β1 (TGF-β1) interacts with the core proteins of the proteoglycans decorin and biglycan (14, 15), with fibronectin (16), and with latent transforming growth factor-β-binding protein (17), also a component of the ECM (18, 19). Tumor necrosis factor-α and IL-7 bind to laminin and fibronectin (20–22) and PDGF (forms AB and BB) to secreted protein, acidic and rich in cysteine/osteonectin (23, 24). Degradation of latent transforming growth factor-β-binding protein and matrix-bound insulin-like growth factor binding protein-5 by serine proteases up-regulates the availability of the respective growth factors (25, 26).

There is growing evidence that collagens, which are the major components of most extracellular matrices, may as well serve as extracellular ligands for certain growth factors and cytokines, as first demonstrated for active TGF-β1 which can be immobilized on collagen type IV (27). Similarly, PDGF (forms AA, BB, and AB), and HGF interact specifically with collagens (28, 29). The binding of PDGF and HGF to collagens does not interfere with their biological activity, suggesting that these abundant ECM proteins may represent an important biological reservoir for active growth factors (28, 29).

In the present study we demonstrate that interleukin 2 (IL-2) which is one of the most important stimulators and modulators of T cell activation, plays a major role in the pathophysiology of various immune-mediated diseases such as rheumatoid arthritis (30), multiple sclerosis (31), and transplant rejection (32), can bind reversibly to collagens. This binding preserves the biological activity of IL-2 and involves primary collagenous consensus sequences.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human recombinant IL-2 was purchased from Biomol (Hamburg, Germany, catalog number 50442), Roche Molecular Biochemicals (catalog number 1147528), and from Eurocetus (Frankfurt, Germany (Pro- leukin™)). All other reagents were either from Merck or Sigma and were the highest purity available. Polystyrene microtiter plates (Immuno- lon 2, Removawell) were from Dynatech (Hamburg, Germany). Native type I, III, IV, V, and VI collagens were isolated from human placenta or skin, and type II collagen was purified from human articular cartilage. Preparation of the native collagens, their isolation, purification, and the biochemical modifications of collagen chains were performed as described before (28, 29, 33–35). Cyanogen bromide (CNBr) peptides were prepared by digestion with CNBr by solubilizing 2 mg of single collagen chains in 1 ml of 70% formic acid at room temperature, flushing the tube 10 min with nitrogen, and adding 2 mg of CNBr, followed by a 4-h incubation at 37 °C and by lyophilization (36). These peptides were purified using gel filtration and ion-exchange fast protein liquid chromatography.

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† Recipient of a Hermann and Lilly Schilling professorship. To whom correspondence should be addressed: Medizinische Klinik I, University of Erlangen-Nuernberg, Krankenhausstr. 12, 91054 Erlangen, Germany. Tel.: 09131-85-3398 (3386); Fax: 09131-85-36003; E-mail: detlef.schuppen@med1.imed.uni-erlangen.de.

The abbreviations used are: ECM, extracellular matrix; CNBr (or CB), cyanogen bromide peptide; HGF, hepatocyte growth factor; IL-2, Interleukin 2; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor β1; PAGE, polyacrylamide gel electrophoresis.
IL-2 binds to immobilized native triple helical collagens and to single collagen chains. Native triple helical collagen (C) types I–VI and single collagen chains of collagen types III, IV, and VI after reduction and alkylation (r/a), as well as chains α1(I) and α2(I) were immobilized on polystyrene microtiter wells at 2 or 4 μg/100 μl for native collagens or collagen chains, respectively, followed by incubation with 1–2 ng (0.0845 to 0.129 nmol) of radiolabeled IL-2 (molecular mass 15.5 kDa). Detergent-blocked polystyrene served as control (p). After three washes bound radioactivity was measured and is expressed as percent of the initially added radioactivity. Shown are the means (± S.E.) of at least five independent experiments performed in triplicate.

