Fluticasone Propionate Inhibits Lipopolysaccharide-Induced Proinflammatory Response in Human Cystic Fibrosis Airway Grafts

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ABSTRACT

Airway inflammation, one of the major factors leading to lung damage in cystic fibrosis (CF) patients, is associated with an abnormal increase in proinflammatory cytokines. In this work, we demonstrate the increased release of the proinflammatory cytokines after lipopolysaccharide (LPS) stimulation: human interleukin (hIL)-8 in CF and non-CF airway xenografts, and hIL-6 and human growth-related oncogene-α (hGRO-α), which could be only analyzed in non-CF xenografts. Under basal conditions, we observed that hIL-8 was higher in CF xenografts compared with non-CF. We also report the anti-inflammatory effect of a glucocorticoid, fluticasone propionate (FP), on CF airway epithelium using a humanized model of airway inflammation developed in nude mice. In CF and non-CF tracheal xenografts, airway inflammation was induced by inoculating Pseudomonas aerugino sa LPS (4 h; 1 µg/ml) in the lumen of the xenografts. FP pretreatment (2 h; 10^{-8} M) followed by P. aerugino sa LPS stimulation induced a significant reduction of LPS-induced hIL-8 release in airway liquid collected from CF and non-CF tracheal xenografts (85 and 80%, respectively). In non-CF tracheal xenografts, FP treatment before LPS stimulation induced a significant decrease in hIL-6 and hGRO-α. From these data, we suggest that FP exerts anti-inflammatory properties that may be appropriate to CF therapy, at an early stage of the disease. In addition, these results demonstrate that the humanized airway model of inflammation provides a relevant tool for analyzing the effects of anti-inflammatory drugs in different diseases in which airway inflammation is implicated.

Cystic fibrosis (CF) is a lethal human autosomal recessive genetic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene (Riordan et al., 1989; Rommens et al., 1989). CF is characterized by pancreatic insufficiency and chronic disease of the respiratory tract, which is manifested by airway obstruction and recurrent infections of the lung that begin early in life (Khan et al., 1995). CF lungs are often infected by Pseudomonas aeruginosa, which plays a major role in the development and progression of pulmonary disease (Konstan et al., 1993). Inflammation is a critical component of the disease in CF. The inflammatory response is characterized by a marked influx of neutrophils into the lung and an increase in inflammatory mediators such as tumor necrosis factor-α, interleukin (IL)-1β, IL-6, and IL-8 (Berger et al., 1991; Konstan et al., 1993; Wilmott et al., 1999, 2001). Clinical studies on nasal and bronchoalveolar lavages have shown high neutrophil counts and increased level of a major chemoattractant, IL-8 (Balough et al., 1995; Khan et al., 1995; Noah et al., 1997), even in the absence of detectable infection. In in vitro and ex vivo studies, we have shown an up-regulation of IL-8 in human CF bronchial glandular cells, which could be due to a dysregulation of nuclear factor-κB (NF-κB) transcription factor (Tabary et al., 1998, 2000).

Whether inflammation is directly related to CFTR and is present very early in CF patients, before any infection, is still the subject of some debate. According to Armstrong et al. (1997), there would be no basic defect that initiates inflam-

ABBREVIATIONS: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; IL, interleukin; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide; FP, fluticasone propionate; DMEM, Dulbecco’s modified Eagle’s medium; AL, airway liquid; hIL, human interleukin; hGRO-α, human growth related oncogene-α.
mation in the absence of any initial airway insult, although they hypothesize that a constitutive abnormality of cytokine regulation within the CF lung could amplify and perpetuate the inflammatory process. The current lack of knowledge on the very early inflammation in CF infants and on the beneficial effects of anti-inflammatory drugs is related, in part, not only to the difficulty of investigating large populations of CF infants but also to the absence of relevant animal models to study the condition. In cfr<sup>m1HGU</sup>/ley t<sup>m1HGU</sup> transgenic mice raised under germ-free conditions, we have previously shown an increased number of inflammatory cells that did not infiltrate the airway surface epithelium but that were more numerous in the tracheal lamina propria of CF mice (Zahm et al., 1997). We have also observed in human fetal tracheae grafts in severe combined immunodeficient mice that mature CF airways are in a proinflammatory state before exposure to an infectious stimulus (Tirouvanziam et al., 2000). After <i>P. aeruginosa</i> infection, we observed a marked leukocyte transepithelial migration similar to that observed in adult CF airway tissue.

