SARS-CoV-2 Omicron variant is attenuated for replication in a polarized human lung epithelial cell model

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Article

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Abstract

SARS-CoV-2 and its emerging variants of concern remain a major threat for global health. Here we introduce a novel infection model based upon polarized human Alveolar Epithelial Lentivirus immortalized (hAELVi) cells grown at the air-liquid interface to estimate replication and epidemic potential of respiratory viruses in the human lower respiratory tract. hAELVI cultures are highly permissive for different human coronaviruses and seasonal influenza A virus and upregulate various mediators following virus infection. Our analysis revealed a significantly reduced capacity of SARS-CoV-2 Omicron variant to propagate in this human model compared to earlier D614G and Delta variants, which extends early risk assessments from epidemiological and animal studies suggesting a reduced pathogenicity of Omicron.

Introduction

Emergence of the human Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in 2020 lead to the global Coronavirus Disease 2019 (COVID-19) pandemic, which caused so far more than 5.7 million deaths worldwide. SARS-CoV-2 is mainly transmitted via virus-containing droplets and aerosols¹. Acute COVID-19 can be divided into up to three stages of disease with increasing severity including the early phase, in which the virus establishes infection in tissues of the upper respiratory tract, which is often associated with no or mild symptoms (stage I). During stage II (pulmonary phase) active viral replication in cells of distal airways can trigger pneumonia including localized inflammation in the lung and hypoxia. In some cases there is progression to even more severe disease such as Acute Respiratory Distress Syndrome (ARDS), shock and cardiac failure as a consequence of systemic hyperinflammation (stage III)². However, the virus may infect the lung also directly after inhalation of virus-containing aerosols³.

Since late 2020, several viral variants of concern (VOC) including Alpha, Beta, Gamma and Delta started to emerge with some of them becoming predominant within a short period of time in distinct geographical regions leading to strong waves of disease⁴. VOCs pose a serious risk to global public health due to an increased transmissibility and escape from adaptive immune responses, which is caused by distinct genotypical changes leading to variations in the viral Spike and other proteins. The most recent VOC termed Omicron, which emerged in countries of Southern Africa in November 2021, encodes at least 30 amino acid changes, 3 small deletions and 1 insertion in the viral Spike gene alone^{5,6}. While the transmission in unvaccinated individuals appears to be comparable to the previously dominating Delta, the Omicron VOC shows immune evasion in fully vaccinated and even booster-vaccinated people, which possibly explains the rapid spread to all continents⁷. From South Africa, it was reported that the Omicron VOC is more likely to infect younger people and cause milder disease⁸. However, it is less well understood what courses of the disease occur in the elderly and other vulnerable groups with increased risk for severe COVID-19.

Severe COVID-19 is characterized by persisting high-level viral shedding in the lower respiratory tract⁹. To assess how emerging respiratory viruses including SARS-CoV-2 variants differ regarding their potential to

propagate in the human lower respiratory tract and thereby induce severe disease, reliable infection models mimicking viral replication and host cell activation in patients as closely as possible are highly desirable. Autopsy studies showed that SARS-CoV-2 infects type I and type II pneumocytes in the lung¹⁰, which are the main cell types building the alveolar epithelium that forms a polarized and tight barrier facilitating fluid homeostasis and oxygen uptake through underlying blood capillaries in the alveolar space. Consistently, lung cells were shown to express SARS-CoV-2 host factors including the receptor angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine subtype 2 (TMPRSS2). Importantly, primary alveolar cells cultured at the air-liquid-interface (ALI) can recapitulate many facets of the alveolar epithelium partially, but quickly lose their *in vivo* phenotype and are poorly permissive for SARS-CoV-2 infection^{10,11}. Several lung derived cell lines are currently in use to investigate SARS-CoV-2 infections, but many of them model the alveolar epithelium imperfectly, either because they lack the ability to form tight junctions (e.g. A549 or NCI-H1299 lines), or are of bronchial origin (e.g. Calu-3, NCI-H441, 16HBE14). Most of them are derived from tumors and may have deregulated homeostasis, in addition. Furthermore, lung organoids derived from primary human lung-specific progenitor or stem cells¹², or explanted human lung tissue have been used to study SARS-CoV-2 infections. The latter model replicates wild-type SARS-CoV-2 only to low levels of around 1×10^4 TCID₅₀¹¹ and both systems require prior ethical approval¹².

