ORIGINAL ARTICLE

Lectin-binding Patterns in Normal Endometrium and Endometrial Adenocarcinoma

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Abstract

Phenotypic aspects of glycoconjugates in human endometrium were examined in association with the menstrual cycle and malignant transformation by horseradish peroxidase (HRP)-labeling method using seven different lectins as a probe. At the light microscopic level, Concanavalin A (ConA), *Ricinus communis* agglutinin (RCA), and wheat germ agglutinin (WGA) stained almost all glandular cells in both normal endometrium and endometrial adenocarcinoma. *Dolichos biflorus* agglutinin (DBA) and soybean agglutinin (SBA) demonstrated markedly enhanced binding in secretory endometrium compared with endometrium in the proliferative phase. *Ulex europeus* agglutinin 1 (UEA-1) strongly stained endometrial adenocarcinoma, especially the well differentiated type, but scarcely stained normal endometrium. The reaction with peanut agglutinin (PNA) was characterized by cytoplasmic staining in the carcinoma cells, whereas its binding was restricted to the apical surface in the normal cells. At the electron microscopic level, the reaction products representing UEA-1 and PNA binding sites were observed in the plasma and Golgi membranes of malignant cells. DBA stained the same membranes in the normal endometrium, with the number of positive cells being markedly increased in the secretory phase. Whereas ConA binding was proved to be positive in the plasma membrane, endoplasmic reticulum, Golgi apparatus and nuclear envelope of normal and malignant cells. These results indicate that sugar components of endometrial glycoconjugates change

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during the menstrual cycle and differ between normal endometrium and endometrial adenocarcinoma.

Key words: lectin-histochemistry, human endometrium, menstrual cycle, endometrial adenocarcinoma, light and electron microscopy

Introduction

Lectins which specifically bind to sugar residues¹ are valuable probes for the histochemical demonstration of carbohydrate moieties on the cell surface as well as in cytoplasmic organelles.²⁻⁴ By application of lectins, cell-associated glycoconjugates have been proved to be closely involved in cell-to-cell interactions,^{5,6} differentiation,⁷ and alterations in hormonal states,⁸⁻¹⁰ as well as in the malignant transformation of various tissues including those of the alimentary tract,¹¹⁻¹³ mammary gland,¹⁴ urinary tract,¹⁵ and others.¹⁶

As the morphological and physiological changes of the uterine endometrium during the menstrual cycle are caused by periodic variations in the blood levels of estrogen and progesterone, the endometrium is well-known to be one of typical target organs in the endocrine system, and, furthermore, to possess the potential to be transformed into adenocarcinoma.¹⁷ Little is known, however, regarding the carbohydrate moieties of human uterine tissue, and most of these histochemical works have been carried out at the light microscopic level only.^{8,18} In the present study, therefore, glycoconjugates in normal endometrium of the proliferative and secretory phase and in endometrial adenocarcinoma were examined by light and also by electron microscopy, using lectins as a probe.

Materials and Methods

Tissue preparation

Twenty-two samples of normal endometrium (15 in the proliferative phase and 7 in the secretory phase), and 45 samples of endometrial adenocarcinoma (21 well-, 16 moderately-, and 8 poorly-differentiated adenocarcinomas) were used. The normal specimens were obtained by surgical resection from normally menstruating women aged 32 to 47 years diagnosed with uterine myoma or early cervical squamous cancer. The endometrial tissues were collected from the uterine body distal to the cervix, and was diagnosed as histopathologically normal. The endometrial adenocarcinoma specimens were obtained by surgical resection, from patients aged 29 to 80 years in clinical stages ranging from I to II as defined according to the classification of the International Federation of Gynecology and Obstetrics.¹⁹

