RESEARCH ARTICLE

Value of serum and bronchoalveolar fluid lavage pro- and anti-inflammatory cytokine levels for predicting bronchopulmonary dysplasia in premature infants

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ABSTRACT. ***Objective:*** **The aim of this study was to determine the value of pro- and anti-inflammatory cytokine levels in both blood and tracheal aspirate (TA) samples that were obtained within 24 h after birth for predicting bronchopulmonary dysplasia (BPD) development in premature infants.** ***Material and methods:*** **Premature infants, who were born before 32 weeks of gestation, weighing less than 1,500 g, and admitted with respiratory distress between September 2009 and December 2010, were enrolled. Tracheal aspirate samples and serum were obtained from all infants on the first day of admittance for evaluation of pro- and anti-inflammatory cytokine levels using ELISA.** ***Results:*** **The study included 102 premature babies of whom 31 (30%) had BPD diagnosed in the follow-up. Mild, moderate and severe BPD was diagnosed in 10 (32%), 14 (45%) and seven (23%) infants, respectively. Both serum and TA sample pro-inflammatory cytokine (TNF-α, IL-1β, IL-6) levels were significantly higher, and anti-inflammatory cytokine (IL-10) levels were significantly lower in infants who developed BPD compared with those who had no BPD. No significant differences were detected in either serum or TA sample pro- and anti-inflammatory cytokine concentrations in preterm infants with BPD in terms of BPD severity. Cut-off values of both serum and TA sample pro- and anti-inflammatory cytokine concentrations for predicting BPD were also determined.** ***Conclusion:*** **It is suggested that higher serum and TA pro-inflammatory cytokine (TNF-α, IL-1β, IL-6) concentrations, along with lower anti-inflammatory cytokine (IL-10) concentrations, might be used for predicting the development of BPD in premature infants with respiratory distress at birth.**

Key words: bronchopulmonary dysplasia, prematurity, pro-inflammatory cytokine, anti-inflammatory cytokine

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that can develop particularly in premature infants and with significant morbidity and mortality [1]. BPD often develops in premature infants who have had little or no respiratory support and low inspired oxygen concentrations during the early postnatal days [2]. BPD affects approximately 10% and 40% of very-low-birth-weight (VLBW) and extremely-low-birth-weight (ELBW) infants, respectively [3]. The highest BPD rates have been reported in infants born at the lowest birth weights and most immature gestational ages [4].

The pathogenesis of BPD is multifactorial and both pre-and postnatal factors including preterm birth, prenatal infection and/inflammation, mechanical ventilation, oxygen toxicity with decreased anti-oxidant mechanisms, patent ductus arteriosus and postnatal infection all contribute to the development of BPD [2, 5].

After initiation of ventilation, there is a neutrophil influx into the airways that is associated with a decrease in the number of circulating neutrophils that was associated with pulmonary edema formation and increased risk of BPD [6, 7]. Several pro-inflammatory cytokines are synthesized by alveolar macrophages, fibroblasts, type II pneumocytes, and endothelial cells upon stimulation by hyperoxia, hypoxia, endotoxins and other bacterial products, and biophysical factors [8]. The increased pro-inflammatory cytokine levels in the airways and pulmonary tissue of preterm infants may be associated with the inability to regulate inflammation through an adequate expression of anti-inflammatory cytokines [6, 9]. Therefore, an imbalance between pro- and anti-inflammatory cytokines has been suggested as an important risk factor for BPD [10]. The cytokines also mediate acute lung injury, exacerbate ventilator-associated lung injury and modulate host

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defenses [11-13]. Increased cytokine concentrations were found in the tracheal aspirate (TA) and serum of premature infants with respiratory distress syndrome (RDS), which are suggested to be predictive of the subsequent development of BPD [14-18].

The aim of this study was to determine the value of pro-[interleukin1-β (IL-1β), IL-6, and tumour necrosis factor-α (TNFα)] and anti-inflammatory (IL-10) cytokine levels in both blood and TA samples that were obtained within 24 h after birth for predicting subsequent BPD development in premature infants.

