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REVIEW ARTICLE

Review Series: Non-Invasive Monitoring of Airway Inflammation in Asthma

Exhaled Nitric Oxide (FeNO) as a Non-Invasive Marker of Airway Inflammation

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ABSTRACT

Nitric oxide (NO), previously very famous for being an environmental pollutant in the field of pulmonary medicine, is now known as the smallest, lightest, and most famed molecule to act as a biological messenger. Furthermore, recent basic researches have revealed the production mechanisms and physiological functions of nitric oxide in the lung, and clinical researches have been clarifying its tight relation to airway inflammation in asthma. On the bases of this knowledge, fractional nitric oxide (FeNO) has now been introduced as one of the most practical tools for the diagnosis and management of bronchial asthma.

KEY WORDS

asthma, cut-off, diagnosis, monitor, non-invasive

INTRODUCTION

Previously known as a toxic molecule listed as an environmental pollutant, nitric oxide (NO) is now known to be the smallest, lightest, and most famed molecule to act as a biological messenger in mammals. NO was first recognized as a physiologically important molecule in the manuscript written by Fruchgott and Zawadzki, entitled "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine", published in Nature in 1980.[1] Initially, the factor released from vascular endothelial cells was named as endothelium derived relaxing factor (EDRF), and large number of scientists had been pursued the true feature of EDRF. In 1987, two groups led by Ignaro and by Moncada, independently discovered and reported that EDRF is a simple gaseous molecule called NO.[2,3] After the discovery, a large amount of studies in field of medicine revealed its many roles in a wide range of pathophysiological status including cardiovascular, immune, metabolic, and neurological diseases. For an ordinary person, it became a very famous physiological mediator of penile erection and opened the door for the treatment of impotence.[4] In 1992, NO was selected as Molecule of the Year for a startlingly simple molecule unites neuroscience, physiology and immunobiology and revised scientists' understanding of how cells communicate and defend themselves.[5] In 1998, the Nobel prize for Physiology or Medicine was awarded to Doctors, Furchgott R, Ignarro L, and Murad F.[6]

In the field of pulmonary medicine, physiological and pathological roles of NO in lung disease have also been investigated. Epithelium dependent inhibition of airway smooth muscle contraction and epithelium dependent relaxation of airway smooth muscle, similar effect of vascular endothelium to vascular smooth muscle, have been reported.[7,8] These phenomena also suggested the existence of epithelium derived relaxing factor (EpDRF).[9] Since NO is also confirmed to be a potent smooth muscle relaxing agent (Fig. 1),[10] several pharmacological studies verifying whether EpDRF is also NO were carried out and confirmed production of NO from airway epithelium.[11] In these processes, measurement systems for NO in exhaled air have been developed.[12] By applying such systems, exhaled NO have been measured in many pulmonary diseases and significant increase

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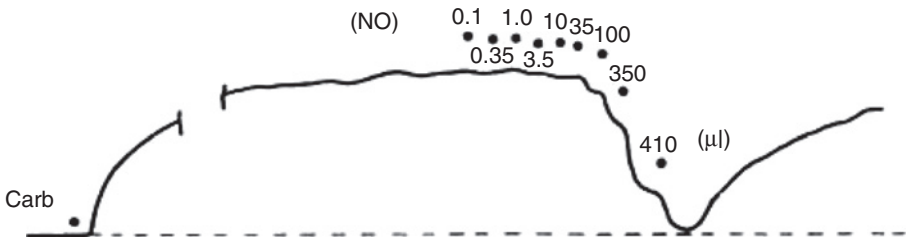

Fig. 1 Airway smooth muscle relaxation induced by nitric oxide (NO). Guinea pig tracheal strip was contracted by stimulated by Carbachol (Carb), then 0.1 to 410 ul of saturated solution of NO (estimated concentration 2 mM) was prepared, and was added to the tissue bath. Clear concentration-dependent relaxation was observed (Adapted from reference 10).

in exhaled NO of the patients with asthma became apparent.[13,14] Since the measurement is noninvasive and effort independent, exhaled NO has been much expected as a new tool for the diagnosis and management of asthma, and large efforts have been devoted on the clinical research.