Staining with lectins for light microscopy

Endometrial specimens were fixed overnight with 10% formalin and embedded in paraffin. Four- μ m sections were cut, deparaffinized with xylene, and rehydrated with a graded series of ethanol. Lectin binding sites were visualized using biotinylated lectins and the avidin-biotin-peroxidase complex (ABC) (Vector Lab. Inc., USA). The sections were first covered with 1% bovine serum albumin (BSA) in 0.01 M sodium phosphate-buffered saline (PBS: 0.14 M NaCl, 2.9 mM NaH_2HPO_4 , 9.0 mM Na_2HPO_4 , pH 7.2). After being rinsed with PBS, the sections were incubated with biotinylated lectins (Vector Lab. Inc., USA; summarized in Table 1) at a concentration of 25 μ g/ml in 0.1% BSA-PBS for 30 minutes at room temperature. The sections were washed in PBS, then incubated with ABC reagent for 30 minutes at room temperature. After being washed again with PBS, the sections were immersed in 3, 3'-diaminobenzidine $\cdot$ 4 HCl (DAB, 0.2 mg/ml)- H_2O_2 (0.005%) for 10 minutes at room temperature. Sections were rinsed in water, counter-staining with Mayer-hematoxylin, dehydrated, cleared, mounted and observed by light microscope.

To confirm the specificity of lectin staining, the sections were preincubated with appropriate hapten sugars (Table 1) at a concentration of 0.1 M and then incubated with biotinylated lectins in the presence of 0.1 M hapten sugars. To detect endogenous peroxidase activity, the sections were incubated with DAB- H_2O_2 solution only. Non-specific binding of ABC reagent was also checked by incubation with ABC reagent, then with DAB- H_2O_2 solution.

Table 1 Lectins and Hapten Sugars Specific for Each Lectin Used

Lectins used	Abbreviation	Specific hapten sugar(s)
Concanavalin A	ConA	α -methyl-D-mannoside
<i>Ricinus communis</i> agglutinin	RCA	lactose
Wheat germ agglutinin	WGA	N-acetyl-D-glucosamine N-acetyl-neuraminic acid
<i>Dolichos biflorus</i> agglutinin	DBA	N-acetyl-D-galactosamine
Soybean agglutinin	SBA	N-acetyl-D-galactosamine
<i>Ulex europaeus</i> agglutinin I	UEA-1	L-fucose
Peanut agglutinin	PNA	D-galactose, lactose

Staining with lectins for electron microscopy

Endometrial specimens were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (21.2 mM NaH_2PO_4 , 80.1 mM Na_2HPO_4 , pH 7.4) for 4 hours at 4°C. After being washed with PBS, frozen sections of 40- μ m thickness were cut. For the stainings

with DBA, UEA-1, and PNA, sections were incubated with HRP-labeled lectins (E.Y. Lab. Inc., USA) at a concentration of 100 $\mu\text{g}/\text{ml}$ for 72 hours at 4°C.³ For the ConA staining, the two-step method was employed.²⁰ In short, the frozen sections were first incubated with ConA (100 $\mu\text{g}/\text{ml}$, Miles-Yeda, Israel) for 72 hours and then with HRP (100 $\mu\text{g}/\text{ml}$, Type II, Sigma, USA) for 1 hour at 4°C. As cytochemical controls, the frozen sections were incubated with lectins in the presence of 0.2 M appropriate hapten sugars (Table 1). After being washed, they were immersed in DAB (0.2 mg/ml)-H₂O₂ (0.005%) for 15 minutes at room temperature, washed with distilled water, and post-fixed with 1% osmium tetroxide. The specimens were dehydrated through a graded series of ethanol and embedded in Epon 812. Ultrathin sections were made, slightly stained with lead citrate, and examined with a JEM-100B transmission electron microscope.

Results

Light microscopic observations

Lectin staining patterns in each specimen were evaluated on the basis of both stain-

INCIDENCE	50~100%	—	+	++	##
	10~50%	—	+	++	++
	~10%	—	+/-	+	+
	negative	—	—	—	—
		negative	weak	moderate	strong
INTENSITY					

Fig. 1 Evaluation criteria for the lectin stainings. The individual lectin reactivity with endometrial tissue sections was scored from (—) to (##) by taking account of both staining intensity and incidence. The staining intensity was graded as negative, weak, moderate or strong, while the incidence of the stained cells was divided into four grades: negative, less than 10%, 10 to 50% and more than 50%. According to these criteria, cases scoring (+) or more were regarded as positive.