PATIENTS AND METHODS

This prospective study was performed in premature infants with respiratory distress who were less than 32 weeks of gestational age and who had been admitted to the Neonatal Intensive Care Unit (NICU) of the Pediatric Department of Uludag University School of Medicine, between September 2009 and December 2010. During the study period, a total of 122 preterm infants, born at less than 32 weeks of gestational age were hospitalized in the NICU and enrolled in the study. However, 20 of them were excluded according to the exclusion criteria. The exclusion criteria were death in the first 28 days of life, presence of maternal clinical and/or histological chorioamnionitis, presence of premature rupture of membranes (PROM), refusal of parental consent, lack of laboratory data, and major congenital abnormalities. The study protocol was approved by the Ethics Committee of Uludag University, School of Medicine. Informed parental consent was obtained for all infants.

Antenatal and postnatal risk factors for BPD development were recorded. A detailed history from mothers, including maternal age, gravidity, medications used in pregnancy, antenatal steroid use, and route of delivery, was obtained. The gestational history of the infants, including birth weight, gestational age, gender, Apgar scores, small for gestational age (SGA), need for resuscitation, presence of neonatal morbidities such as RDS, intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), patent ductus arteriosus (PDA), retinopathy of prematurity (ROP), was obtained and recorded. Duration of ventilation, duration of total supplemental oxygen, and duration of hospital stay of infants were also recorded.

BPD was defined according to the 2001 NICDH consensus statement as requirement for supplemental oxygen for 21 of the first 28 days of life, and was classified into three groups in terms of severity (mild, moderate, severe), depending on the duration and level of supplemental oxygen and mechanical ventilatory support at 36 weeks postmenstrual age [1]. RDS, IVH, NEC and ROP were diagnosed according to the defined criteria in the literature [19-22]. PDA was visualized by two-dimensional echocardiograms of the parasternal, short-axis view at the level of the aortic valve or higher, and then a color Doppler flow of the ductus was imaged. Gestational age was evaluated by maternal dates and confirmed by the modified Ballard examination [23]. The infants were intubated and they were initially followed-up during synchronized, intermittent, mechanical ventilation (SIMV) with an inspiratory time of 0.35-0.40 s, positive inspiratory pressure over 20 mmHg, positive end-expiratory pressure between 4-6 cm H_2O, and respiratory rate of 40/minute. If indicated, assist-control ventilation and high-frequency ventilation were also used. Infants were extubated to nasal, continuous positive airway pressure (CPAP) as soon as possible. The surfactant used in this study was beractant (Survanta; Abbott Laboratories, North Chicago, IL, USA), a modified bovine lung extract. During the study period, Vitamin A prophylaxis was not routinely given to infants.

An early blood sample from the umbilical cord was collected for cell count at birth via an indwelling umbilical catheter within the first 4 h of life; and also late samples at postnatal days 3, 7, 14 and 21 were obtained from all survivors still at our NICU. The sample consisted of 2 mL blood that was obtained at the same time as the withdrawal ordered by the attending physician for routine laboratory examinations. The blood was collected in a heparinized syringe and processed immediately. EDTA-plasma samples were separated by centrifugation and stored at -70°C until analysis.

TA samples were collected on day 1. This collection was made at least 2 h after the first dose of surfactant, during the period of six to 24 h after birth, usually between eight and 12 h. The infant was positioned in a supine posture with the head in the midline. Then, 1 mL of saline was instilled into the endotracheal tube, two to four manual breaths were provided by a self-inflating bag, and the airway was suctioned once or twice with a suction catheter. The catheter was rinsed with 1 mL of saline, and the aspirate was collected into sterile traps. The infant's heart rate, respiratory rate, and oxygen saturation were monitored and allowed to stabilize during the suctioning procedure. TA samples were kept on ice and processed within 1-2 h.

Concentrations of cytokines (IL-1β, IL-6, TNFα and IL-10) in both blood and TA samples were measured using ELİSA kits (Biosource, Belgium) according to the manufacturer's recommendations. The lowest detection levels for IL-1β, IL-6, TNFα and IL-10 were 0.35 pg/mL, 2 pg/mL, 0.7 pg/mL and 1.6 pg/mL, respectively. Standard curves and quantitative levels for these cytokines were determined.