**Methods**

**Coating of Microtiter Plates with Collagens, Single Collagen Chains, and α1(I)-derived Cyanogen Bromide (CNBr) Peptides—**Coating of microtiter plates and calculation of coating efficiencies were performed as described before (28, 29). Briefly, native collagens and collagen chains were immobilized on polystyrene microtiter wells at concentrations of 2–4 μg/100 μl/well for binding studies, and at 50 ng/200 μl/well for inhibition experiments. Purified collagen α1(I)-derived cyanogen bromide peptides were coated at 1 μg/μl/well. Immobilization was done in 50 mM ammonium bicarbonate, pH 9.6, overnight at 4 °C, followed by three washes with phosphate-buffered saline (PBS), pH 7.4. Unspecific binding sites were blocked with PBS, containing 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate), for 1 h at 4 °C. Coating efficiencies for 2 μg/well of native collagens and collagen chains ranged between 21 and 48% (28, 29).

**Radiolabeling and IL-2 Binding Assay—**IL-2 was radiolabeled with the 125I-labeled Bolton-Hunter reagent (PerkinElmer Life Sciences) according to the manufacturer’s recommendations. 125I-Labeled IL-2 was separated from free iodine by a Sepharose G-25 column (PD 10, Amersham Pharmacia Biotech) in PBS, containing 0.05% Tween 20 as described (28, 29). Incorporated radioactivity ranged between 20,000 and 30,000 cpm/μg 125I-1.IL-2. Precipitation with trichloroacetic acid (10% w/v) in the presence of 200 μg of borine serum albumin/200 μl, usually yielded 90–98% of protein-bound radioactivity. Purity of radiolabeled IL-2 was demonstrated by SDS-PAGE and autoradiography.

For binding studies 1–2 ng of 125I-1-IL-2 in 100 μl of PBS, 0.05% Tween was added to the collagen-coated wells incubated for 2 h at room temperature, and finally, after three washes in binding buffer (PBS, 0.05% Tween 20), radioactivity bound to the collagen-coated wells was measured in a γ-counter (Berthold, Bad Wildbach, Germany).

For ligand blot, 2 μg of CNBr peptides from single collagen chains α1(I), α2(I), and α1(III), and pepsin-resistant triple helical fragments of collagen types IV and VI were separated by SDS-PAGE and blotted to nitrocellulose. The blots were blocked with PBS, 0.3% Tween 20 overnight at 4 °C, washed three times in binding buffer, and incubated with ~50 ng of 125I-1-IL-2 diluted in 20 μl of binding buffer (50,000 cpm/ml) for 2 h at room temperature, followed by three washes with binding buffer before air-drying and autoradiography.

**Inhibition Experiments—**Since solubilized triple helical, native collagen types I, III, V, and VI may rapidly form fibrils in neutral buffers, reproducible results from inhibition experiments were only obtained by using single chains from these collagens, either after heat denaturation or after reduction and alkylation. Furthermore, CNBr peptides of collagen chains α1(I) were used for inhibition experiments. 1–2 ng of 125I-1-IL-2 and sequential dilutions of collagen chains or collagen α1(I)-CNBr peptides were preincubated in a total volume of 350 μl for 2 h at room temperature in detergent-blocked polypropylene tubes. 100 μl of the mixture was then added in triplicate to the collagen-precoated microtiter wells. After a further 2 h of incubation and three washes with binding buffer, bound activity was measured as described above.

**Saturation Binding Experiments—**For saturation binding studies increasing amounts of unlabeled IL-2 (0–10 μg) were added to 2 ng (~50,000 cpm) of the labeled cytokine in a final volume of 100 μl of binding buffer and incubated for 2 h at room temperature in microtiter wells that were precoated with 200 ng of native triple helical collagens or single collagen chains. To exclude different binding affinities of radiolabeled *versus* unlabeled IL-2, binding experiments were also performed by using the radiolabeled IL-2 up to a concentration of 20 ng (~500,000 cpm) per well. The resultant binding curves showed a superimposable pattern when compared with those obtained with 2 ng of radiolabeled IL-2 and increasing amounts of unlabeled cytokine (data not shown).