Table 2 Binding of a Variety of Labeled Lectins to Normal and Malignant Endometrium

Lectins	Normal endometrium						Endometrial adenocarcinoma																		
	P(15)			S(7)			G1(21)			G2(16)			G3(8)												
	-	+	##	-	+	##	-	+	##	-	+	##	-	+	##										
	-	+	##	-	+	##	-	+	##	-	+	##	-	+	##										
ConA	0	0	6	6	3	0	0	4	2	1	0	0	1	12	8	0	0	2	9	5	0	0	2	4	2
RCA	0	0	8	7	0	0	0	4	3	0	0	0	0	14	7	0	0	2	8	6	0	0	0	6	2
WGA	0	0	3	7	5	0	0	0	1	6	0	0	0	6	15	0	0	0	4	12	0	0	0	2	6
DBA	13	2	0	0	0	0	2	0	4	1	17	2	1	1	0	16	0	0	0	0	7	1	0	0	0
SBA	5	9	1	0	0	1	2	3	1	0	6	6	2	7	0	3	7	4	1	1	6	0	2	0	0
UEA-I	6	6	3	0	0	7	0	0	0	0	1	0	5	10	5	5	2	3	2	4	4	1	1	2	0
PNA	0	1	6	7	1	1	2	2	2	0	3	4	4	7	3	3	2	3	3	5	0	2	1	3	2

Scoring from (-) to (##) is based on the criteria shown in Fig. 1. Numbers in parentheses indicate numbers of cases examined.

P: Proliferative phase, S: Secretory phase.

G1: well-differentiated type, G2: moderately-differentiated type, G3: poorly-differentiated type.

Fig. 2 Positive rates for normal endometrium and endometrial adenocarcinomas stained with ConA (a), WGA (b), RCA (c), DBA (d), SBA (e), UEA-1 (f) and PNA (g). The positive rate for each lectin is indicated by the percentage of the positive cases in the 15 cases of proliferative (P) and the 7 of secretory normal endometrium (S) and in the 21 of well-differentiated (G1), the 16 of moderately-differentiated (G2) and the 8 of poorly-differentiated endometrial adenocarcinomas (G3).

ing intensity and incidence of positive endometrial glandular cells or cancer cells. The intensity was graded on an arbitrary scale into negative, weak, moderate, and strong, and the incidence was classified into four grades: negative, less than 100%, 10 to 50%, and more than 50%, according to the percentage of positively stained cells within the endometrial glandular or cancer cells in each section. By combining both intensity and incidence, the reactivity of each specimen was scored from (–) to (+++) as shown in Fig. 1; the overall results are summarized in Table 2 according to these criteria. Specimens with a score greater than or equal to (+) were designated as positive cases, and the respective positive rate for each lectin staining was defined as the percentage of the positive cases to the total number of specimens, i.e., normal and malignant cases. These rates are summarized in Fig. 2.

ConA, RCA, WGA

The positive rates for normal endometrium and endometrial adenocarcinoma stained with ConA, RCA and WGA were 100% based on the above criteria (Fig. 2a-c). ConA diffusely stained the cytoplasm as well as the plasma membranes (Fig. 3a, b), while RCA (Fig. 3c) and WGA (Fig. 3d) strongly stained the apical surface and the cytoplasm, especially in the apical region. No significant difference in the staining pattern was recognized among the proliferative, secretory, and malignant endometrium.

DBA

Endometrium in the proliferative phase was essentially negative except for two cases which showed very faint staining along the apical surface of a few cells (Table 2 and Fig. 4a). These specimens were evaluated as negative according to the criteria set-out above. The positive rate for the secretory endometrium, however, was 71.4% (5/7) (Fig. 2d). DBA positive sites in the secretory endometrium were seen along the apical surface and in the supranuclear or entire cytoplasm of the glandular cells (Fig. 4b). In endometrial adenocarcinoma, DBA weakly stained only 9.5% (2/21) of the well-differentiated cases (Fig. 2d).

SBA

The positive rates for proliferative and secretory endometrium were 6.7% (1/15) and 57.1% (4/7), respectively (Fig. 2e). As for the positive sites, SBA weakly stained only the apical surface of some endometrial glands in the proliferative phase (Fig. 4c), while in the secretory phase the supranuclear or entire cytoplasm was positively stained in addition to the apical surface (Fig. 4d). In some adenocarcinoma cells, SBA rather strongly stained the apical surface, with a few cancer cells showing positive cytoplasmic staining as well. The positive rates for well-, moderately- and poorly-differentiated adenocarcinomas were 42.9% (9/21), 37.5% (6/16) and 25% (2/8), respectively (Fig. 2e).