The SPSS 16.0/Windows program was used for data analyses. Descriptive statistics were given as mean, standard deviation and percentage. The differences between groups were evaluated with chi-square tests for qualitative data and with the Mann-Whitney U and t-tests for quantitative data. Values of $p<0.05$ were considered to be significant. Receiver operating characteristic (ROC) analyses were performed with the MedCalc version 9.3.9.0 statistical program. Values of $p<0.05$ were considered significant.

RESULTS

A total of 102 infants (48 male, 52 female) were included in the study. The mean gestational age of the infants was 28.6 ± 1.4 weeks (range; 24 to 32 weeks), and mean birth weight was 986 ± 461 g (range; 520 to 1,435 g). Surfactant treatment was given to 56 infants (55%) who were diagnosed with RDS. Thirty-one infants (30%) received a diagnosis of BPD during follow-up. *Table 1* shows the demographic features and frequency of neonatal morbidities within the study population.

Table 1
Demographic features of the study population and frequency of neonatal morbidities.

	Study population n=102
Gestational age (week) mean ± SD	28.6 ± 1.4
Birth weight (gram) mean ± SD	986 ± 461
Gender (male) n(%)	48 (47)
Small for gestational age n(%)	29 (28)
Multiple gestation n(%)	43 (42)
Cesarean delivery n (%)	76 (75)
Antenatal steroid treatment n (%)	52 (51)
Apgar minute[1] mean ± SD	4.8 ± 1.7
Apgar minute[5] mean ± SD	6.9 ± 1.5
Duration of ventilation (day) mean ± SD	16.5 ± 8.0
Duration of total oxygen treatment (day) mean ± SD	27.5 ± 15.1
Neonatal morbidities	
Respiratory distress syndrome n (%)	56 (55)
Bronchopulmonary dysplasia n (%)	31 (30)
Necrotizing enterocolitis n (%)	17 (17)
Intraventricular hemorrhage n (%)	17 (17)
Retinopathy of prematurity n (%)	18 (18)
Patent ductus arteriosus n (%)	15 (15)
Neonatal sepsis n (%)	26 (25)

Of the 31 infants with BPD, 16 (52%) were male. Mild, moderate and severe BPD was diagnosed in 10 (32%), 14 (45%) and 7 (23%) infants, respectively. The mean gestational age and birth weight values for infants with BPD were 25.7 ± 1.7 weeks and 815 ± 111 g, respectively. The infants with BPD had significantly lower gestational age and birth weight compared with those without BPD. The duration of mechanical ventilation and total oxygen treatment were significantly higher in infants with BPD. Also, the incidence of neonatal morbidities including RDS, and PDA, were significantly higher in infants with BPD compared with those without BPD. A five-day, postnatal steroid treatment was given to infants with moderate and severe BPD. *Table 2* shows the comparison of infants with and without BPD in terms of demographic features and other neonatal morbidities.

Mean umbilical cord pro-inflammatory cytokine (TNF-α, IL-1β, IL-6) levels were significantly higher, and anti-inflammatory cytokine (IL-10) levels were significantly lower in the BPD group compared with those who had no BPD. *Table 3* shows the comparison of mean pro- and anti-inflammatory cytokine concentrations in infants with and without BPD. Similarly, mean TA sample pro-inflammatory cytokine concentrations that were obtained within 24 h after birth were significantly higher, and anti-inflammatory cytokine concentrations were significantly lower in infants who developed BPD compared with those who did not develop BPD (*table 4*). No significant differences were detected in either serum or TA sample pro- and anti-inflammatory cytokine concentrations of preterm infants with BPD in terms of BPD severity (*table 5*).

The cut-off values of both serum and TA sample pro- and anti-inflammatory cytokine concentrations for predicting BPD were determined by ROC analysis and the most significant values were obtained for TA IL-6 and TNF-α concentrations (*table 6*).