Here we report on a novel model in polarized human Alveolar Epithelial Lentivirus immortalized (hAELVi) cell cultures¹³ to study severe infections of SARS-CoV-2 and other respiratory viruses in the lower human respiratory tract. The immortalized hAELVi cells were derived from type I pneumocytes¹³ that cover the vast majority of the lung surface. Using filter supports, hAELVi cells can be differentiated under ALI conditions to a stratified, polarized epithelial cell layer with high barrier functions. This appears to be advantageous as respiratory cells polarized under ALI conditions resemble authentic body cells in their gene expression patterns more closely compared to cells grown in submerged cultures¹⁴. The hAELVi cells are commercially available and many aspects of their structure, physiology and responses to exterior stimuli have been characterized, but to the best of our knowledge there is no report on their use in virus research.

Results

Polarized hAELVi ALI cultures express SARS-CoV-2 host factors

hAELVi cells were seeded and grown on permeable filter inserts under liquid-liquid conditions for 72 hours, after which cells were further incubated for up to 28 days under ALI (Fig. 1a). Cell cultures started to develop transepithelial electrical resistance (TEER) after 10 days under ALI with a high maximum value of $6000 \Omega \cdot \text{cm}^2$ approximately 21 days after seeding. Similar dynamics were observed for the permeability of the paracellular transport marker sodium fluorescein, which progressively decreased through day 14 indicating the presence of a tight epithelium (Fig. 1b).

Cells developed under ALI conditions from a rather cubic shape and in many regions multilayered organization at day 0 to a pseudostratified or stratified columnar epithelium at day 21 (Fig. 1c; Supplemental Fig. 1). The epithelial cells revealed many short apical microvilli (Supplemental Fig. 2a) and a fibrous secretion (Supplemental Fig. 3c) released by vesicles present in the apical zone of their cytoplasm. Cilia or their basal bodies could be detected exceptionally in single cells (Supplemental Fig. 2b). Multilamellar bodies, which are typical for alveolar type II cells, were not detected. In summary, the cell layer at later stages morphologically resembled epithelium of the terminal bronchiolar segments of the lower respiratory tract, including also non-ciliated Clara cells.

Immunoblot and ELISA analyses showed that hAELVi cells upregulated expression of the ACE2 receptor and the host protease TMPRSS2 through the 28 days period grown under ALI conditions (Fig. 1d, e). In contrast, expression of the MERS-CoV receptor protein DPP4 decreased during the polarization process (Fig. 1e).

hAELVi ALI cultures are a highly-productive infection model for various respiratory viruses

We proceeded to investigate the hAELVi cultures as hosts for the three human coronaviruses (CoV) SARS-CoV, MERS-CoV and an early SARS-CoV-2 from 2020 (D614), as well as seasonal influenza A virus. Growth curve analyses of progeny viruses harvested in apical washes of the ALI cultures revealed a high permissiveness of the polarized hAELVi cultures with viral titers peaking at 48h p.i. at 5×10^7 pfu/ml for SARS-CoV-2, 2×10^8 pfu/ml for SARS-CoV and higher than 1×10^9 pfu/ml for seasonal IAV (Fig. 2a). Peak titers of MERS-CoV were up to two orders of magnitude lower compared to SARS-CoV-2. Confocal laser-scanning microscopy confirmed the infection by SARS-CoV-2 in mostly separated cells at 24h p.i.. At 48 h p.i. we detected infected cells in larger clusters indicating ongoing viral cell-to-cell spread (Fig. 2b). Thin section electron microscopy demonstrated the presence of large numbers of viral particles at the surface of (Fig. 2c) and within (Supplemental Fig. 3) single cells or small groups of cells intermingled between non-infected epithelial cells. Extracellular virus particles were linked by fine fibrous material (Fig. 2c), which was present at the surface of all cells (Supplemental Fig. 3c). All ultrastructural hallmarks of coronavirus replication, such as double-membrane vesicles, budding into membrane compartments and release at the cell surface, were visible in the infected cultures (Supplemental Fig. 3).