UEA-1

UEA-1 faintly stained the apical surface of 20% (3/15) of the cases in the proliferative phase (Figs. 2f and 5a), while no positive reaction was seen in the secretory phase. In endometrial adenocarcinoma cells, UEA-1 strongly stained the entire or supranuclear cytoplasm as well as the apical surface (Fig. 5b). Staining was marked in well-differentiated adenocarcinomas, but was less conspicuous in poorly differentiated cases. The positive rates for well-, moderately- and poorly-differentiated adenocarcinomas were 95.2% (20/21), 68.8% (11/16) and 37.5% (3/8), respectively (Fig. 2f).

PNA

The positive rates for proliferative and secretory endometrium were 93.3% (14/15)

Fig. 3 Light microscopic observations of human endometrium stained with ConA (a, b), RCA (c) and WGA (d).

a) Normal endometrium in the proliferative phase. ConA diffusely stains the cytoplasm as well as the plasma membrane. $\times 170$. b) Well-differentiated adenocarcinoma. The diffuse cytoplasmic staining for ConA is also seen in all of the cancer cells. $\times 170$. c) Normal endometrium in the secretory phase. RCA strongly stains the apical surface and the cytoplasm. $\times 140$. d) Well-differentiated adenocarcinoma. WGA also stains the apical surface and the cytoplasm. $\times 170$.

Fig. 4 Light microscopic observations of normal endometrium stained with DBA (a, b) and SBA (c, d).

a) Proliferative phase. DBA very faintly stains the apical surface in a few cells. This case was evaluated as negative. $\times 170$. b) Secretory phase. The apical surface and the cytoplasm are strongly positive in almost all cells. $\times 140$. c) Proliferative phase. SBA stains the apical surface only. $\times 170$. d) Secretory phase. Both the apical surface and cytoplasm are strongly positive for SBA. $\times 140$.

Fig. 5 Light microscopic observations of normal and malignant endometrium stained with UEA-1 (a, b) and PNA (c, d, e, f). $\times 170$.

a) Proliferative phase. UEA-1 faintly stains the apical surface. b) Well-differentiated adenocarcinoma. The cytoplasm is intensely positive for UEA-1, as well as the apical surface. c) Proliferative phase. PNA stains only the apical surface. d) Well-differentiated adenocarcinoma. PNA stains the cytoplasm and basolateral plasma membrane in many cancer cells as well as the apical surface. e) Poorly-differentiated adenocarcinoma. PNA strongly stains the cytoplasm. f) Well-differentiated adenocarcinoma. An example of the cytochemical controls. Incubated with PNA in the presence of 0.1 M D-galactose. Positive reaction is completely inhibited (cf. Fig. 5d).

and 57.1% (4/7), respectively (Fig. 2g), with the binding sites being observed only along the apical surface (Fig. 5c). In some adenocarcinoma cells, the cytoplasm and the plasma membrane were positively stained in addition to the apical surface (Fig. 5d, e). Positive cytoplasmic staining was recognized in 10 of 21 well-, 6 of 16 moderately- and 6 of 8 poorly-differentiated adenocarcinomas. Regarding the differentiation grading of endometrial cancers, the positive rates for well-, moderately- and poorly-

Fig. 6 Electron microscopic observations of normal endometrial glandular cells stained with DBA. N: nucleus, G: the Golgi apparatus.

a) Proliferative phase. DBA stains the apical membrane (arrow) and the Golgi apparatus in a few cells of an endometrial gland. The lateral plasma membrane is also positive with very weak intensity. The cells positive and negative for apical staining are clearly distinguishable. $\times 3,800$. b) Secretory phase. The plasma membrane and the Golgi apparatus are strongly stained. Note that a few lamellae in the cis region (arrowheads) are devoid of reaction products. $\times 8,000$.

differentiated adenocarcinomas were 66.7% (14/21), 68.8% (11/16) and 75% (6/8), respectively (Fig. 2g).

Cytochemical controls

All positive reactions were inhibited when the specimens were incubated with lectins in the presence of appropriate hapten sugars (Fig. 5f). Neither non-specific binding of ABC reagent nor endogenous peroxidase activity was observed.