Table 2
Comparison of infants with and without bronchopulmonary dysplasia in terms of demographic features and neonatal morbidities

Characteristic features	**Infants with BPD n=31**	**Infants without BPD n=71**	**p value**
Male gender n (%)	16 (52)	33 (46)	0.65
Gestational age (week) mean ± SD	**25.7 ± 1.7**	**29.9 ± 1.3**	**0.02**
Birth weight (g) mean ± SD	**815 ± 111**	**1,094 ± 193**	**0.02**
Maternal preeclampsia n(%)	12 (38)	24 (33)	0.63
Antenatal steroid treatment n(%)	16 (52)	35 (49)	0.83
Cesarean delivery n (%)	25 (80)	49 (69)	0.22
Apgar score at minute 1 mean ± SD	4.4 ± 1.7	5.4 ± 1.9	0.11
Apgar score at minute 5 mean ± SD	6.1 ± 1.5	7.5 ± 1.2	0.06
Duration of ventilation (day) mean ± SD	**28.5 ± 11.5**	**5.5 ± 5.0**	**0.0001**
Duration of total oxygen treatment (day) mean ± SD	**45.3 ± 22.1**	**10.1 ± 6.5**	**0.0001**
Neonatal morbidities n(%)			
Respiratory distress syndrome (RDS) n (%)	**17 (54.8)**	**21 (29.5)**	**0.02**
Necrotizing enterocolitis (NEC) n (%)	6 (19)	9 (13)	0.2
Intraventricular hemorrhage (IVH) n (%)	6 (19)	7 (10)	0.18
Retinopathy of prematurity (ROP) n (%)	8 (26)	8 (11)	0.06
Patent ductus arteriosus (PDA) n (%)	**7 (22)**	**6 (8)**	**0.05**
Neonatal sepsis n(%)	9 (29)	17 (24)	0.5

Table 3
Mean umbilical cord cytokine levels in infants with and without BPD

Cytokines (serum)	Infants with BPD n=31	Infants without BPD n=71	p value
TNF- α mean ± SD (pg/mL)	600.7 ± 364.8	213.7 ± 174.6	<0.001
IL-1β mean ± SD (pg/mL)	1,769.7 ± 1,104.9	821.5 ± 270.7	0.007
İL-6 mean ± SD (pg/mL)	4,534.4 ± 2,803.5	899.5 ± 509.2	<0.001
İL-10 mean ± SD (pg/mL)	4.4 ± 4.0	52.8 ± 42.9	<0.001

TNF-α: tumour necrosis factor-alpha, IL-1β: interleukin 1-beta, IL-6: interleukin 6, IL-10: interleukin 10

Table 4
Mean tracheal aspirate cytokine levels in infants with and without BPD

Cytokines (serum)	Infants with BPD n=31	Infants without BPD n=71	p value
TNF- α mean ± SD (pg/mL)	422.4 ± 364.8	130.8 ± 82.6	<0.001
IL-1β mean ± SD (pg/mL)	339.9 ± 245.8	193.6 ± 153.9	0.002
İL-6 mean ± SD (pg/mL)	2,579.8 ± 2,524.5	274.9 ± 241.7	<0.001
İL-10 mean ± SD (pg/mL)	3.3 ± 2.6	32.7 ± 26.6	0.001

TNF-α: tumour necrosis factor-alpha, IL-1β: interleukin 1-beta, IL-6: interleukin 6, IL-10: interleukin 10

Table 5
Mean serum and tracheal aspirate cytokine levels in infants with BPD in terms of BPD severity.

Serum umbilical cord cytokine levels	Severity of BPD	n	Mean ± SD (pg/mL)	p value
TNF- α	Mild	10	612.2 ± 240.1	0.47
	Moderate	14	641.1 ± 463.5	
	Severe	7	694.3 ± 333.6	
IL-1β	Mild	10	2,957.0 ± 4539.3	0.75
	Moderate	14	2,673.3 ± 4880.3	
	Severe	7	2,718.4 ± 2239.0	
İL-6	Mild	10	4,255.9 ± 2848.4	0.79
	Moderate	14	5,272.2 ± 5121.5	
	Severe	7	4,940.9 ± 3557.4	
İL-10	Mild	10	1.4 ± 1.3	0.38
	Moderate	14	3.8 ± 3.1	
	Severe	7	2.2 ± 3.7	
Tracheal aspirate cytokine levels				
TNF- α	Mild	10	407.2 ± 172.1	0.08
	Moderate	14	314.1 ± 185.7	
	Severe	7	546.0 ± 384.3	
IL-1β	Mild	10	327.5 ± 128.7	0.82
	Moderate	14	286.7 ± 146.2	
	Severe	7	405.6 ± 305.5	
İL-6	Mild	10	1,815.8 ± 1211.5	0.40
	Moderate	14	2,248.7 ± 1529.2	
	Severe	7	3,675.12 ± 2734.6	
İL-10	Mild	10	2.7 ± 1.3	0.22
	Moderate	14	4.7 ± 3.0	
	Severe	7	2.6 ± 2.2	