**Lymphocyte Proliferation Assay—**To determine biological activity of collagen-bound IL-2, a modified IL-2 bioassay was used (37). Briefly, a mouse T-lymphocyte cell line (CTLL-2, ATCC TIB 214) was cultured in 80-cm² flasks containing RPMI 1640, glutamine (2 mM), mercaptoethanol (50 μl), IL-2 (Roche Molecular Biochemicals, 0.05 units/ml), supplemented with penicillin (107 units/ml), streptomycin (10 mg/ml), and 10% fetal calf serum (Biochrom, Berlin, Germany) under standardized conditions (37 °C, 8% CO₂) in a humidified atmosphere. Collagen I was coated on microtiter wells at a concentration of 2 μg/100 μl/well (0.3 cm²) overnight at 4 °C. Wells were blocked with polyvinyl alcohol (1 mg/ml) in PBS, 0.05% Tween 20 for 1 h followed by extensive washing with PBS/Tween. IL-2 in PBS, 0.05% Tween 20 was added at increasing concentrations to the wells and incubated for 2 h at room temperature. After 2 h unbound IL-2 was removed by washing with PBS/Tween, followed by three washes with PBS. 100 μl of the IL-2-dependent CTLL-2 cells in the logarithmic growth phase (200,000 cells/ml), were plated in IL-2-deficient medium on uncoated (control) or collagen-coated wells that had been preincubated with different concentrations of IL-2. In parallel, IL-2 added to the CTLL-2 cells served as a positive control. Cells were then cultured for 20 h, and DNA synthesis was measured by [H]thymidine incorporation during the last 4 h.

**Statistical Analysis—**Binding data are expressed as mean ± S.E. Dissociation constants and the number of binding sites obtained by saturation experiments were analyzed according to the method of Scatchard (28, 29, 38).

**RESULTS**

IL-2 binds to Native Collagen Types I–VI, Single Collagen Chains, and Collagen Chain-derived CNBr Peptides—Native triple helical collagen types I–VI and heat-denatured or reduced and alkylated single chain collagen types I, III, IV, and VI bound between 22 and 34% (for native collagens) and 18–29% (for denatured collagens) of radiolabeled IL-2 (1–2 ng) when immobilized on polystyrene microtiter wells (Fig. 1). Furthermore, collagens with the decreasing order, type I > III > VI > their respective constituent chains, were shown to be the...
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Fig. 2. Differential binding of radiolabeled IL-2 to collagen α1(I)-derived cyanogen bromide peptides. CNBr peptides were immobilized on polystyrene (p) microtiter wells at 1 μg/100 μl. Binding assays were performed as described in Fig. 1. Shown are the means (±S.E.) of four independent experiments performed in triplicate.

Fig. 3. Preferential binding of IL-2 to a subset of collagen peptides, transferred to nitrocellulose. CNBr peptides (2 μg/lane) from collagen chains α1(I) (lanes 1 and 8), α2(I) (lanes 2 and 9), and α1(III) (lanes 3, 4, 10, and 11) and pepsin-resistant fragments of collagen types IV (lanes 5, 6, 12, and 13) and VI (lanes 7 and 14) were separated by SDS-PAGE and blotted to nitrocellulose in duplicate. Blocking of unpecific binding sites with 0.3% Tween 20 in PBS was followed by either washing with Amido Black (lanes 1–7) (A) or incubation with 50 ng of 125I-IL-2 for 2 h, three washes with PBS, air-drying, and autoradiography (lanes 8–14). (B). Disulfide bonds of peptides on lanes 4, 6, 7, 11, 13, and 14 were reduced prior to electrophoresis. Molecular masses (in kDa) of the CNBr peptides or of the pepsin-resistant fragments are as follows: 62, the dimeric α2(CB3-5); 54, 52, and 50, α(V); α(VI); α(III), respectively; 50, P2 fragment of α2(IV); 31, α2(CB5) or α1(CB5); 30, α1(CB7); 28, α1(CB8); 25, α1(II-ICB8); 24, α1(IIIICB8-50; α1(IVCB6); and 14, α1(CB3). Note that only a subset of CNBr peptides interacts with radiolabeled IL-2 and that some oligomeric peptides such as the dimeric α2(CB3-5) or the trimeric (nonreduced) α1(IIIICB9 that are hardly detected by protein staining react strongly with 125I-IL-2 by autoradiography only intact IL-2 was identified (data not shown).

