TAP-inhibiting proteins US6, ICP47 and UL49.5 differentially affect minor and major histocompatibility antigen-specific recognition by cytotoxic T lymphocytes

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Abstract

CTLs specific for hematopoietic system-restricted minor histocompatibility antigens (mHags) can serve as reagents for cellular adoptive immunotherapy after allogeneic stem cell transplantation (SCT). In the HLA-mismatched setting, CTLs specific for hematopoietic system-restricted mHags expressed solely by the non-self ‘allo’ HLA molecules could be used to treat relapse after HLA-mismatched SCT. The generation of mHag-specific allo-HLA-restricted CTLs requires antigen-presenting cells (APCs) expressing low numbers of endogenous peptides to avoid co-induction of undesired allo-HLA reactivities. In this study, we exploited viral evasion strategies to generate APCs expressing a controlled set of endogenous peptides. Herpesviruses persist lifelong following primary infection due to expression of viral gene products that hamper T-cell recognition of infected cells. The herpesvirus-derived proteins US6, ICP47 and UL49.5 down-regulate endogenous antigen presentation in human APCs via inhibition of the transporter associated with antigen processing. EBV-transformed B cell lines transduced with retroviral vectors encoding US6, ICP47 or UL49.5 exhibited a stable decrease in cell-surface HLA class I expression and were protected from lysis by mHag-specific CTLs. Exogenous addition of mHag peptide fully restored target cell recognition. UL49.5 showed the most pronounced inhibitory effect, reducing HLA class I expression and mHag-specific lysis up to 99%. UL49.5 also significantly diminished allo-HLA reactivities mediated by allo-HLA-specific CTLs. In conclusion, UL49.5 could be a powerful new tool to study and modulate endogenous antigen presentation.

Introduction

CTLs specific for the minor histocompatibility antigens (mHags) HA-1 or HA-2 are potent reagents for adoptive immunotherapy of leukemia after allogeneic HLA-matched mHag-mismatched stem cell transplantation (SCT) (1). CTL responses directed against HA-1 and HA-2 are specific for hematopoietic system-derived cells including leukemic cells and their progenitors (2–5). mHag-specific CTLs can be generated in vitro using peptide-pulsed or mHag-transduced autologous dendritic cells (6, 7). CTLs can also be targeted to mHags presented in the context of non-self ‘allo’ HLA molecules by the use of allogeneic HLA-mismatched antigen-presenting cells (APCs) (8). These CTLs may serve as reagents for the treatment of relapsed leukemia after HLA-mismatched SCT. However, the generation of mHag-specific allo-HLA-A2-restricted CTLs is hampered by adventitious expansion of broad allo-HLA-A2-specific T cells present in the T-cell repertoire of HLA-A2neg individuals (8). Such allo-HLA-reactive T cells are directed at a variety of endogenous peptides presented by allo-HLA molecules (9) and are potentially harmful to the patient. Minimizing the

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number of peptides presented by the allogeneic APC may reduce the induction of undesired allo-HLA reactivities.

Endogenous peptide presentation is affected by inhibition of the transporter associated with antigen processing (TAP). TAP transports cytosolic peptides into the endoplasmic reticulum (ER) (10), where they are loaded onto HLA class I molecules linked to TAP through tapasin (11). In the absence of functional TAP, most HLA class I molecules are not loaded with peptides and are eventually redirected to the cytosol where they are degraded by proteasomes (12–14). Consequently, HLA class I molecules on the cell surface of a TAP-deficient cell will present only a limited number of signal sequence-derived peptides that can serve as ligands for allo-HLA-reactive T cells (15). Exogenous addition of peptides stabilizes these HLA class I molecules, thereby restoring antigen presentation in a peptide-specific manner (16, 17). A TAP-inhibited allogeneic APC that has been exogenously loaded with the peptide of choice may retain the capacity to stimulate CTLs specific for the added peptide, without adventitious co-stimulation of CTLs specific for other endogenous peptides. We aimed at investigating whether HLA-A2\textsuperscript{bos} APCs, transduced with a TAP-inhibiting protein and pulsed with mHag peptides, can indeed elicit mHag-specific but not allo-HLA-specific CTL responses. If so, such APCs would be suitable antigen-specific stimulators for the in vitro induction of mHag-specific allo-HLA-A2-restricted T cells.

