Delivery of Human Factor IX in Mice by Encapsulated Recombinant Myoblasts: A Novel Approach Towards Allogeneic Gene Therapy of Hemophilia B

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A potentially cost-effective strategy for gene therapy of hemophilia B is to create universal factor IX-secreting cell lines suitable for implantation into different patients. To avoid graft rejection, the implanted cells are enclosed in alginate-polylysine-alginate microcapsules that are permeable to factor IX diffusion, but impermeable to the hosts' immune mediators. This nonautologous approach was assessed by implanting encapsulated mouse myoblasts secreting human factor IX into allogeneic mice. Human factor IX was detected in the mouse plasma for up to 14 days maximally at ~4 ng/mL. Antibodies to human factor IX were detected after 3 weeks at escalating levels, which were sustained throughout the entire experiment (213 days). The antibodies accelerated the clearance of human factor IX from the circulation of the implanted mice and inhibited the detection of human factor IX in the mice plasma in vitro. The encapsulated myoblasts retrieved periodically from the implanted mice up to 213 days postimplantation were viable and continued to secrete human factor IX ex vivo at undiminished rates, hence suggesting continued factor IX gene expression in vivo. Thus, this allogeneic gene therapy strategy represents a potentially feasible alternative to autologous approaches for the treatment of hemophilia B.

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MATERIALS AND METHODS

Tissue culture conditions: Mouse C2C12 myoblasts were obtained from American Type Culture Collection (ATCC; Rockville, MD; #CRL 1772) and cultured under standard conditions in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The myoblasts were passaged at a cell density of 2-3 x 10^5 cells/cm^2. The myoblasts were harvested and seeded at 2 x 10^4 cells/cm^2 on 60-mm diameter tissue culture dishes. After 24 hours, the dishes were washed with sterile PBS to remove non-attached cells. The dishes were then supplemented with 2 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The dishes were incubated at 37°C with 5% CO2.

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co's modified essential medium (DMEM) containing glucose (4.5 g/L), 1% penicillin and streptomycin (GIBCO, Burlington, Canada), and 10% fetal bovine serum (GIBCO). After reaching confluency, cells were cultured in DMEM medium containing 2% horse serum for 2 to 3 days to induce differentiation into myotubes (which have distinct microscopic morphology). Myoblasts in microcapsules were cultured in vitro in 100-mm dishes, with 3 mL of capsules per 10 mL of medium under the same conditions.

**Transfection of myoblasts with hFIX cDNA**. Mouse C2C12 myoblasts were transfected by the calcium phosphate precipitation method with pLIXSNL vector (a gift from Dr A. Dusty Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) containing the hFIX cDNA under the control of the viral LTR promoter and the Neo gene conferring resistance to G418. Additionally, pKL444-hFIX was used similarly to transfect C2C12 myoblasts. This vector was obtained by cloning the hFIX cDNA as a 1.6-kb HindIII fragment from pLNcIXL, a gift from Dr A. Dusty Miller, into the HindIII site of pKL444 downstream from the human β-actin promoter. Individual recombinant clones were isolated after selection in G418 (400 µg/mL). Except where otherwise indicated, clone MLhFIX-5D5 from transfection with pLIXSNL was used throughout this study for its vigorous growth and good level of hFIX expression. Clone M/hFIX-D6 was selected from the transfection with pKL444-hFIX.

**Enclosure of recombinant myoblasts in microcapsules**. Encapsulation of recombinant myoblasts was performed as described earlier with minor modifications. Briefly, a suspension of cells was mixed with 1.5% potassium alginate (Kelmar, Kelco Inc, Chicago, IL) in a syringe and extruded through a 27 G needle with a syringe pump (39.3 ml/min). The alginate suspension was mixed with 1.5% potassium alginate (Kelmar, Kelco Inc, Chicago, IL) in a syringe and extruded through a 27 G needle with a syringe pump (39.3 ml/min). The alginate-PLL layer was chemically cross-linked with poly-L-lysine hydrobromide (PLL, mol wt: 15,000 to 30,000, Sigma, St Louis, MO) for 6 minutes and then with another layer of alginate. Finally, the remaining free alginate core was dissolved with sodium citrate for 6 minutes to yield microcapsules of ~500 µm in diameter with an alginate-PLL-alginate membrane containing cells.