PRODUCTION AND FUNCTION OF NO IN THE AIRWAYS

In the respiratory tract, NO is produced by a wide variety of cell types and is generated through conversion of L-arginine to L-citrulline by the action of nitric oxide synthase (NOS). Three isoforms of NOS are known: neuronal NOS (NOS I or nNOS), inducible NOS (NOS II or iNOS), and endothelial NOS (NOS III or eNOS).[15] Two isoforms, nNOS and eNOS, are expressed constitutively, but iNOS is not normally expressed in most tissues but is induced in several types of cells by pro-inflammatory cytokines.[16-18] All three types of NOS isoforms are known to be expressed in the lung. Endothelial NOS (eNOS) immunoreactivity is found in endothelial cells of pulmonary vessels. In addition, it is constitutively expressed in human bronchial epithelium and in type II pneumocytes.[19,20] NO produced by eNOS and released from endothelial cells in the pulmonary circulation is speculated to regulate vascular basal tone and counteract hypoxic vasoconstriction.[21] Neuronal NOS (nNOS) is expressed in human airway nerves, including those present in the airway smooth muscle,[22,23] and is estimated to be a major mediator of the inhibitory non-adrenergic non-cholinergic nervous (iNANC) system.[24] Co-localization with vasoactive intestinal polypeptide (VIP) is also observed.[25] Nerves distributed to submucosal glands also contain nNOS and NO regulates secretory function of the glands.[22,26] In the lung, iNOS (or NOS II) is known to be expressed in macrophages,[27] epithelial cells,[28-30] type II pneumocytes,[31,32] endothelial cells,[33] airway and vascular smooth muscle,[34] mast cells,[35] neutrophils,[36] chondrocytes,[23] and fibroblast.[37] Usually, iNOS in these cells is expressed when stimulated by endogenous mediators such as chemokines and cytokines, and by exogenous stimulant such as bacterial toxins, viruses, allergens, etc. Constitutive expression of iNOS in airway epithelial cells and rapid loss of its expression after removal of the epithelial cells from the in vivo airway environment were reported only in humans and suggest that the expression is dependent upon conditions and/or factors present in the airway.[38] iNOS derived NO is also speculated to regulate both airway smooth muscle tone and inflammatory responses.

MEASUREMENT OF EXHALED NO

The presence of NO in the exhaled air of humans was demonstrated by chemiluminescence, diazotization and mass spectrometry in 1991.[12] Thereafter, several measurement systems have been developed. The most commonly used system is chemiluminescence, and in Japan, two types are available; NOA280i (Severs, GE Analytical Instruments, Boulder, USA) and NA623N (Chest MI, Tokyo, Japan). These can be applied for both online and offline measurement of fractional exhaled NO (FeNO) in ppb. With these two types, we can get almost the same FeNO values.[39-42] It is known that FeNO is strongly affected by expiratory flow rate, FeNO levels in dead space air are high, and those in nasal cavities are very high. Therefore, several cautions should be kept in mind to get reasonable FeNO values. These cautions are included in American Thoracic Society (ATS) and European Respiratory Society (ERS) recommendations for the measurement of FeNO,[43,44] and following these recommendations is very important when FeNO is measured with these analyzers.

Another NO measurement system is the electrochemical method. The merits of the system are its compact size and portability. In Japan, two types of analyzers, NIOX MINO (Aerocrine, Stockholm, Sweden) and NObreath (Bedfont Scientific, Kent, UK), are available. There are some differences in FeNO levels measured by these analyzers when compared to a chemiluminescence analyzer. Differences of FeNO levels measured by different analyzers have been examined and conversion equations are also

available.[39-42] Several attempts to separate alveolar NO from airway NO have been performed by measuring FeNO at multiple exhalation flow rates,[45-48] however, it seems very difficult to apply such methods to clinical medicine and they might be useful as research tools.