In addition to the results of the polystyrene assay, binding of IL-2 to the collagen chains could also be demonstrated by ligand blot experiments. Triple helical collagens (after denaturation), single collagen chains, and their cyanogen bromide peptides, once transferred to nitrocellulose after SDS-PAGE, bound radiolabeled IL-2 as shown by autoradiography. In accordance with the enzyme-linked immunosorbent-type assay, IL-2 bound only to some of the cyanogen bromide peptides, although protein staining demonstrated a comparable transfer of all collagen chains and cyanogen bromide peptides to nitrocellulose (Fig. 3).

Due to their characteristic molecular weight, these cyanogen bromide fragments could be identified as α1(I)CB6 (20 kDa) and α1(I)CB8 (28 kDa), whereas binding was weaker for α1(I)CB3 (30 kDa) and an uncleaved dimeric peptide of α1(I) (52 kDa). Additional IL-2-binding peptides were CB3 or CB5 (both 51 kDa) and the dimeric uncleaved CB3-5 (62 kDa) of the α2(I) chain, α1(IIIICB9 (migrating as a trimer of 72 kDa before and a monomer of 24 kDa after reduction), as well as pepsin-resistant fragments of the α2 chain of collagen type IV (120, 60, and 50 kDa) and of the three α-chains of collagen type VI (Fig. 3).

Single Collagen Chains and α1(I)-derived CNBr Peptides Inhibit Binding of IL-2 to Immobilized Collagen—Incubation of IL-2 with the constituent chains of collagen type I, namely α1 and α2, with native collagen types III and IV, previously immobilized at 50 ng/200 μl/well, in the presence of increasing amounts of solubilized collagen chains or their CNBr peptides resulted in a dose-dependent inhibition of the IL-2-collagen interaction. Different collagen chains were able to inhibit IL-2 binding to their respective homotypic but also heterotypic immobilized collagens (Fig. 4), demonstrating the potential of collagen chains for cross-inhibition. The inhibition varied slightly when different combinations of soluble collagen chains and collagenous substrates were used. As an example, 10 μg/ml of the reduced and alkylated chains of type VI collagen inhibited the binding of IL-2 to the immobilized homotypic chains and to reduced and alkylated collagen type III up to 90%, whereas they blocked binding of IL-2 to the immobilized α1 or α2 chains of collagen type I only by 70% (Fig. 4A). Results with the α1 chain of collagen type I as inhibitor were similar (Fig. 4B).

To define further the potential binding sites on the α1 chain of collagen type I, CNBr peptides were used as inhibitors (Fig. 4C). Clearly, CB6 and to a lower extent CB8, but not CB7 or CB3, were able to inhibit IL-2 binding to the immobilized α1(I) chain. Thus, the inhibition experiments with single collagen chains and CNBr peptides indicate a shared or a similar binding site for IL-2 on different collagen chains. Furthermore, this binding site appears to be a collagenous primary structure which for α1(I) is restricted to peptides α1(I)CB6 and α1(I)CB8.