To assess the hAELVi model for early immune activation by the investigated viruses we analyzed the supernatants of infected cells for the presence of type I and III interferons (IFN), as well as cytokines and chemokines by ELISA. Interestingly, SARS-CoV-2 infection triggered secretion of IFN- β and λ 1-3 at moderate levels, whereas IAV induced a more pronounced induction of IFN λ 1-3 (Fig. 2d). SARS-CoV-2 and SARS-CoV induced distinct sets of proinflammatory cyto- and chemokines with SARS-CoV upregulating a larger set of immune factors including CXCL1, G-CSF, IL8, MIF and CXCL10. In contrast, SARS-CoV-2 infection only triggered modest secretion of MIF and CXCL10 from infected cells (Fig. 2e).

Attenuated replication of SARS-CoV-2 Omicron variant

Finally, we utilized the hAELVi model to compare propagation of the recently emerged SARS-CoV-2 Omicron variant to a SARS-CoV-2 D614G isolate and the Delta variant. The analysis showed similar replication kinetics for the D614G and Delta viruses with highest titers between $2.5 - 5.5 \times 10^7$ PFU/ml at 48h (MOI = 0.3) and $1.9 - 2.3 \times 10^7$ PFU/ml at 72h p.i. (MOI = 0.3), respectively. Interestingly, infectious titers of the Omicron variant were significantly reduced by up to 2 orders of magnitude at 48h p.i. compared to Delta (Fig. 2f). This effect was even more pronounced following infection with a 10-fold decreased MOI with the Omicron variant replicating to 1078-times lower titers compared to Delta at 48h p.i. (Fig. 2g). Collectively, these findings demonstrate an attenuated growth phenotype of the Omicron variant in a human ALI infection model of the distal lung in comparison to previous SARS-CoV-2 including the Delta.

Discussion

The alveolar space of the human lung can be heavily affected in severe COVID-19 or influenza, but analyses of viral replication and pathophysiological processes is limited by the inability to systematically sample infected cells of the alveolar epithelium in severely diseased patients *intra vitam*. In our study we present several pieces of evidence that cultures of polarized hAELVi cells grown under ALI can model productive SARS-CoV-2 infections in the lower respiratory tract under controlled experimental conditions. This includes virion morphogenesis and release, cell-to-cell spread, accumulation of high viral titers, as well as moderate induction of antiviral type I and III IFNs.

Besides active viral replication it has been shown that induction of regulatory cytokines plays a critical role in the pathogenesis of COVID-19¹⁵. Our study showed that hAELVi cultures maintain the capacity to react towards a viral stimulus. Upregulation of various proinflammatory mediators in this model is in line with previous analyses suggesting significant activation of cytokines and chemokines including IL-8, CXCL1, CXCL5 and CXCL10 in human lung explants infected with SARS-CoV *ex vivo*, whereas SARS-CoV-2 induced only a smaller subset of these mediators¹⁶. Moreover, virus-infected hAELVi cultures did upregulate type I and type III IFN suggesting their suitability to scrutinize in future studies the role of these antiviral and antiproliferative cytokines to tissue damage observed in the lower respiratory tract of patients with severe to critical COVID-19¹⁵. The possibility to establish co-cultures with macrophages-like cells raises the opportunity to extend the hAELVi infection model for future investigations of the cross-talk between epithelial and myeloid cells¹⁷.

In the present study we compared propagation of recently emerged Omicron and the earlier D614G and Delta variants in the polarized human hAELVi lung cell cultures infected as a proxy for viral fitness in the distal lung. Our analysis demonstrated an attenuated growth phenotype of Omicron in comparison to the earlier variants as viral titers increased only in a delayed or reduced manner. Reduced fitness of Omicron in the hAELVi model might involve a decreased usage of TMPRSS2 protease¹⁹ expressed in these cultures. Significantly, these findings mirror conclusions on reduced replication and disease severity caused by Omicron infections in hamster and mouse models^{18,19} and recapitulate and extend recent

results obtained in explanted human lung tissue²⁰. Overall, these experimental findings could well explain epidemiological observations showing a reduced likelihood for hospitalization of COVID patients infected with SARS-CoV-2 Omicron compared to Delta^{7,8,20}. Collectively, we suggest that hAELVi cultures are a novel experimental human infection model that is well suited for fast phenotypic evaluation of emerging viral variants and to inform Public health strategies in a timely manner.