Electron microscopic observations

When HRP-labeled DBA was applied to normal endometrium in the proliferative phase, it stained the apical membrane and the Golgi apparatus in only a few cells of the endometrial glands, with the lateral plasma membrane also showing a very weak reaction, and those cells with positive apical staining were clearly distinguishable from those without it (Fig. 6a). In the secretory phase, DBA positive cells were markedly increased in number and almost all of the glandular cells were strongly stained, with binding sites being observed along the plasma membrane and in the Golgi apparatus, particularly, in the intermediate to trans lamellae of the Golgi stacks (Fig. 6b). No positive reaction to DBA staining was seen in the endoplasmic reticulum, nuclear envelope or mitochondria.

Endometrial adenocarcinoma was examined using UEA-1- and PNA-conjugate. Both lectins strongly stained the Golgi apparatus and the plasma membrane (Figs. 7 and 8). In particular, the reaction products of UEA-1 were observed significantly on the glycocalyx along the apical cell surface in the well-differentiated type (Fig. 7a); and in the poorly-differentiated type, the plasma membrane along the intercellular luminal surface was rather strongly stained with PNA (Fig. 8).

In addition to the Golgi apparatus and the plasma membrane, ConA stained the intracellular membranes, such as the endoplasmic reticulum and nuclear envelope in both normal endometrium and endometrial adenocarcinoma (Fig. 9).

In the control study, lectin binding was inhibited by the addition of the appropriate hapten sugars (Fig. 10).

Discussion

The finding of the present stainings were evaluated by the special format including both staining incidence and intensity. Recently, a number of histochemical studies²¹⁻²³ using various ligands have been performed, but definitive criteria for the evaluation of staining patterns have yet to be established. As the present staining profiles showed heterogeneity not only among different sections but also within the same section, especially in the malignant tissues and even in the normal ones, the specimens examined were classified according to a combination of both incidence and intensity of positive

Fig. 7 Electron microscopic observation of well-differentiated endometrial adenocarcinoma cells stained with UEA-1.

a) UEA-1 reaction is positive in the abundant glycocalyx, as well as along the apical plasma membrane. $\times 17,000$. b) UEA-1 strongly stains the stacked cisternae and vesicles of the Golgi apparatus in addition to the lateral plasma membrane (arrowheads). $\times 20,000$.

Fig. 8 Electron microscopic observation of poorly-differentiated endometrial adenocarcinoma cells stained with PNA. PNA intensely stains the Golgi apparatus and the plasma membrane, the intercellular luminal surface (arrows) being rather strongly positive. $\times 13,000$.

Fig. 9 Electron microscopic observation of poorly differentiated adenocarcinoma cells stained with ConA. ConA stains the Golgi apparatus, endoplasmic reticulum, nuclear envelope and plasma membrane (arrowheads). $\times 16,000$.

Fig. 10 An example of the cytochemical controls at the electron microscopic level. Secretory endometrial cells stained with DBA in the presence of 0.2 M N-acetyl-D-galactosamine. The reaction is inhibited even in the apical membrane. (cf. Fig. 6b) $\times 3,500$.

cells. In addition, only specimens with less than a 10% incidence and weak intensity were considered negative in order to ensure data accuracy. Based on these criteria, the present study clearly showed that the staining patterns of DBA and SBA changed during the menstrual cycle, and those of UEA-1 and PNA were different between normal endometrium and endometrial adenocarcinoma.

As for DBA and SBA, which specifically bind to N-acetyl-D-galactosamine,¹ both lectins more strongly and more frequently stained the endometrium in the secretory phase than in the proliferative phase. The increased binding of DBA was also confirmed by electron microscopy. These results indicate that DBA- and SBA-specific glycoconjugates vary with the hormonal state, and suggest that endometrial glycoconjugates may be related to the interaction between blastocysts and endometrial cells in the process of implantation, since complex carbohydrates on the cell surface are implicated as mediators of cell adhesion⁵ or cell recognition.⁶ Using acetone fixed cryostat sections and the fluorescein isothiocyanate labeling method, Lee and Damjanov⁸ reported that SBA showed increased reactivity with the human pregnant endometrium compared with that in the proliferative phase. Their results support our findings, but it should be kept in mind that implanting blastocysts modify the sugar components in rabbit endometrial epithelium,¹⁰ and further, that the fixative and staining procedures different between their study and ours.²⁴