Saturation Binding Studies and Estimated Affinities of the IL-2-Collagen Interaction—When increasing amounts of unlabelled IL-2 were incubated with a constant amount of 125I-IL-2 (~1 ng/well), binding to immobilized collagens was saturable (see Fig. 5 for collagen type III and the α(I) chain). Saturation was reached between 150 and 250 ng of IL-2/100 μl on 200 ng/well of immobilized collagen types I and III and on 400 ng/well of the immobilized α1(I) chain.

By taking into account the coating efficiencies of the respective immobilized collagens (see “Experimental Procedures”) and the amount of IL-2 needed to reach saturation, Scatchard analysis (38) yielded binding sites of comparable affinity on the tested collagens, with dissociation constants (Kd) of ~10^-8 mol/l. Based on these data, 1 mol of immobilized collagen type I and III and 1 mol of the α1(I) chain were estimated to bind approximately 6, 4, and 1 mol of IL-2, respectively.
Collagen-bound IL-2 Stimulates DNA-synthesis of a T-lymphocyte Cell Line—IL-2 bound to collagen type I stimulated DNA synthesis of a mouse T-lymphocyte cell line (CTLL-2 cells), as measured by [3H]thymidine incorporation, whereas no DNA synthesis was observed with comparable amounts of IL-2 bound to polystyrene alone (Fig. 6). The collagen-bound IL-2 was ~5-fold less biologically active than “free” IL-2 (Fig. 6).

**DISCUSSION**

We demonstrated that IL-2 can bind to immobilized collagen types I–VI in vitro. Preferential binding was observed to the most abundant fibrillar collagen types I and III. Binding was saturable and yielded dissociation constants of $\sim 10^{-9}$ M, a range similar to that found for many other protein–protein interactions, particularly among molecules of the ECM (39), and comparable to that described for the interaction of PDGF and HGF with collagens (28, 29).

The interaction of IL-2 with native as well as denatured collagens could be inhibited by single collagen chains, and cross-inhibition experiments suggested one or more collage-

nous consensus binding sites for IL-2. That indeed a limited number of collagenous consensus sequences may be involved is further supported by the results of ligand blots and inhibition experiments performed with smaller collagen peptides, derived from digestion with cyanogen bromide or pepsin. These experiments show only few of the peptides, e.g. $\alpha$1(II)CB6 and $\alpha$1(II)-CB9 that bind radiolabeled IL-2 with high affinity, whereas other peptides of similar size, such as $\alpha$1(II)CB7 and $\alpha$1(III)CB8, are not (or minimally) reactive in ligand blots and inhibition experiments (Figs. 3 and 4). The peptides that interact with IL-2 are those that also bind the growth factors PDGF and HGF, albeit with different preferences (as judged by the inhibitory potential or the relative intensities of the autoradiographic bands) (28, 29), indicating overlapping binding sites on collagens for IL-2, PDGF, and HGF. We could indeed show cross-competition among the three growth factors for the studied collagen types and collagen chains (data not shown).

As discussed before (28, 29), a disadvantage of solid phase assays is the potential of epitope masking by interaction of the collagens with the polystyrene matrix. Thus we cannot exclude that we underestimated the binding sites and obtained a lower calculated number of IL-2 molecules bound to single chain compared with triple helical collagen.

A necessary precondition for binding experiments is the purity of the used ligands, since minimal contamination of collagens with other ECM components, e.g. glycosaminoglycans and proteoglycans, can invalidate the results. Such interference could be excluded, since the collagens that were used for these binding experiments had been subjected to rigorous purification procedures. Furthermore, pretreatment with several en-

zymes excluded contaminating glycosaminoglycans, proteoglycans, or glycoproteins as discussed elsewhere (28, 29). Since an interaction of IL-2 with glycosaminoglycans, e.g. heparin, was described in a single study (9), inhibition experiments with up to 250-fold molar excess of heparin (relative to immobilized collagen) were performed, which did not show inhibition of IL-2 binding to collagen types I, III, or IV (data not shown).