**Implantation of microcapsules into mice**. Normal male C57BL/6 mice (Charles River, Montreal, Canada) were implanted with microcapsules enclosing factor IX-secreting myoblasts. The microcapsules were washed in Hank’s solution (GIBCO) just before implantation. The animals were anesthetized using a small-animals anesthetic machine (Med-Vet, Toronto, Ontario) providing a controlled amount of isofluorane (Anaquest, Mississauga, Ontario), oxygen, and nitrous oxide. The implantation procedure was done using intravenous (IV) catheter (Angiocath, 16 G) introduced into the peritoneal cavity through a valvular course to avoid regurgitation. The whole procedure took about 5 minutes, and the animals were soon freely mobile in their cages. Animals were implanted either with 3 mL (8 × 10⁶ cells) or 18 mL (5 × 10⁷ cells) of microcapsules (90% packed capsule volume in Hanks) given in four separate doses over 2 successive days. The increase in abdominal volume as a result of the implantation dissipated with time, returning to normal size in 48 hours. All experiments and techniques were performed according to Canadian Animal Ethics guidelines.

**Immunosays**. Blood samples were taken periodically from the tail vein through heparinized capillary tubes. Plasma was obtained and stored at -20°C. Both hFIX and anti-hFIX antibodies were detected by enzyme-linked immunosorbent assay (ELISA). For the detection of hFIX, microtiter plates were coated at 37°C for 2 hours with 200 ng/well of monoclonal antibody against hFIX FXX008 (Boehringer Mannheim, Montreal, Canada) in 100 µL of 0.1 mol/L Na₂CO₃ pH 9.6. After blocking with 5% blotto (skim milk powder) at 4°C for 18 hours, each well was incubated with 100 µL of mouse plasma (1:5 dilution in 5% blotto) at 37°C for 1 hour. Human FIX bound to the wells was detected by incubating with rabbit anti-hFIX (Hoecht, Montreal, Canada) diluted 1:1,000 in 5% blotto at 37°C for 1 hour, followed by goat anti-rabbit IgG-AP (BRL, Burlington, Canada) diluted 1:1,000 in 5% blotto at 37°C for 1 hour. The substrate para-nitrophenylphosphate (Sigma) at 1.5 mg/mL was added, incubated for 37°C for 1 hour, and the absorbance at 405 nm determined using a Titertek Multiskan PLUS MKII (Labsystems, Helsinki, Finland) ELISA plate reader. Pooled human plasma (containing 5 µg of factor IX/mL) was used as the standard for hFIX. For detection of antihuman factor IX antibodies raised in mice, Nunc maxisorb microtiter plates (GIBCO) were coated with 50 ng of purified hFIX in 100 µL of 0.1 mol/L Na₂CO₃, pH 9.6, per well and incubated at 37°C for 2 hours. The wells were washed with phosphate-buffered saline/Tween (PBS/T; 0.1 mol/L Na₂HPO₄, 0.15 mol/L NaCl, 0.05% Tween 20, pH 7.4), and then blocked with 5% blotto (skim milk powder) in PBS/T at 4°C for 18 hours. After washing, 100 µL of mouse plasma (diluted 1:300 in 5% blotto) per well was incubated at 37°C for 2 hours. After washing, 100 µL/well of goat antimouse IgG conjugated to alkaline phosphatase (Promega, Madison, WI) diluted 1:1,000 in 5% blotto was incubated at 37°C for 1 hour. Following four washes, 100 µL of para-nitrophenylphosphate (Sigma) at a concentration of 1.5 mg/mL was added to each well as a chromogenic substrate. After incubation at 37°C for 1 hour, the absorbance of each well at 405 nm was read using a Titertek Multiskan PLUS MKII ELISA plate reader. Plasma of mice immunized with 15 µg of purified hFIX given in three doses with Freund's adjuvant was included as a positive control in each assay.