Methods

Cell culture

For cultivation of hAELVi cells, cell culture flasks were coated prior to application of cells using huAEC Coating solution (inSCREENex, INS-SU-1018-100ml). hAELVi cells were cultivated in huAEC Medium (inSCREENex, INS-ME-1013-500ml). Prior to seeding of hAELVi cells on transwell filters (ThinCert, pore size 0.4 μ m, Greiner), inserts were coated by adding huAEC coating solution according to the manufactures protocol. Approximately 2.3×10^5 (12-well: Fig. 1b-e and Fig. 2a-e) or 0.7×10^5 cells (24-well: Fig. 2f-g) were seeded into the apical chamber of the filter insert and initially incubated at 37°C and 5% CO₂ under liquid-liquid-conditions for 3 days following further cultivation under ALI up to 28 days. Based on the analyses of barrier integrity, morphological analysis and expression of cellular host factors, the polarized hAELVi cells were used for infection experiments after an incubation period of at least 21 days under ALI. Vero E6 cells were propagated in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1x non-essential amino acids and 1 mM sodium pyruvate. MDCKII cells were cultivated in Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. All cells were incubated at 37°C with 5% CO₂ in a humidified atmosphere.

Infection of hAELVi cells and virus titration on Vero E6 cells

Cells were infected with SARS-CoV-2 D614G (hCoV-19/Germany/BW-RKI-N-0001/2020, GISAID accession: EPI_ISL_481253), SARS-CoV-2 Delta (ENA project PRJEB50616; sequence ID IMSSC2-206-2021-00148), SARS-CoV-2 Omicron (hCoV-19/Germany/BE-RKI-I-353502/2021, GISAID accession: EPI_ISL_7116918), SARS-CoV Frankfurt-1 (Genbank accession number FJ429166.1), MERS-CoV EMC/2012 (Genbank: JX869059) or Influenza A/Panama/2007/1999 virus (Genbank: DQ487333-DQ487340). For infection, cells were washed with D-PBS once and inoculated with virus diluted in D-PBS/0.3% BA in the apical chamber. Following incubation for 1 h at 37°C, cells were washed apically with D-PBS and fresh medium was added to the basolateral compartment of the filter insert. For replication analysis, 10% of the basolateral supernatant was harvested at indicated time points and refilled with fresh culture medium. To collect apical samples 50 μ l (24-well) or 100 μ l D-PBS (12-well), respectively, were used to perform apical washes at 37°C for 30 min. Supernatants were stored at -80°C until titration by standard Plaque Assay on Vero E6 cells (CoV) or MDCKII cells (influenza A virus) using Avicel overlay was performed to quantify infectious virus particles.

Ethical declaration

SARS-CoV-2 viruses were isolated from naso- or oropharyngeal swabs on VeroE6 or Caco-2 cells in the context of routine diagnostics by RKI units FG17 and ZBS1, which were approved by the ethics committees of Charité-Universitätsmedizin Berlin Ethical Board (Reference EA2/126/11) and the Berlin Medical Association (Eth-40/20).

Preparation of cell lysates and immunoblot analysis

Cells were washed twice with ice-cold D-PBS before lysis buffer (10 mM Tris/HCl (pH 7,5), 150 mM NaCl, 0.5 mM EDTA, 1% NP-40 including protease inhibitor) was added to the apical chamber of the filter insert and incubated for at least 30 min at 4°C. Cells lysates were centrifuged at 15000 x g and 4°C for 10 min and supernatants were stored at -20°C until further processing. 50 µg of each sample was separated by reducing SDS-PAGE under denaturing conditions and transferred onto nitrocellulose membrane by semi-dry western blotting. Detection of ACE2 was performed by incubation with anti-hACE2 antibody (R&D Systems (AF933); 1:500) and suitable secondary antibody coupled to horseradish-peroxidase (HRP) (1:10,000; Agilent Technologies, Santa Clara, USA). Equal loading of samples was controlled with immunostaining of actin. SuperSignal™ WestDura Extended Duration Substrate was added to the membrane and the resulting chemiluminescence was detected using an Advanced Fluorescence Imager (Intas).