One limitation of the model of mature fetal airway xenografts is related to the difficulty involved in procuring CF human fetal tissue. Moreover, the grafted trachea is a closed pouch that is therefore different from the in vivo situation, and cannot be easily used to analyze the effect of anti-inflammatory drugs. In contrast, the open trachea model in nude mice that we recently developed using human adult epithelial cells (Dupuit et al., 2000) regenerates a well differentiated airway epithelium allowing, in the present study, the collection of tracheal liquid 1) under basal conditions; 2) after <i>P. aeruginosa</i> infection, we observed a marked leukocyte transepithelial migration similar to that observed in adult CF airway tissue.

Materials and Methods

**Drugs and Bacterial Stimulus.** FP was generously provided by GlaxoSmithKline (Uxbridge, Middlesex, UK). A stock solution of FP (10<sup>-3</sup> M) in 99.5% ethanol was prepared. FP was diluted in serum-free DMEM/F-12 medium (Invitrogen, Paisley, UK) to a 10<sup>-8</sup> M working stock solution. The stock solutions were further diluted in DMEM/F-12 medium to achieve the 10<sup>-6</sup> M concentration used. <i>P. aeruginosa</i> LPS (Calbiochem, San Diego, CA) was dissolved in serum-free DMEM/F-12 medium to a final working solution containing 1 µg/ml of <i>P. aeruginosa</i> LPS.

**Human Airway Tissues and Epithelial Cell Dissociation.** Samples from 12 subjects were included in the study. Human airway tissues were obtained from nasal polypectomy of six CF patients (1 ΔF508/G55E, 1 ΔF508/Q1042X, 1 ΔF508/2372 del 8, 1 ΔF508/2894 ins AG, and 2 ΔF508/ΔF508, aged 6–10 years). Control tissues were collected from bronchial cancers resections of six non-CF patients (aged 58–74 years). Immediately after nasal polypectomy or airway surgical resection, the tissues were transferred to the laboratory in Hank's HEPES salt (Invitrogen) supplemented with antibiotics (200 U/ml penicillin and 200 µg/ml streptomycin). The solution also contained colimycin (3 × 10<sup>3</sup> IU/ml) for all CF tissues. Collected tissues were then dissociated by 0.1% pronase (Sigma-Aldrich, St. Louis, MO) digestion for 12 h, and the dissociated human airway epithelial cells were resuspended at a density of 1 to 2 × 10<sup>6</sup> cells/ml in an hormonally defined culture medium (RPMI 1640; Invitrogen) supplemented with 1 µg/ml insulin, 1 µg/ml transferrin, 10 ng/ml vitamin A, 200 U/ml penicillin, 200 µg/ml streptomycin, 50 µg/ml gentamicin, and 2.5 µg/ml amphotericin. The dissociated human airway epithelial cells were then inoculated into xenograft lumens (see below).

**Experimental Model of Tracheal Grafts in Nude Mice.** Humanized xenografts were prepared as described previously (Dupuit et al., 2000). Briefly, tracheae of adult Wistar rats (weighing 220–250 g) (Charles River France, Saint-Aubin-Lès-Elbeuf, France) were frozen at −80°C and thawed (three cycles) to remove the rat surface epithelium. The rat tracheae were tied at their ends to sterile polyethylene tubing and stored at −80°C until instillation with dissociated human airway cells. After instillation, the rat tracheae were implanted subcutaneously, two per mouse, into the flanks of anesthetized (i.p. injection of 40 mg/kg pentobarbital sodium) nude mice. The mice were maintained under pathogen-free conditions and the tracheal xenografts flushed twice per week to remove cell debris from the lumen. Tracheae were maintained for 5 weeks in the nude mice to obtain a well differentiated human airway epithelium (Fig. 1). To develop an inflammatory state, <i>P. aeruginosa</i> LPS (1 µg/ml) was inoculated for a 4-h period. The <i>P. aeruginosa</i> LPS and FP solutions were directly inoculated into the lumen of the xenografts.