**Retrieval of implanted microcapsules**. Animals were anesthetized with isoflurane, and sterile saline forcefully flushed through a small abdominal incision into the peritoneal space to create a current carrying out the capsules. If the animal was to be maintained, the abdominal wound was closed with surgical sutures (2-0). The whole procedure took less than 10 minutes to complete. The retrieved capsules were then resuspended in warm phosphate buffered saline and washed several times to eliminate free peritoneal macrophages and lymphocytes, which were usually retrieved with the capsules. The hFIX secretion by the encapsulated cells was determined by sampling media aliquots at timed intervals. For releasing the encapsulated cells, a sample of microcapsules was placed on a slide with trypan blue and, after gentle pressure was applied with a coverslip, the microcapsules were broken to release the enclosed cells. The number of cells/capsule was counted and cell viability determined by trypan blue exclusion. A sample of released cells was regrown in culture. Once confluent, the cells were assayed again for hFIX secretion in vitro.

**Clearance of hFIX from mouse plasma**. Cohorts of mice were each injected intraperitoneally (IP) once with 6 µg of purified hFIX. Animals were bled at regular intervals for 24 hours after the injection, and the blood samples assayed for hFIX by ELISA.

**Western blotting**. Mouse C2C12 and MLhFIX-SDS recombinant cells (4 × 10⁶ cells) were harvested after a 24-hour culture in the absence of serum, resuspended in 100 µL of lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.02% sodium azide, 100 µg/mL phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL aprotinin, 1% Triton X-100), and the suspension freeze-thawed three times to lyse the cells. After centrifugation (Eppendorf) for 30 minutes at 14,000 rpm, the supernatant was stored until needed. Proteins in the cell culture medium of harvested cells were precipitated with 0.1% trichloroacetic acid. Samples were incubated at 4°C for 1 hour and centrifuged (Eppendorf) for 5 minutes at 14,000 rpm. The pellet was washed twice with acetone, vacuum dried, and resuspended in loading buffer before electrophoresis. Protein samples were subjected to electrophoresis in a 12.5% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The proteins were then transferred onto nitrocellulose paper (Bio-
Fig 1. Secretion of recombinant hFIX by transfected cells. Samples prepared from cell lysate or culture media of 4 x 10^5 MLhFIX-5D5 cells and nontransfected C2C12 myoblasts were separated by electrophoresis. The proteins were transferred onto nitrocellulose membrane and reacted against immunoaffinity-purified sheep anti-hFIX IgG with purified hFIX (500 ng) as the positive control. Markers are expressed in kD.

RESULTS

Secretion of human factor IX from encapsulated myoblasts. Forty-eight C2C12 clones transfected with pLIXSNL were isolated after G418 selection and screened for their hFIX secretion and vigorous growth. Clone MLhFIX-5D5 secreted on average 304 ± 49 ng of factor IX per 10^6 cells/24 hours as myoblasts and 173 ± 47 ng/24 hours as myotubes derived from 10^6 undifferentiated myoblasts. Western blotting analysis with affinity purified sheep anti-hFIX IgG showed that recombinant hFIX of Mr ≈ 60,000 was the primary species of hFIX secreted from the transfected MLhFIX-5D5 clone. Human FIX was not detected in cell lysate of the transfected cells nor in the media from the nontransfected C2C12 cells (Fig 1). On encapsulation, the recombinant myoblasts continued to secrete hFIX at a rate of 285 ng/10^6 cells/24 hours as determined 24 hours postencapsulation.

Delivery of human factor IX in mice. Normal C57BL/6 mice (n = 4) were implanted IP with microcapsules containing 5 x 10^6 viable recombinant myoblasts. A maximum of 3.8 ng/mL of human FIX was detected in plasma on day 2, decreasing thereafter to undetectable levels by day 14 (Fig 2). A second group of mice (n = 2) implanted with microcapsules containing 8 x 10^5 viable recombinant myoblasts had 1.5 and 1.1 ng hFIX/mL of mouse plasma on day 2, and hFIX was undetectable by day 7 (Fig 2). The third group of control mice (n = 4) injected IP with 5 x 10^6 unencapsulated recombinant myoblasts had no detectable hFIX in their plasma. Thus, without the protection of the microcapsules, the nonautologous recombinant cells were unable to deliver hFIX in vivo.