When UEA-1 was used for staining, the positive rate for endometrial adenocarcinoma was higher than that for normal endometrium. Only the apical surface of the proliferative endometrium was faintly stained, while not only apical and but also cytoplasmic staining was seen in endometrial adenocarcinoma, with staining intensity and incidence being markedly enhanced. Enhanced UEA-1-reactions associated with malignancy were demonstrated in colon adenocarcinoma¹¹ and esophageal squamous cell carcinoma.¹³ The present result suggests that UEA-1-reactive glycoconjugates also accumulate in the endometrial adenocarcinoma cells.

UEA-1 had been believed to recognize H(O)-antigen, i.e., a blood type substance with a structure containing a fucose linked α 1, 2 to a galactose as terminal residues.²⁵ A recent immunohistochemical investigation using anti-H(O) monoclonal antibody suggested that oligosaccharides with H(O) blood type activity increased in endometrial adenocarcinoma,²³ showing a similar tendency to the result obtained from the present UEA-1 staining. It is unlikely, however, that all of the binding sites of UEA-1 are the same as the site recognized by the monoclonal antibody, since UEA-1 is thought to bind to various oligosaccharide structures bearing terminal fucose residues, in addition to the H(O) structure.²⁶

PNA binding was restricted to the apical surface in normal endometrium, while not only the apical surface but also the cytoplasm was positively stained in half of the malignant cases (22/45 cases). In particular, this proportion was relatively high in the

poorly-differentiated type (6/8 cases). As a positive cytoplasmic reaction has been reported also in other malignant tissues such as colon¹² and breast cancer,¹⁴ cancer cells are perhaps characterized by the cytoplasmic stainability of PNA. Furthermore, positive reactions along the lateral or basal plasma membrane of some cancer cells might reflect a deviation in cell-polarity from normal, since changes in polarity can be detected by differences in lectin binding sites.²⁷

There are some reports that other lectins such as ConA,¹⁵ RCA⁹ or DBA¹⁶ are appropriate for the investigation of tumor characteristics in various organs. As far as uterine endometrium is concerned, UEA-1 and PNA may be regarded as indicators of malignant transformation, i.e., as morphological phenotypic tumor markers. UEA-1, therefore, is expected to be particularly useful as a marker for the highly-differentiated endometrial adenocarcinoma, and PNA for the poorly-differentiated type.

At the electron microscopic level, DBA, UEA-1 and PNA, which are specific for N-acetyl-D-galactosamine, L-fucose and D-galactose, respectively,¹ stained the Golgi apparatus. As intracellular membranes such as the Golgi apparatus and endoplasmic reticulum are reported to be sites of the biosynthesis and processing of glycoproteins,²⁸⁻³⁰ and as the subcellular locations of lectin binding are believed to correspond to these processing sites,^{4,16} the present electron microscopic findings may reflect that sugar residues such as N-acetyl-D-galactosamine, L-fucose and D-galactose are incorporated into the growing oligosaccharide chains on the Golgi membranes. The further detailed observation revealed that DBA bound to the intermediate to trans lamellae of the Golgi stack in the secretory endometrial cells, and that the reaction products of DBA showed a distinct polarity in its localization within the Golgi stack. Furthermore, ConA, specific for D-mannose,¹ binds to the endoplasmic reticulum and the Golgi apparatus. These findings most probably reflect that D-mannose is incorporated in the endoplasmic reticulum during the first step of glycosylation and the mannose containing glycoproteins are then processed in the Golgi apparatus.^{28,29} It seems unlikely that the sites of saccharide processing in the endometrial glandular cells, and even in the adenocarcinoma cells differ from those in various other kinds of cells, as the same binding patterns at the electron microscopic level are obtained in other tissues.^{3,4,13,16}

It is revealed biochemically that saccharide synthesis and processing are catalyzed by a series of glycosyltransferases,²⁹ and that these enzymes change during the oestrous cycle³¹ and during the course of malignant transformation.³² Also in the human endometrium, therefore, such changes are not surprising. Whether or not the change in the lectin binding is correlated with a change in the glycosyltransferase activities requires future study for an answer.

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