Three different proteins have been described so far that specifically inhibit peptide translocation by TAP in human cells. The human cytomegalovirus-encoded US6 is an ER-resident protein that blocks conformational changes within the transporter complex required for ATP binding and thus peptide translocation (18, 19). ICP47 is a herpes simplex virus type 1- and type 2-encoded protein that associates with cytosolic domains of the TAP complex, thereby acting as a high-affinity competitor for peptide binding (20–23). Recently, the UL49.5 protein encoded by the bovine herpesvirus type 1 has been identified as a potent inhibitor of TAP (24). UL49.5 inactivates TAP by arresting the transporter in a translocation-incompetent conformation and mediating its degradation by proteasomes.

We investigated these three TAP inhibitors for their individual capacity to block endogenous antigen presentation by APCs. To this end, we transduced EBV-transformed lymphoblastoid cell lines (EBV-LCLs) with retroviral vectors encoding US6, ICP47 or UL49.5. The effects of these viral TAP inhibitors on cell-surface HLA class I expression and on endogenous peptide presentation were investigated for each individual sample a secondary control was performed using with streptavidin–PE (Becton Dickinson) in appropriate dilution. The HLA specificities of these human mAbs (all IgG) were determined by complement-mediated cytotoxicity assays against large (n > 240) panels of serologically typed PBMCs. The human mAbs were purified by protein A chromatography (Pharmacia, Uppsala, Sweden) and biotin labeled (Pierce, Rockford, IL, USA) following the manufacturer’s instructions. The reactivities of biotin-labeled human mAbs were validated by flow cytometry. All biotin-conjugated human mAbs showed homogeneous, HLA allele-specific staining on CD3-positive cells.

Table 1. HLA class I and mHag phenotyping of the EBV-LCLs used in this study

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Flow cytometric analyses
HLA class I cell-surface expression was determined by labeling with biotinylated human HLA-specific mAbs counterstained with streptavidin-PE (Becton Dickinson) in appropriate dilutions. For each individual sample a secondary control was included consisting of staining with streptavidin-PE only. Gates were set on vital lymphocytes according to their typical pro_helper_free.html) upstream of the internal ribosomal entry site (IRES) and enhanced green fluorescent protein (GFP). Retrovirus production and transduction of EBV-LCLs were performed as described (http://www.stanford.edu/group/nolan/protocols/pro_helper_free.html).

Cell lines
EBV-LCLs Modo and Hodo (Table 1) were transduced with retroviral vectors to generate the following stable GFP-positive cell lines: Modo-control and Hodo-control (containing a retroviral vector without insert), Modo-US6, Modo-ICP47, Modo-UL49.5 and Hodo-UL49.5. GFP-positive cells were selected by a FACS Vantage cell sorter (Becton Dickinson, San Jose, CA, USA) to ensure homogenous and comparable expression of the various TAP inhibitors. All EBV-LCLs were cultured in Iscove’s modified Dulbecco’s medium (IMDM) containing 5% FCS.

In vitro generation of mHag- and allo-HLA-specific CTL clones is documented in detail elsewhere (25, 26). Clone #1 was kindly donated by J. H. F. Falkenburg (Leiden University Medical Center). All CTL clones were cultured in IMDM containing 10% pooled human serum and 25 U ml\(^{-1}\) IL-2 (Cetus, Emeryville, CA, USA).

Synthetic peptides and human mAbs
HA-1, HA-2 and HY peptides were synthesized according to their reported sequences (27–29). Where stated, EBV-LCLs were pulsed with 10 \(\mu\)g ml\(^{-1}\) of relevant mHag peptides for 1 h at 37°C.

Hybridomas producing human mAbs SN607D8 (anti-HLA-A2/A28), VTM1F11 (anti-HLA-B7/B27/B60) and GV5D1 (anti-HLA-A1/A9) were generated as described previously (30). The HLA specificities of these human mAbs (all IgG) were determined by complement-mediated cytotoxicity assays against large (n > 240) panels of serologically typed PBMCs. The human mAbs were purified by protein A chromatography (Pharmacia, Uppsala, Sweden) and biotin labeled (Pierce, Rockford, IL, USA) following the manufacturer’s instructions. The reactivities of biotin-labeled human mAbs were validated by flow cytometry. All biotin-conjugated human mAbs showed homogeneous, HLA allele-specific staining on CD3-positive cells.