Development of anti-hFIX antibodies in mice. Because the delivery of xenogeneic hFIX into mice could elicit an antibody response, production of anti-hFIX antibodies was monitored. At 2 to 4 weeks postimplantation, low titer anti-hFIX was detected in mice with either encapsulated or nonencapsulated recombinant cells. However, only the mice implanted with encapsulated cells subsequently showed sustained antibody titer until the end of the experiment (day 213), while the titer of anti-hFIX in mice receiving unencapsulated cells remained at the initial low levels (Fig 3).
sustained antibody titer in the plasma of mice implanted with microcapsules suggested a basal ongoing hFIX delivery in vivo, although not enough to be detectable in plasma after day 14. The presence of antibodies against hFIX in plasma of implanted mice was confirmed by Western blotting. Plasma from mice implanted with microcapsules for 63 to 213 days showed antibodies that reacted specifically with purified hFIX while preimplantation plasma showed no reaction (Fig 4). This finding was reproduced when C57BL/6 mice were implanted with encapsulated myoblasts transfected with a β-actin promoter-driven hFIX construct (Fig 4).

Detection of hFIX in mouse plasma. It was possible that the circulating anti-hFIX antibodies accelerated the clearance of the recombinant hFIX delivered into mice to render hFIX undetectable in the ELISA test. To test this hypothesis,

![Graph](image1)

**Fig 2.** Human FIX delivered to mouse plasma. Four normal C57BL/6 mice (+, ⬤, ⬤, ⬤) were implanted IP with $5 \times 10^6$ and two mice (△, □) with $8 \times 10^5$ microencapsulated recombinant myoblasts (MLhFIX-5D5). Four control mice (+, ○, △, ○) were injected with $5 \times 10^6$ unencapsulated MLhFIX-5D5 myoblasts. Human FIX secretion into mouse plasma was detected by ELISA with a sensitivity of $\geq 200$ pg/mL of human factor IX.

![Graph](image2)

**Fig 3.** Development of anti-hFIX antibodies in the implanted mice. Four C57BL/6 mice (+, ⬤, ○, △) were implanted with $5 \times 10^6$ microencapsulated MLhFIX-5D5 and four control mice (+, ○, △, ○) were injected with $5 \times 10^6$ unencapsulated recombinant myoblasts. One mouse (+) died on day 78 from accidental anesthetic overdose. Antibodies against hFIX in plasma were detected by ELISA. The ratio of absorbance for a particular day and mouse (Dx/Do) was used to indicate antibody titer. Plasma from mice immunized with purified hFIX (Dx/Do = 9.17 ± 0.39) was included in all assays as a positive control.
we injected 6 μg of purified hFIX into three mice (mice B, C, and D from Fig 3) that had been implanted with encapsulated recombinant myoblasts on day 213 postimplantation when hFIX was no longer detectable in plasma (group A), and into three mice implanted with encapsulated mouse Ltk-fibroblasts not secreting hFIX (group B). As a control, 6 μg of hFIX was injected IP into three naive mice (group C).