TNF-α: tumour necrosis factor-alpha, IL-1β: interleukin 1-beta, IL-6: interleukin 6, IL-10: interleukin 10

DISCUSSION

This study showed that premature infants who developed BPD had significantly higher serum and TA sample pro-inflammatory cytokine concentrations with significantly lower anti-inflammatory cytokine concentrations that were obtained within 24 h of life. However, these concentrations were not associated with the severity of BPD. Cut-off values of both serum and TA sample pro- and anti-inflammatory cytokine concentrations for predicting BPD were also determined.

Table 6
Cut-off levels of pro- and anti-inflammatory cytokine concentrations in both serum and TA samples for predicting BPD development.

Cytokine	Cut-off levels (pg/mL)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC	p value
TNF-α (Serum)	≥108.5	60	100	100	62.5	0.82	0.0001
IL-1β (Serum)	≥109.9	50	100	100	57.1	0.69	0.001
IL-6 (Serum)	≥898.6	60	100	100	62.5	0.74	0.0013
TNF-α (TA sample)	≥65	73.3	100	100	78.9	0.86	0.0001
IL-1β (TA sample)	≥218.6	93.3	66.6	73.7	90.9	0.82	0.0001
IL-6 (TA sample)	≥339.5	73.3	93.3	91.7	77.8	0.90	0.0001

TA: tracheal aspirate, PPV: positive predictive value, NPV: negative predictive value, AUC: area under curve

The highest BPD rates have been reported among infants born at the lowest birth weights and most immature gestational ages [4, 24]. Our finding was also in accordance with these data as the majority of our cases who developed BPD had significantly lower birth weight and gestational age.

Oxygen toxicity is associated with both hydroxyl radicals and peroxidation of membrane lipids, so it is an important, contributing factor for the development of BPD in premature infants [25]. Our data, showing significantly longer durations of ventilatory therapy and higher total oxygen treatment in infants with BPD were also in agreement with the literature.

Cytokines are the principal regulators of cell interactions and drive their functions. Inflammatory cytokines such as IL-2, IL-6, IL-8 and TNF-α induce the inflammatory processes, recruiting and stimulating the inflammatory cells. Anti-inflammatory cytokines such as IL-4, IL-10, TGF-β1 have anti-inflammatory and profibrotic actions [26]. Cytokines also participate in lung development, mediate acute lung injury, and exacerbate ventilator-associated lung injury [11, 12]. Several pro-inflammatory cytokines are synthesized by alveolar macrophages, fibroblasts, type II pneumocytes, and endothelial cells upon stimulation by hyperoxia, hypoxia, endotoxins and other bacterial products, and biophysical factors [8]. The increased pro-inflammatory cytokine levels in the airways and pulmonary tissue of preterm infants might be associated with the inability to regulate inflammation through an adequate expression of anti-inflammatory cytokines [6, 9]. Therefore, available data suggest that the imbalance between pro-inflammatory and anti-inflammatory cytokine levels could serve as an important risk factor for BPD and lung injury [10].

Pro-inflammatory cytokines bind to specific cell-surface receptors and act as mediators in the immune response [27]. Several studies have shown increased protein levels and high mRNA expression of pro-inflammatory cytokines including IL-1, IL-6, IL-8 and TNF-α in airway secretions and bronchoalveolar cells of premature infants who developed BPD [14-18].