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forward- and side-scattering characteristics. All flow cytometric analyses were performed on a FACSCalibur with CellQuest software (Becton Dickinson). Results are expressed as the mean fluorescence intensity (MFI) of two samples. MFI = [mean fluorescence sample 1 – mean fluorescence of secondary control] + [mean fluorescence sample 2 – mean fluorescence of secondary control]/2. Raw data are shown for single representative samples.

Cytotoxicity assays

Cytotoxicity was evaluated by incubating 2500 ^{51}Cr-labeled target cells with serial dilutions of CTLs for 4 h. Supernatants were harvested for gamma counting. Percent specific lysis = (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100%. Results are expressed as the mean of duplicate samples and shown for an effector:target (E:T) ratio of 10:1 unless stated otherwise.

Statistics

Statistical analyses were performed using unpaired t-tests for data derived from a single experiment and paired t-tests for data pooled from multiple experiments. P values < 0.05 were considered to be significant. Data pooled from multiple experiments were standardized for statistical analysis as follows: fluorescence (in fluorescence units) = [(mean fluorescence of sample – mean fluorescence of secondary control)/(mean fluorescence of mock control – mean fluorescence of secondary control)] × 100%; lysis = mean percent lysis/mean percent lysis mock. Error bars represent standard errors of the mean.

Results

Effects of US6, ICP47 and UL49.5 on HLA class I cell-surface levels

EBV-LCLs derived from HLA-A2pos, HLA-B60pos donor Modo (Table 1), were retrovirally transduced with US6, ICP47 or UL49.5 or with an empty control vector to evaluate the effects of the three TAP inhibitors on HLA class I expression and antigen presentation. Cell-surface levels of HLA-A2 and HLA-B60 were analyzed using HLA allele-specific human mAbs (Fig. 1A and B). The HLA-A2 expression of EBV-LCLs transduced with US6, ICP47 or UL49.5 decreased with 63, 57 and 73%, respectively; the HLA-B60 expression with 80, 82 and 99%, respectively, compared with the empty vector-transduced EBV-LCL (P < 0.05). These low HLA class I cell-surface levels remained consistent upon continuous in vitro culture (data not shown). No difference in HLA-A2 or HLA-B60 expression could be observed between untransduced and empty vector-transduced EBV-LCLs.

Effects of US6, ICP47 and UL49.5 on mHag-specific target cell recognition

To determine whether the down-regulation of HLA class I cell-surface expression resulted in a decrement of functional recognition by mHag-specific CTLs, the transduced Modo EBV-LCLs were used as target cells in cytotoxicity assays. Four different CTL clones with previously established specificity for the mHags (HLA-) A2/HA-1, A2/HA-2, A2/HY or B60/HY were used as effector T cells (Fig. 2A). The Modo EBV-LCLs naturally express each of these mHags (Table 1). All CTL clones exhibited a significantly diminished recognition of TAP inhibitor-transduced EBV-LCLs as compared with empty vector-transduced EBV-LCL (P < 0.05). Inhibition of target cell lysis ranged from 70 to 87% for US6, 77 to 89% for ICP47 and 85 to 99% for UL49.5 for the various CTL clones. Increasing the E:T ratio did not restore target cell recognition (Fig. 2B), indicating consistent blocking of endogenous mHag peptide translocation and HLA loading by TAP inhibitors. No difference could be detected between untransduced and control-transduced EBV-LCLs for any of the CTL clones tested.

TAP-inhibiting effects by US6, ICP47 and UL49.5 were statistically analyzed by pooling the data on HLA-A2 and HLA-B60 expression as well as the data on mHag recognition by the various CTL clones from the experiments described above. This analysis showed significant differences for decrement of HLA class I expression between US6 and UL49.5 (P = 0.0264) and ICP47 and UL49.5 (P = 0.0006), but not between US6 and ICP47 (P = 0.6474). Similarly, decreases in mHag-specific lysis differed significantly between US6 and UL49.5 (P = 0.0005) and ICP47 and UL49.5 (P = 0.0346), but not between US6 and ICP47 (P = 0.1355). These results indicate that UL49.5 is consistently more effective in down-regulating endogenous mHag presentation.