Cytokine ELISA

Basolateral supernatants of infected cells were collected at indicated time points and stored at -80°C until further processing. If necessary, samples were diluted in the corresponding culture medium prior to ELISA measurement. Samples were analyzed using the R&D DuoSet ELISA Kits DY9345 (IFNα2), DY814 (IFNβ), DY7246 (IFNλ1), DY1587 (IFNλ2), DY5259 (IFNλ3), DY279 (CCL2), DY270 (CCL3), DY271 (CCL4), DY275 (CXCL1), DY254 (CXCL5), DY214 (G-CSF), DY206 (IL-6), DY208 (IL-8), DY289 (MIF), DY201 (IL-1β), DY266 (CXCL10) according to the manufacturer's instructions. For collective heat map presentation of all analyzed cytokines, mean values of x-fold inductions relative to mock-infection of three independent experiments were calculated for each cytokine. Individual concentrations of all cytokines are shown in Supplementary Fig. 4.

For quantification of host factor expression cell lysates were prepared as previously described, total protein amount was measured using -BCA-Kit- according to the manufacturer's instructions and 100µg of protein lysate was analyzed using the R&D DuoSet ELISA Kits DY933-05 (ACE2), DY1180 (DPP4) and the commercially available ELISA kit ABIN6960140 (TMPRSS2) according to the corresponding manufacturers protocol.

Detection of viral antigen by laser-scanning confocal fluorescence microscopy

Cells were washed once with D-PBS, fixed with 3.7% formaldehyde in D-PBS for 15 min at RT and further incubated for 10 min at RT with 10 mM ammonium chloride/D-PBS. Subsequently, cells were permeabilized for 7 min with 0.5% TritonX-100/D-PBS at RT, followed by blocking of cells with 3% BSA/D-PBS for at least 1 h at RT. Between each of these steps, the samples were washed twice with D-PBS.

For staining of specific proteins, filter inserts were stamped out with 6 mm biopsy punches and incubated with primary antibodies diluted in 3% BSA/D-PBS at 4°C overnight. After washing three times with D-PBS for at least 5 min, cells were incubated with secondary fluorescence conjugated antibodies for 1 h at RT in the dark. Cells were subsequently washed twice with D-PBS and nucleus stained by incubating cells with DAPI for 10 min. Fluorescence laser-scanning microscopy was performed using a Zeiss LSM 780 confocal microscope with corresponding ZEN software and a Plan-Achromate 20x (NA 0.8) objective.

Thin section electron microscopy (EM)

hAELVi cells were fixed on their filter substrate with a mixture of 1% formaldehyde and 2.5% glutaraldehyde in HEPES (0.05 M, pH 7.4) for at least 2 h at RT. Filters were punched out with a skin punch and were post-fixed with osmium tetroxide, tannic acid, uranyl acetate and embedded in epon²¹. Ultrathin sections were collected on naked grids and examined with a transmission electron microscope (Tecnai Spirit, Thermo Fisher Scientific Inc.) operated at 120 kV acceleration voltage. Images were recorded with a CMOS camera (Phurona, EMSIS). Overview images of the epithelium were recorded from carbon coated resin block-faces after sectioning with a diamond knife using a scanning electron microscope (Teneo VS, Thermo Fisher Scientific Inc.) and the T1 detector at 2 kV.