As shown in Fig 5, hFIX was gradually cleared from the circulation of the naive mice (group C) from 41 ng/mL at 3 hours to 19 ng/mL at 24 hours (a clearance rate of ~50% from 3 hours to 24 hours). In mice implanted with capsules containing non-hFIX-secreting cells (group B), the level of hFIX detected was initially lower than that of the naive mice (24 ng/mL at 3 hours), indicating that the implanted capsules may have an absorption effect in reducing the initial amount of hFIX delivered to the plasma. However, once it had been delivered to the plasma, the exogenous hFIX was also cleared to about 50% of its level at 3 hours (from 24 ng/mL at 3 hours to 19 ng/mL at 24 hours), indicating that microcapsules containing non-hFIX-secreting cells had no effect on the clearance rate of exogenous hFIX. In contrast, in mice implanted with hFIX-secreting capsules (Group A), only residual levels of hFIX (<6 ng/mL) were detected throughout the 24-hour period without decline. In fact, one mouse (C) had undetectable hFIX even at 3 hours, while another mouse (D) had barely detectable hFIX at 3 hours (<1 ng/mL), and undetectable levels thereafter. Although the time course did not permit a determination of the circulating half-life of hFIX, these results indicate that the clearance of hFIX in plasma is accelerated in mice with encapsulated hFIX-secreting cells, thus suggesting that antibodies may play a role in increased clearance of hFIX from plasma.

We also investigated the in vitro effect of anti-hFIX antibodies in the ELISA assays for hFIX (Fig 6). Human normal plasma containing 5 μg/mL of hFIX was added to mouse plasma samples that either did or did not contain anti-hFIX antibodies. The amount of detectable hFIX in the sample
was dramatically reduced by anti-hFIX antibodies from a mouse immunized with hFIX. The same effect was observed with plasma from mice implanted with microencapsulated hFIX-secreting Mj/hFIX-D6 cells matched in anti-hFIX titer to mice B, C, and D in Figs 2 and 3. The amount of hFIX in plasma was determined by ELISA. Error bars indicate average ± SEM.

**Fig 6.** Determination of hFIX by ELISA in the presence of anti-hFIX antibodies. Serial dilutions of pooled human plasma (considered to contain 5 µg/mL of hFIX) were added to mouse plasma samples from (A) naive mice (controls), (B) mice immunized with purified hFIX, and (C) mice implanted with encapsulated Mj/hFIX-D6 cells secreting hFIX for 3 weeks at an anti-hFIX antibody titer similar to that of mice B, C, and D in Figs 2 and 3. The amount of hFIX in plasma was determined by ELISA. Error bars indicate average ± SEM.

DISCUSSION

This study demonstrated that C2C12 myoblasts engineered to secrete hFIX can be implanted into normal mice to deliver hFIX systemically (Fig 1). Although C2C12 myoblasts have been used to deliver hFIX in mice even at therapeutic levels, either immune suppression of the allogeneic hosts or use of nude mice was necessary to prevent graft rejection. Our protocol clearly demonstrated the efficacy of immuno-isolation to protect allogeneic cells from such rejection. Therefore, the feasibility of using nonautologous cells to deliver F IX provides a potential new approach for the genetic treatment of hemophilia B and an alternative to ex vivo protocols using myoblasts, keratinocytes, or fibroblasts for autologous implantation or in vivo protocols using viral particles for direct injection.

To treat hemophilia B successfully using gene therapy, both physiologically relevant levels and long-term delivery of biologically active hFIX into the circulation are necessary. The maximum level of hFIX delivered in the present vector–myoblast combination, was only ~4 ng/mL (Fig 2) or 0.1% of normal physiologic level in humans. Hence, for this protocol to become clinically relevant, the level of expression must be improved by using more efficient vectors, and/or stronger and more appropriate promoter-enhancers. However, delivery of even 3 to 10 ng/mL F IX in hemophilia B dogs reduced the whole blood clotting time by 50%. Therefore, the low level of F IX delivered by the current protocol may be an effective prophylactic treatment for hemophilia B, even though a physiologic level of F IX delivery is yet to be achieved.

The requirement for sustained hFIX delivery was more difficult to attain, as the level of F IX delivered in mice had decreased to undetectable levels by day 14 (Fig 2). Similar transient delivery of hFIX has been reported using various protocols for hFIX delivery in vivo. An in vivo downregulation of viral promoter (Mo MLV 5' LTR in MLhFIX-5D5) may be responsible for the transient expression. Inhibition in vivo of LTR-driven FIX or FVIII expression has been reported for fibroblasts and hematopoietic cells and was suggested for keratinocytes secreting F IX. Dai et al also described a similar pattern of dog F IX delivery in mice using myoblasts, when expression of the F IX cDNA was under the viral LTR promoter control. However, a long-term delivery of F IX in vivo (>180 days) was achieved when a myoblast-specific enhancer (MCK) was introduced in the retroviral construct. Thus, a judicious choice of promoter/enhancer elements may be critical for the long-term success of gene therapy protocols based on recombinant cells.