Effects of exogenous peptide addition on recognition of TAP-inhibited target cells

TAP inhibitory proteins affect HLA class I expression because the absence of endogenous peptide renders cell-surface HLA class I molecules unstable. Yet, HLA class I cell-surface expression is not completely abrogated. Exogenously added peptides can bind to these HLA class I molecules. To investigate whether sufficient HLA class I molecules remain for functional mHag presentation, we loaded TAP inhibitor-transduced EBV-LCLs with mHag peptides. Hereto, HLA-A2pos HA-1pos Modo EBV-LCLs transduced with US6, ICP47, UL49.5 or an empty vector were pulsed with various concentrations of HA-1 peptide. An EBV-LCL derived from HLA-A2pos HA-1neg donor H6 (Table 1) was included as a control (Fig. 3). Addition of HA-1 peptide to TAP-inhibited EBV-LCLs restored recognition by A2/HA-1-specific CTLs in a dose-dependent manner to the level observed for the control target cell H6. Addition of non-specific peptide had no effect (data not shown). Thus, even low numbers of HLA molecules appear to be sufficient for functional mHag-specific recognition; an observation in line with the functional recognition of low copy numbers of mHags. Thus, upon functional inhibition of TAP, the target cell can still be pulsed exogenously with any HLA-binding peptide of interest; one of the original aims of our study.

Effects of TAP inhibition on allo-HLA-A2-specific target cell recognition

As mentioned above, TAP inhibition does not abrogate cell-surface HLA class I expression completely. Thus, TAP-inhibited EBV-LCLs may still present peptides on the cell surface that might be recognized by allo-HLA-specific CTLs.

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To test the latter proposition, we compared allo-HLA recognition of empty vector-transduced EBV-LCLs with TAP-inhibited EBV-LCLs. EBV-LCLs derived from HLA-A2<sup>pos</sup> donor Modo and transduced with US6, ICP47 or UL49.5 or with an empty vector were used as targets in a cytotoxicity assay. As effector T cells, we used two allo-HLA-A2-specific CTL clones (designated clone #1 and clone #2). Clone #1 was shown to be TAP dependent in earlier experiments (data not shown), whereas clone #2 is known to be TAP independent (31). The HLA-A2<sup>pos</sup> TAP-deficient cell line T2 was included as a control. Two E:T ratios are shown for the transduced Modo EBV-LCLs i.e. 10:1 and 1:1 (Fig. 4). TAP-dependent CTL clone #1 exhibited a significantly diminished recognition of ICP47- and UL49.5-transduced EBV-LCLs as compared with empty vector-transduced EBV-LCL for both E:T ratios (P < 0.05). No significant inhibition of lysis was observed for US6 in this experiment (P = 0.05). Of ICP47 and UL49.5, the latter was again the more potent inhibitor (P < 0.05 E:T 1:1, P = 0.33 E:T 10:1). Allo-HLA-A2 recognition of UL49.5-transduced Modo EBV-LCL was reduced by 90% for E:T ratio 1:1 but only by 47% for E:T ratio 10:1. In comparison, at E:T ratio 10:1, mHag-specific recognition of UL49.5-transduced Modo EBV-LCLs was abrogated almost completely (Fig. 2A). Apparently, UL49.5 does not inhibit the presentation of peptides recognized by TAP-dependent allo-HLA-A2-specific CTLs completely. Allo-HLA-A2 recognition by TAP-independent CTL clone #2 was not affected by any of the TAP inhibitors.