**Induction of <i>P. aeruginosa</i> LPS Inflammation.** Inclusions were performed with a standardized volume of solution (70 µl) which covered the entire surface of the trachea and thus ensured that all epithelial cells were in contact with inoculating solutions. Seventy microliters of serum-free DMEM/F-12 medium (4 h) or LPS (1 µg/ml; 4 h), with or without preincubation for 2 h in 10<sup>−8</sup> M FP, was instilled into the lumen of the xenografts. After incubation with the different media (2 h with FP and 4 h with LPS), airway liquids were collected and the level of secretion of proinflammatory cytokines was measured.

**Staining and Histology of Tracheal Xenografts.** After incubation with different media as described above, tracheae were removed and embedded in optimum cutting temperature compound and stored at −80°C. Transverse cryosections (5 μm in thickness) of tracheal xenografts were prepared on a microtome (model 2800 Frigocut; Reichert-Jung, NuBlock, Germany), transferred onto gelatin-coated slides, air-dried, and stored at −20°C. These cryosections were stained with hematoxylin-eosin and used for histological analysis.

**Collection of Airway Liquid (AL) from Tracheal Xenografts Lumens.** The developing airway epithelium in tracheae was maintained in constant contact with the different solutions described above. After incubation with each of the solutions, the volume of the recovered AL was measured and then frozen at −80°C for further cytokine analysis.

**Quantification of Cytokines by Enzyme Immunoassay.** Human interleukin (hIL)-8, hIL-6, and growth related oncogene-α (hGRO-α) levels were quantified in the liquids recovered from tracheal lumens using an enzyme-linked immunosorbent assay carried out according to the manufacturer’s instructions (Biosource International, Camarillo, CA). The limit of detection for each of these cytokines was 5, 2, and 2 pg/ml for hIL-8, hIL-6, and hGRO-α, respectively. To standardize these results, the total protein content in the AL was quantified using the Bradford (1976) method. Results are expressed as picograms per milliliter per milligram of total protein.
Human bronchial xenografts. Tracheae of male Wistar rats were frozen at −80°C and thawed three times to remove surface epithelial cells. Inner diameters of tracheae were approximately 2 mm, and their length was around 1.5 cm. Each rat trachea was tied aseptically at its distal end to polyethylene tubing, and dissociated human airway cells (1–2 × 10⁶ cells) were inoculated into the lumen of the trachea. Such montages were then implanted, two per mouse, into the flanks of recipient nude mice anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Xenografts were flushed twice per week to remove cellular debris. Fully differentiated human airway epithelium could be observed 5 weeks after implantation into the nude mice.

Statistical Analysis. Values are expressed as the mean ± S.D. for results from six animals for in vivo experiments and analyzed using nonparametric tests (Mann-Whitney and Wilcoxon tests). The level of statistically significant differences was defined as *P < 0.05 and **P < 0.01.

Results

Histology of CF and non-CF Airway Tissues. Before dissociating the CF and non-CF airway tissues, we characterized the percentage of surface mucous cells and the degree of inflammation in the surface epithelium and lamina propria by semiquantitative analysis performed on CF and non-CF paraffin-embedded tissue sections (Table 1). Compared with CF nasal polyps, the degree of mucous hyperplasia and inflammation observed in non-CF tissues did not seem to be different. The CF and non-CF tissues exhibited enlarged interstitium with edema, capillary congestion, and inflammatory cell infiltrates, reflecting an inflammatory state. We also observed a large variation in the percentage of surface airway mucous cells in both CF and non-CF tissues.