Determination of transepithelial electrical resistance (TEER) and Transepithelial Transport of Fluorescein in hAELVi cell monolayers

To determine TEER values, the apical chamber of cultured hAELVi cells was refilled to LCC levels with DPBS and the MillicellR ERS (electrical resistance system) volt was used for measurement. To obtain $\Omega \cdot \text{cm}^2$ the surface area of the transwell insert was multiplied with the measured TEER values. To assess the transport of sodium fluorescein across the cellular monolayer, fresh culture medium was added to the basolateral acceptor chamber and culture medium containing 1mg/ml sodium fluorescein was added into the apical chamber of the insert. Every 30 minutes for 3h one fifth of the basolateral culture medium was taken and directly replaced with fresh huAEC medium. Optical density of samples was determined at 486nm including a sodium fluorescein standard curve (0, 5, 10, 15, 20, 30, 40, 50 $\mu\text{g/ml}$).

Statistical analysis

All statistical analyses were done by using non-paired, non-parametric Kruskal-Wallis test (* $p < 0.05$) and performed using GraphPad Prism Software Version 9.1.0. Results were presented as mean $\pm$ standard error (SEM). For heat map presentation, mean values of x-fold inductions relative to mock-infection of three independent experiments were calculated for each cytokine.

Declarations

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

C.M., J.S. and D.B. planned and conducted the experiments and analyzed the data. G.H. and M.L. performed electron microscopic analysis. C.M., J.S., M.L., A.N., R. D. and T.W. contributed to the interpretations and conclusions presented. C.M., J.S., M.L. and T.W. wrote the manuscript. All authors participated in editing the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper.

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Figures

Figure 1

hAELVi cell air-liquid-interface cultures as model of the distal lung. (A) Schematic representation of hAELVi air-liquid-interface (ALI) cultures. (B) Measurement of trans-epithelial electrical resistance (TEER; n=5) and permeability of sodium fluorescein across the cellular monolayer (n=4) of hAELVi cells growing under ALI up to 28 days. (C) Electron microscopic analysis of hAELVi cells grown at the ALI for 0 or 21 days. Data are representative for two independent experiments. Bar: 20 μ M (D) Western Blot analysis of ACE2 expression of hAELVi cells grown under ALI for 0, 7, 14, 21 or 28 days, respectively. (E) Detection of ACE2, DPP4 and TMPRSS2 in cell lysates of hAELVi cells grown under ALI for 0, 7, 14, 21 or 28 days, respectively using ELISA. Experiments were performed for n=2.

Figure 2

Infection of hAELVi cell air-liquid-interface cultures with highly pathogenic coronaviruses. (A-G) hAELVi cells grown under ALI for 21 days were used for infection experiments with highly pathogenic coronaviruses. (A) Cells were infected with SARS-CoV-2 D614G, SARS-CoV, MERS-CoV and IAV at MOI of 0.3 and further incubated under ALI conditions at 37°C. Progeny viruses were collected by washes from the apical compartment at indicated time points and titrated using standard plaque assay on VeroE6 cells. Replication analysis was performed for n=3 in technical duplicates. (B) Cells were infected with SARS-CoV-2 at MOI 1 and were processed for analysis by confocal laser-scanning fluorescence microscopy to detect the viral spike protein at 24h and 48h p.i. (green channel). Nuclei were stained with DAPI (blue channel). Data are representative for two independent experiments. Bar: 50 μ M (C) Thin section electron microscopy of a SARS-CoV-2 infected epithelial cell shows cluster of coronavirus particles at the cell surface (upper image) and a small cluster at higher magnification. Infection was performed with MOI of 3 and cells were fixed 24h p.i.. Data are representative for two independent experiments. Bar: 200nm (D+E) Cells were infected with SARS-CoV-2, SARS-CoV, MERS-CoV and IAV at MOI of 1 followed by collection of the basolateral fluid at 48h p.i. for ELISA detection of type I and III IFN (D) or the indicated cyto- and chemokines (E). Experiments were performed for three independent experiments in technical duplicates. (F+G) For growth curve analyses, cells were infected with MOI of 0.3 (F) or MOI 0.03 (G) with SARS-CoV-2 variants D614G, Delta or Omicron, respectively. Apical washes were

performed at indicated time points and titrated using standard plaque assay. Replication analysis was performed for n=2 in duplicates. Statistical analysis was done by using Kruskal-Wallis test, * p<0.05.

Supplementary Files

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