IL-2, one of the best studied cytokines, plays a central role in inflammation. It is produced by activated T cells and acts in an autocrine and/or paracrine manner on T, B, and natural killer cells. IL-2 signaling promotes T cell survival by up-regulation of Bcl-2 but can also induce T cell tolerance via elimination of autoreactive T cells through IL-2-induced apoptosis (40). IL-2 exerts this effect through a specific and high affinity trimeric receptor complex ($K_d$ of $10^{-11}$ M), consisting of an $\alpha$, a $\beta$, and a $\gamma$-subunit, of which the $\alpha$- and $\beta$-subunits and the heterodimers ($\alpha/\beta$ and $\beta/\gamma$) can bind IL-2 independently, but with lower affinity ($K_d$ of $10^{-7}$, $10^{-8}$, and $10^{-9}$, respectively) (41, 42). Considering the pluripotent effects of IL-2 in immune regulation, our finding of a specific interaction of IL-2 with collagens and predominantly collagen types I, III, and VI, which repre-

sent the most abundant components of normal and fibrotic extracellular matrices, should have biological significance. Thus, these collagens could modulate the local availability and

**Fig. 4.** Single chains of collagen types I and VI and CNBr peptides of the $\alpha$1(I) chain inhibit binding of IL-2 to various immobilized collagen chains. A. 1–2 ng of $^{125}$I-IL-2 (0.065 to 0.13 nmol) were preincubated with increasing concentrations of CV1ra and then added to various collagen chains (see inset), immobilized at 200 ng/well, for another 2 h, followed by determination of bound radioactivity. Further inhibition experiments were performed using the $\alpha$1(I) chain on various immobilized collagen chains (B) or different CNBr peptides of $\alpha$1(I) on immobilized $\alpha$1(I) (C). For the molecular masses of the proteins and peptides refer to Fig. 3. Binding is expressed as the percentage of bound radioactivity in the presence of inhibitor relative to the bound radioactivity in the absence of inhibitor. Shown are results of a representative of four (three for CNBr peptides) experiments performed in triplicate.

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activity of this cytokine by serving as low affinity stores for IL-2, which may be picked up by invading T cells that carry the high affinity receptor. This would implicate that the pattern of ECM determines, at least in part, the activation of resident or invading lymphocytes by engaging their integrin receptors (43) as well as by presenting IL-2. This hypothesis is supported by our cell culture experiments in which proliferation of the IL-2-dependent cell line CTLL-2 was stimulated when seeded on immobilized collagens preincubated with IL-2 (Fig. 6).

Although collagen-bound IL-2, compared with equimolar doses of soluble IL-2, is less biologically active in our in vitro system, the bioavailability of collagen-bound IL-2 in vivo may be augmented by matrix degradation, as occurs during inflammation and wound repair (44–46). In this line collagens do not merely serve as a store for IL-2 but simultaneously can up-regulate the IL-2 receptor via engagement of integrins, since collagens as well as fibronectin were shown to stimulate expression of IL-2 receptor p55 and p75 mRNA in murine lymphocytic M4 T cells, to a level comparable to that induced by IL-2 itself (47). Furthermore, it is well established that integrin and growth factor receptor cross-talk potentiates the mitogenic response in various cell types, including lymphocytes (48). This was shown for prolonged lymphocytespreading, which is integrin-dependent, resulting in the up-regulation of the mitogen cytokine IL-2 and in S phase entry (49).

In summary, our study demonstrates a specific binding of IL-2 to interstitial collagens, which may be exploited to modulate the local availability and activity of this cytokine in wound repair and inflammation. Since binding of IL-2 to collagens is mediated by cross-inhibitory consensus sequences, competitive collagenous peptides, their nonpeptidic analogues, or the design of collagenous peptide carriers for IL-2, e.g., for use in local cancer therapy, may be envisaged.

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