Histology of CF and non-CF Tracheal Xenografts before and after P. aeruginosa LPS-Induced Stimulation. The histological aspect of tracheal xenografts was analyzed 5 weeks after they had been implanted into nude mice, with microscopic images showing that denuded rat tracheae had become repopulated with human surface epithelial cells (Fig. 2A). We observed a pseudostratified columnar epithelium with secretory and ciliated differentiation (Fig. 2B) similar to that observed in human airway tissue. We did not observe any histological difference between the CF and the non-CF tracheal xenografts (Fig. 2, C and D). CF and non-CF tracheal xenografts were also analyzed after a 4-h incubation with P. aeruginosa LPS. In the presence of LPS (4 h; 1 μg/ml), no increase was observed in the number of mucous cells in stimulated CF and non-CF tracheal xenografts compared with unstimulated controls. Furthermore, compared with control, no efflux of host inflammatory cells through the airway epithelium was observed after a 4-h exposure of the xenografts to LPS (Fig. 2, E and F).

Collection of Human AL from non-CF and CF Tracheal Xenografts. At the beginning of the experiment, the tracheal lumen was inoculated with a standardized volume (70 μl) of solution that filled entirely the lumen and therefore covered the human airway epithelial cells developing on the surface of the rat trachea. After a 4-h incubation period, we collected the human AL from non-CF and CF xenografts and measured the volume obtained. Under basal conditions, we observed a significant (P < 0.01) difference in AL volume between CF and non-CF tracheal xenografts. The volume of AL in non-CF xenografts was 1.6-fold higher on average than that in CF xenografts (Fig. 3). After stimulation with LPS (4 h; 1 μg/ml), a decrease of AL volume in CF as well as in non-CF xenografts was observed but the difference was not significant. After pretreatment with FP (2 h; 10⁻⁸ M) fol-

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<td>Characterization of the airway tissues before dissociation and reimplantation in the tracheal xenografts</td>
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<td>CF genotypes are indicated. The percentage of mucous cells and the degree of inflammation (airway inflammatory cells, enlargement of interstitium, and capillary edema) are indicated.</td>
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<th>CF Airway Tissues</th>
<th>ΔF508/G85E</th>
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<td>Degree of inflammation</td>
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N.D., not determined.
allowed by *P. aeruginosa* LPS (4 h; 1 μg/ml) incubation, the mean of AL volume in non-CF and CF tracheal xenografts was similar to that collected in control xenografts.

**Cytokine Content of AL from CF and non-CF Grafts.** The lumen of fully differentiated CF and non-CF xenografts was first inoculated with a 70-μl volume of serum-free DMEM/F-12 medium. After a 4-h incubation, the human AL was collected and the level of secretion of proinflammatory cytokines measured. For this control condition, the hIL-8 secretion was significantly (*P < 0.05*) higher with a 6-fold increase in CF compared with non-CF tracheal xenografts (Fig. 4).

To mimic bacterial stimulation in CF patients and to analyze the response induced in the airway epithelium, we stimulated the human airway tracheal xenografts with *P. aeruginosa* LPS, a well-known endotoxin in CF pathology. Human non-CF and CF airway tracheal xenografts exposed to *P. aeruginosa* LPS showed approximately a 10-fold increase (*P < 0.05*) in hIL-8 secretion compared with tracheal xenografts only exposed to control culture medium (Fig. 5).

**Fig. 2.** Morphology of human airway surface epithelium regenerated in tracheal xenografts in nude mice. Light micrographs of cross sections stained by hematoxylin at 5 weeks after implantation in nude mice. The epithelium is characterized by ciliated, secretory basal cells and glandular structures (A) and is similar to a normal human pseudostratified airway surface epithelium (B). No histological difference could be observed between non-CF (C) and CF (D) epithelium in tracheal xenografts. After *P. aeruginosa* LPS stimulation, migration of inflammatory cells through the airway epithelium in non-CF and in CF tracheal xenografts could not be observed (E and F). Scale bars, 10 μm.
Using this humanized model of airway inflammation, we analyzed the effect of an anti-inflammatory compound, FP, on the secretion of hIL-8. Pretreatment of grafts with FP (2 h; 10^{-8} M) followed by stimulation with *P. aeruginosa* LPS (4 h; 1 μg/ml) resulted in a decrease of hIL-8 secretion in CF and non-CF tracheal xenografts, of 85 and 80% respectively, compared with tracheal xenografts exposed to *P. aeruginosa* LPS alone. The level of hIL-8 after pretreatment with FP was approximately the same as that recorded under control conditions as described above (Fig. 5).