Effects of UL49.5 on allo-HLA-A1-specific target cell recognition

Inhibition of TAP has a stronger effect on HLA-A1 expression than on HLA-A2 expression (32, 33). Therefore, we also evaluated allo-HLA-A1-specific recognition of UL49.5-transduced EBV-LCLs. To that end, we retrovirally transduced EBV-LCLs derived from HLA-A1<sup>pos</sup> donor Hodo with UL49.5 or with an empty control vector. UL49.5-transduced Hodo EBV-LCL showed significantly decreased cell-surface HLA-A1 expression (P < 0.05) and decreased susceptibility to lysis by an HLA-A1-restricted mHag-specific CTL clone (data not shown).
UL49.5- and control-transduced Hodo EBV-LCLs were then used as targets for an allo-HLA-A1-specific CTL clone (designated clone #3) as effector cell. E:T ratios 10:1 and 1:1 are shown for the transduced Hodo EBV-LCLs (Fig. 5). Allo-HLA-A1-specific recognition was significantly decreased for UL49.5-expressing Hodo EBV-LCLs as compared with control-transduced EBV-LCLs ($P < 0.05$). However, down-regulation of allo-HLA-A1-specific lysis was not complete, similar to that of allo-HLA-A2-specific lysis (Fig. 4). Taken together, these findings indicate that retroviral transduction of APCs with UL49.5 diminishes but not abrogates major allo-HLA recognition in a TAP-dependent fashion.

**Discussion**

In this study, we investigated the capacity of three virus-derived proteins that specifically inhibit peptide translocation by TAP, to block minor and major histocompatibility antigen-specific recognition. Our results show that mHag-pos EBV-LCLs transduced with retroviral vectors encoding US6, ICP47 or UL49.5 all exhibit a stable decrease in cell-surface HLA class I expression and are protected from lysis by mHag-specific CTL clones. Antigen presentation can be fully restored by exogenous addition of specific mHag peptides, demonstrating that cells transduced with viral TAP inhibitors can be used as functional APCs. Transduction of EBV-LCLs with TAP inhibitors also inhibits allo-HLA-A1- and allo-HLA-A2-specific recognition, albeit to a lesser extent than mHag-specific recognition. Herewith, our scientific challenge to modify the peptide repertoire of a particular APC using viral TAP inhibitors, thereby creating an opportunity to direct the CTL response toward defined, e.g. tumor-associated specificities, is verified.

From the three TAP inhibitors we analyzed, UL49.5 is the most potent. It reduces mHag-specific lysis of EBV-LCLs to the level observed for the TAP-deficient cell line T2 (data...
 Whereas US6 blocks conformational changes required for ATP binding and peptide translocation (10) and ICP47 competes for peptide binding (13), UL49.5 inhibits essential conformational changes at a later phase of the translocation cycle, thereby fully blocking the transport of peptide. In addition, UL49.5 targets TAP for proteasomal degradation causing disintegration of the MHC class I peptide-loading complex (12, 32, 33), a phenomenon not observed for US6 or ICP47 (19, 21–24). UL49.5’s ‘double-strike policy’ ensures optimal down-regulation of TAP and thus better protection from the host immune response against the type 1 bovine herpesvirus encoding this protein.

Earlier studies have investigated the effect of TAP inhibition by ICP47 on allo-HLA recognition and reported decreased lysis of ICP47-transduced target cells by sensitized lymphocytes (34, 35). We are the first to look at the effect of TAP inhibition on allo-HLA recognition at a clonal level. The allo-HLA-A1- and allo-HLA-A2-specific CTL clones used in this study recognize as yet undefined endogenous peptides that associate with HLA-A1 or HLA-A2 (25, 36). Allo-HLA-A2-specific CTL clone #1 does not lyse HLA-A2pos TAP-deficient cell line T2, but does lyse T2 reconstituted with TAP (data not shown). These data imply that cell-surface expression of the peptide recognized by clone #1 is dependent on the presence of functional TAP. Yet, recognition of an HLA-A2pos EBV-LCL transduced with UL49.5 by clone #1 is only partially inhibited, while recognition of the same EBV-LCL by a mHag-specific CTL clone is almost completely abrogated.