IL-1 is a pro-inflammatory cytokine that activates lymphocytes and increases the expression of adhesion molecules [27]. In a mouse model of BPD, perinatal expression of IL-1β in epithelial cells of the lung caused a lung disease closely resembling BPD by disturbing capillary development, inhibiting vascular endothelial growth factor production in the lungs, inducing several chemokines and inflammatory proteins, and also causing goblet cell hyperplasia and bronchial smooth muscle hyperplasia [28]. Rindfleisch *et al*. [29] also suggested that a relative imbalance between IL-1β and IL-1 receptor antagonist in lung lavages might contribute to prolonged inflammation in BPD. Kotecha *et al*. [14] reported significantly higher IL-1β concentrations in bronchoalveolar lavage fluid of infants with BPD. In two studies by the same group, increased levels of IL-1β were detected in TA samples of premature infants who later developed BPD [9, 30]. In a recent study, Ambalavanan *et al*. [31] studied 25 cytokines in blood samples that were obtained within 4 h of life, and reported increased IL-1β concentrations in infants who developed BPD. In another study, IL-1 in lung contents was reported to be increased after exposure to oxygen, and treatment with IL-1 receptor antagonist showed a lung-protective effect [32]. Our findings showing increased IL-1 levels in both cord blood and TA samples from premature infants who later developed BPD, are in good agreement with the literature.

IL-6 also increases inflammatory cytokine production [27]. Similar to IL-1, several studies reported increased IL-6 concentrations in TA samples from infants who subsequently developed BPD [14, 30, 33, 34]. Also, in a recent study, increased serum IL-6 levels within 4 h of life were shown in infants who later had BPD [31]. Therefore, our data including significantly higher serum and TA sample IL-6 concentrations immediately after birth in infants who later developed BPD, are in agreement with those found in the literature.

TNF-α is a pro-inflammatory cytokine that appears during the early phases of the inflammatory response. It enhances leukocyte endothelial activation and activates macrophages [27]. Also, it was suggested that activation of nuclear factor kappa B, by IL-1β and TNF-α activation, disrupted the normal expression of fibroblast growth factor-10 and inhibited lung morphogenesis [35]. Similar to other pro-inflammatory cytokines, TNF-α was found to be increased in TA samples from infants who subsequently developed BPD [30, 36-38]. To our knowledge, only one study did not find an association between serum TNF-α levels and BPD development [31]. However, in our study, concentrations of TNF-α in both blood and TA samples from premature infants who subsequently developed BPD, was significantly higher than those who did not develop BPD.

An imbalance between pro- and anti-inflammatory cytokine levels could serve as an important risk factor for the development of BPD [10]. The increased pro-inflammatory cytokine levels in the airways and lung tissue

of preterm infants with BPD might reflect an inability to regulate inflammation by adequate expression of anti-inflammatory cytokines [6]. IL-10 was shown to protect lung by inhibiting pro-inflammatory cytokine production and upregulating IL-1 antagonist [39]. Although IL-10 was found in the airway secretions of term infants, it could not be detected in secretions of preterm infants and this was explained by the relative inability of lung macrophages in preterm infants to produce IL-10 [40]. However, there are conflicting data about early and late IL-10 concentrations in preterm infants who subsequently developed BPD. Although some studies reported higher IL-10 levels in both blood and TA samples that were obtained on the first day of life [18, 31], some reported lower levels on the first day of life [9, 41, 42]. In the present study, significantly lower IL-10 levels were found in both serum and TA sample of premature infants who subsequently developed BPD. Our findings were also in agreement with the data of Mahieu *et al*. [36] who reported lower IL-12 levels as an anti-inflammatory cytokine in TA samples that were obtained within 2 h of birth in preterm infants. Therefore, all these data suggest that serial measurement of IL-10 concentrations, in a large sample of preterm infants after birth, are warranted in order to elucidate the association between IL-10 concentrations and the development of BPD.

Some studies detected cut-off levels of several cytokines in both blood and TA samples for predicting BPD in premature infants [18, 43]. In our study, cut-off levels of the cytokines in blood and TA samples for predicting BPD were determined. The most significant values were determined for TA sample IL-6 and TNF-α levels. Therefore, we suggest that these cut-off values may be used to predict infants who may subsequently develop BPD.

In conclusion, pro- and anti-inflammatory cytokine concentrations in both umbilical cord and TA samples that were obtained on the first day of life in premature infants could be used for predicting the development of BPD. For the same purpose, cut-off values of these cytokine concentrations were also determined. However, increased pro- and decreased anti-inflammatory cytokine concentrations were not associated with the severity of BPD. Future studies including larger number of infants and a greater number of cytokines with serial measurements in both blood and TA samples in premature infants are required to define the exact association between cytokine concentrations and BPD.

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