Due to the small volume of AL recovered from tracheal CF xenografts, we could only measure hIL-8 secretion in the AL from these grafts, and not hIL-6 and hGRO-α as well, which was possible in non-CF tracheal xenografts. After *P. aeruginosa* LPS stimulation, the secretion of hIL-6 and hGRO-α in non-CF tracheal xenografts was significantly (*P < 0.01) increased (953.9 ± 91.5 and 10,068.7 ± 963.2 pg/ml/mg of protein, respectively) compared with that measured in non-CF tracheal xenografts exposed to control culture medium (277.5 ± 42.9 and 4,252.6 ± 483.2 pg/ml/mg of protein, respectively).

After FP pretreatment of non-CF xenografts, the levels of hIL-6 and hGRO-α secretion were significantly (*P < 0.01) reduced (445.3 ± 92.4 and 4125.7 ± 1463.1 pg/ml/mg of protein, respectively) compared with that measured after *P. aeruginosa* LPS stimulation alone.
Discussion

In the present study, we analyzed the in vivo effects of *P. aeruginosa* LPS-induced inflammatory stimulation on cytokine secretion by collecting luminal tracheal fluids from humanized airway xenografts before and after a 2-h pretreatment with FP. We first analyzed the control (basal) inflammatory state in airway epithelium from the humanized xenografts from both CF and non-CF. The xenografts had not previously been exposed to bacteria

Under basal condition, the results demonstrated that hIL-8 secretion is significantly higher in CF tracheal xenografts compared with non-CF tracheal xenografts. In contrast, the volume of AL collected was significantly lower in CF tracheal xenografts. After stimulation with *P. aeruginosa* LPS, we observed an increase in the level of hIL-8 secretion in CF and non-CF tracheal xenografts compared with xenografts exposed to control culture medium alone. FP was shown to decrease the level of LPS-induced hIL-8 secretion in CF and non-CF xenografts compared with xenografts exposed to LPS alone. These results are consistent with the increased level of basal IL-8 secretion identified in cultures of CF airway epithelial cells (Kammouni et al., 1997; Tabary et al., 1998, 1999; Bonfield et al., 1999) and in CF bronchoalveolar fluids (Balough et al., 1995; Khan et al., 1995; Noah et al., 1997). More recently, we used an in vivo model of human CF fetal trachea grafted into severe combined immunodeficient mice, and we demonstrated that the AL IL-8 content from CF grafts was increased compared with non-CF grafts (Tirouvanziam et al., 2000). The up-regulation of IL-8 in CF cells may be explained by a dysregulation of the complex NF-κB and its inhibitor factor IkBα (Tabary et al., 2000), occurring early in the development of CF airways before the onset of any infection.

The volume of AL collected from CF xenografts was lower than that measured in non-CF xenografts. This result is supported by the study of Zhang et al. (1996) where a higher rate of fluid absorption in CF has been also reported in in vivo human bronchial xenografts. These findings coupled with our results suggest a mechanism by which increased fluid absorption in CF airway epithelium leads to dehydration of mucus and impaired mucociliary clearance. They also support the recent results from Tarran et al. (2001a,b), describing a profound decrease in nasal airway surface liquid volume of CF mice.