There are several possible explanations for this observation. First, TAP inhibition by UL49.5 gene transfer may not be complete. If CTL clone #1 expresses a TCR of high affinity, a very low peptide copy number per cell will be sufficient to trigger a lytic response. Alternatively, if the peptide recognized by CTL clone #1 is present at a greater peptide copy number per cell or displays a greater affinity for the TAP transporter than mHag-derived peptides, its presentation may be relatively preserved in a TAP-inhibited, but not completely blocked setting. Assuming that TAP inhibition by UL49.5 gene transfer is complete, the continuous presence of peptide recognized by CTL clone #1 could be explained by an alternative route of antigen presentation. Lautscham et al. (37) showed recently that hydrophobic peptides may be processed via a proteasome-dependent, TAP-independent pathway. Peptides of intermediate hydrophobicity that were normally TAP dependent showed inappropriate presentation in TAP-negative cells when expressed by minigenes (38). The partial inhibition of allo-HLA-A2-specific lysis by CTL
clone #1 as compared with a near complete arrest of mHag-specific lysis could thus be the result of differences in hydrophobicity between the relevant peptides.

Because inhibition of allo-HLA recognition by UL49.5 is incomplete, our original aim of generating antigen-specific stimulators for the induction of mHag-specific allo-HLA-restricted T cells is not achieved. Yet, we do show that UL49.5 effectively abrogates CTL recognition of relevant target cells and that addition of a chosen peptide efficiently restores peptide-specific CTL recognition. UL49.5 thus facilitates preferential presentation of a target sequence, enabling the direction of CTL responses toward a desired target epitope. These observations could offer interesting new possibilities for immunomodulation.

The results of our study are relevant to other areas of research as well. First, UL49.5 may be helpful in elucidating the ‘alloresponses’ that still hamper SCT across HLA barriers. Studies of the ‘allopeptides’ recognized by allo-HLA-specific CTLs are complicated by two factors. The number of potential ligands is large, because the allo-HLA-specific CTL repertoire has not been selected to ignore self-peptides presented by allo-HLA molecules. In addition, CTLs specific for viral peptides bound by self-HLA molecules have been shown to exhibit cross-reactivity with allo-HLA molecules, rendering the precise target antigen difficult to establish (39). Several known human cytomegalovirus-encoded proteins block cell-surface expression of HLA class I molecules completely (40). US3 retains HLA class I molecules in the ER (41, 42), while US2 and US11 target HLA class I allele heavy chains for degradation in the cytosol (14, 43). Each of these proteins affects a defined set of HLA class I alleles (42, 44, 45). The characteristics of US3, US2 and US11 can therefore be used to abrogate antigen presentation and thus cross-reactive T-cell recognition for a selected set of HLA class I alleles expressed by a particular APC. The peptide repertoire presented by the remaining HLA alleles can then be modified by UL49.5. Thus, US2, US3 and US11 together with UL49.5 constitute a powerful viral toolbox facilitating studies of allopeptides’ recognition patterns.

Second, impairment of TAP, and thus of antigen presentation, is frequently observed in human tumors (46), allowing tumors to escape from immune surveillance by CTLs (47, 48). Recently, it was shown that an alternative repertoire of peptide epitopes emerges at the surface of murine cells with impaired function of TAP (49). These peptides most likely derive from the ER but are not normally loaded into MHC class I due to the presence of more abundant TAP-dependent peptides. Because they are absent on normal cells, these ‘new’ peptides may act as immunogenic neo-antigens and can be exploited as targets for immunotherapy against TAP-deficient tumors. The potent TAP inhibitor UL49.5 might be used to elicit the presentation of these peptide epitopes and aid the study of the TAP-independent peptide repertoires of human tumor cells.

In summary, we here show novel functional characteristics of the recently described varicellovirus-derived TAP inhibitor UL49.5. UL49.5 down-regulates HLA class I expression and inhibits mHag-specific CTL responses more efficiently than US6 and ICP47. UL49.5 also reduces allo-HLA reactivity, thus providing a new tool to study fundamental aspects of allo-HLA reactivity in general and the TAP-dependent and independent peptide repertoires in particular.

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**Abbreviations**

- APC: antigen-presenting cell
- EBV-LCL: EBV-transformed lymphoblastoid cell line
- ER: endoplasmic reticulum
- E.T: effector:target
- GFP: green fluorescent protein
- HA: hemagglutinin
- IMDM: Iscove's modified Dulbecco's medium
- MFI: mean fluorescence intensity
- mHag: minor histocompatibility antigen
- SCT: stem cell transplantation
- TAP: transporter associated with antigen processing

**References**

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