Microcapsules containing the recombinant cells retrieved after 213 days of implantation secreted hFIX at similar rates as those of the preimplantation microcapsules.
DELIVERY OF FACTOR IX IN MICE WITH MICROCAPSULES

Day 0

Day 28

Day 84

Day 213

<table>
<thead>
<tr>
<th>Post-implantation</th>
<th>Day 0</th>
<th>Day 28</th>
<th>Day 84</th>
<th>Day 213</th>
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<td>123±15</td>
<td>128±29</td>
<td>125±25</td>
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<td>hFIX expression (ng/10⁶ cells/24 h)</td>
<td>285±72</td>
<td>301±104</td>
<td>226±51</td>
<td>211±39</td>
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</table>

Fig 7. Retrieval of microcapsules postimplantation. Microcapsules were retrieved from the abdominal cavities of implanted mice by lavage at various time points postimplantation. The integrity and appearance of the microcapsules were recorded with dark-field photomicrography. The retrieved capsules were washed, kept under regular tissue culture condition overnight, and assayed for rates of hFIX secretion into the media the next day. For viability and cell count, the microcapsules were ruptured to release the cells as described in Material and Methods. Cells regrown in vitro from the retrieved microcapsules secreted hFIX at preimplantation levels (data not shown). Scale Bar = 500 μm.

(Fig 7). Our data indicated that the microcapsules remained permeable to FIX in vivo after prolonged implantation and that the encapsulated myoblasts-myotubes retained their capacity to secrete recombinant gene products at an undiminished rate. The presence of antibodies against xenogeneic hFIX delivered in vivo was demonstrated by Palmer et al.¹⁷ and thought to be responsible for the disappearance of hFIX from plasma of normal mice.¹⁵ We present data to show that, after day 14, anti-hFIX antibodies were detected at sustained titers for at least 200
days (Fig 3). When exogenous hFIX was delivered to these sensitized mice implanted with hFIX-secreting microcapsules, hFIX was cleared from the circulation rapidly (Fig 5). Furthermore, the presence of anti-hFIX antibodies in the plasma samples of the implanted mice also led to a gross underestimation of hFIX in plasma, as determined by ELISA (Fig 6). Hence, both in vitro and in vivo effects could have led to an underestimation of hFIX delivery in vivo.

The use of myoblasts for allogeneic implantation to deliver FIX appears a promising approach. As shown in Fig 7, encapsulated cells implanted in vivo remained viable for up to 213 days, with a net increase in the number of cells per capsule over time. Thus, the myoblasts not only withstood the encapsulation process, but they also thrived and proliferated to a limited degree within the microcapsules in the peritoneal cavity, an unusual environment for these cells. Although the C2C12 myoblasts can be tumorigenic when implanted into immunologically compromised nude mice (unpublished observation, 1995), no tumor was detected in the current series of normal mice used. Other investigators have also used recombinant mouse myoblasts injected into mouse leg muscles to deliver FIX.15-21 The levels of hFIX delivered in the present study by microcapsules by day 2 were comparable to those reported by others,15-21 once expression of FIX in vitro by the recombinant myoblasts and the number of implanted cells were taken into account. Hence, the peritoneal cavity appears to be a supportive environment for the secretion of hFIX by encapsulated myoblasts. We have used a similar allogeneic gene therapy protocol to deliver mouse growth hormone and partially correct the defect of the growth hormone-deficient dwarf mice.14 While the long-term durability of the alginate hydrogel material has to be improved,17 current human clinical trials using this14 and alternate acrylate membranes35 will provide important insight into the use of immuno-isolation devices for cellular transplant.

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Delivered Factor IX in Mice with Microcapsules


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Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B

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