These authors also observed an increase in the size and number of secretory cells in the tissues they examined. We did not observe such changes in the airway epithelium of CF xenografts, which seemed to be histologically unaffected compared with non-CF tracheal xenografts. Furthermore, despite the heterogeneity that we observed on the native tissue, no morphological differences in epithelium developed in tracheal xenografts were observed. The similar histological appearance between the CF and the non-CF airway xenografts is in agreement with the notion that significant disease is not seen in CF lung before infections and already reported in a previous article (Tirouvanziam et al., 2000). When CF tracheal xenografts were challenged with *P. aeruginosa* LPS, we observed neither migration of inflammatory cells, mucus hypersecretion, nor epithelial desquamation. In contrast to other studies, we inoculated only a low *P. aeruginosa* LPS concentration (1 μg/ml) in the xenograft lumen and for only a short period of time (4 h). The LPS concentration used in the present study was approximately equal to that reported in bronchoalveolar lavage in adult respiratory distress syndrome (1–1.5 pg/ml) (Martin et al., 1994). From the LPS concentrations reported in the literature for bronchoalveolar lavage, we could hypothesize that, for pathological situations, the LPS concentration approximates 1 μg/ml. In our study, contrary to mice models challenged with *P. aeruginosa* in agar beads (Wilmott et al., 2000) or human airway grafts infected with *P. aeruginosa* (Tirouvanziam et al., 2000), we did not observe a leukocyte transepithelial migration. Although Tirouvanziam et al. (2000) reported a proinflammatory state (increased IL-8) without any histological difference in CF and non-CF grafts, they have shown that a recruitment of inflammatory cells and epithelial cell detachment were observed when the CF grafts were exposed to *P. aeruginosa* (not seen in non-CF grafts). We hypothesize that we did not observe an inflammatory cell transmigration in CF grafts after LPS stimulation because the soluble virulence factors like LPS are not able to induce leukocyte transepithelial migration and/or epithelial exfoliation as do alive *P. aeruginosa* bacteria. The short incubation period (4 h) and relatively low concentration of *P. aeruginosa* LPS used in our study may likely explain the absence of leukocyte migration despite the induction of proinflammatory mediators. Our experimental conditions probably mimic a situation of early and mild airway inflammation.

For the first time, a model of mild airway inflammation in well differentiated human airway epithelium developed in nude mice is described. This model allowed us to analyze the effects of an anti-inflammatory molecule, fluticasone propionate, on proinflammatory cytokine release. After FP treatment, we demonstrated a decrease of hIL-8 secretion induced by *P. aeruginosa* LPS in the AL collected from CF and non-CF grafts. In fact, airway inflammation through proinflammatory cytokines is a central feature of the pathophysiology of CF patients and represents a major challenge to the treatment of CF patients. Glucocorticoids are well known as very potent anti-inflammatory drugs. FP is one of the most efficient molecules able to reduce the inflammatory response and has been shown to attenuate inflammation in several respiratory inflammatory diseases such as asthma, nasal polyposis, rhinitis, and acute sinusitis (Scadding, 2000). FP possesses highly lipophilic properties that allow the drug to attain high pulmonary concentrations. Moreover, the high affinity of FP with the glucocorticoid receptor permits FP to have a prolonged anti-inflammatory effect. In vitro studies have shown that FP is very efficient in inhibiting the release of proinflammatory mediators from human bronchial epithelial cells (Wang et al., 1997; Ek et al., 1999). FP has also been reported to inhibit more potently granulocyte macrophage colony-stimulating factor from nasal polyp epithelial cells than other glucocorticoids such as budesonide, beclomethasone dipropionate, and triamcinolone acetonide (Mullol et al., 2000).

Many investigations have been made into the molecular and cellular actions of steroids. The anti-inflammatory action of corticosteroids is shown to be mediated by the inhibition of activation of transcription factors such as activator protein-1 and NF-κB (Adcock and Caramori, 2001), which are implicated in the transcription of inflammatory genes. We have recent in vitro data that demonstrate that FP can suppress...
NF-κB activation via inhibition of IκB kinases, that is, via activation of IκBo, the major inhibitor of NF-κB (Escotte et al., 2001).

In adult CF patients, short-term fluticasone therapy had no evident effect on clinical and sputum parameters (Dauletbaev et al., 1999), and other studies showed that young CF patients did not improve symptom scores, lung function, or sputum inflammatory markers when treated with inhaled FP compared with placebo (Balfour-Lynn et al., 1997). One possible explanation is that, due to sputum hyperviscosity in CF patients, the penetration of FP through the viscous mucus to the mucosal tissue is very difficult. In the present study, grafts were rinsed two times per week to remove cellular debris and this is likely to have also removed some of the mucus. Under such conditions, FP could access directly and penetrate into the epithelial cells.

In conclusion, our results suggest the use of models such as that of human CF and non-CF airway grafts to investigate the early inflammatory status of the airway epithelium and the response to bacterial stimuli. This study confirms that inflammation occurs very early in CF airways and demonstrates that FP may prevent the release of inflammatory mediators after P. aeruginosa LPS-induced inflammation.

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