NO AND AIRWAY INFLAMMATION IN ASTHMA

Asthma is a syndrome characterized by the presence of two physiological characteristics, reversible airflow limitation and airway hyperresponsiveness with respiratory symptoms. However, a recent advance in asthma research revealed the importance of airway inflammation existing behind these physiological characteristics. According to such progress in the concept of asthma, diagnosis and treatment strategies have been changing dramatically. One of the most prominent examples is the introduction of anti-inflammatory therapy with ICS, resulting in the pronounced the improvement in control and quality of life of the patients, and a dramatic reduction in the number of emergency room visit and deaths of the patients in Japan.

The recognition of the important roles of airway inflammation in asthma also promoted the development of new technology to evaluate airway inflammation in asthma. In these processes, a FeNO measurement was recognized as the most anticipated candidate. In early 1990s, significant increase in FeNO of ICS naïve patients with asthma, and decreased FeNO after ICS treatment was revealed,[13,14] suggesting the relationship between airway inflammation and elevation of FeNO. Hamid *et al*. applied immune-histochemical methods for bronchial biopsy specimen to investigate the presence of NOS in asthma. Immunoreactivity to iNOS was observed in the epithelium and some inflammatory cells in 22 of 23 biopsies from asthmatics, but in only 2 of 20 controls.[49] Guo *et al*. also examined iNOS expression by mRNA and protein assay and revealed that human airway epithelium has abundant expression of iNOS due to continuous transcriptional activation of the gene in vivo, and that individuals with asthma have higher than normal NO concentrations and increased iNOS mRNA and protein due to transcriptional regulation through activation of STAT1.[50] In addition, they revealed decreased expression of iNOS mRNA in asthmatics receiving ICS.[50] Redington *et al*. also examined iNOS expression in the airway epithelium and revealed enhanced expression in asthmatic subjects and regulation by corticosteroid treatment.[51]

The regulation mechanisms of iNOS expression are far from full elucidation. Although abundant expression of iNOS is observed in human airway epithelium, it will instantly disappear when these cells are cultured ex vivo,[38] suggesting the existence of in vivo factors or stimuli in the airway. In other types of cells, iNOS expression is only observed after stimulation with cytokines such as IFN-γ, IL-1β, and/or TNF-α.[52] Guo *et al*. revealed that a combination of IFN-γ/IL-4, which occurs naturally in lung epithelial lining fluid, leads to maintenance of iNOS expression in human airway epithelium through production of soluble mediators and stabilization of mRNA.[53] Alving and Malinovschi suggested a possible model of iNOS regulation of human airway from the results of recent studies[54] (Fig. 2). In healthy subjects (Fig. 2a), continuous expression of iNOS is maintained by IFN-γ, which normally exists in the respiratory tract. In this process, induction of unidentified soluble mediators by IFN-γ and the subsequent activation of the JAK/STA pathway are considered to be important.[55] In asthmatic airways (Fig. 2b), different regulation mechanisms are estimated (Fig. 2b). Initially, Th2 cytokines such as IL-4 and IL-13 were recognized to down-regulate iNOS expression.[56] However, several recent studies revealed that IL-4 and IL-13 actually induce iNOS expression in human airway epithelial cells in reasonable medium conditions through the STAT-6 pathway.[50,53,57,58]

There are several epidemiological evidences suggesting the relation between allergic airway inflammation and increased FeNO. Saito *et al*. examined FeNO levels, pulmonary function, and serum total and antigen specific IgE levels in 278 normal school children aged 10 to 12.[59] There are statistically significant positive correlations between FeNO and total IgE or mite specific IgE, and significant negative correlations between FeNO and FEV1/FVC. In addition, FeNO was determined by means of multiple logistic regression analysis to be the best predictor for recurrent wheeze, suggesting the relationship between allergic airway inflammation and FeNO levels. They also obtained the same results when they examined 280 normal adults aged 18 to 82 who received annual health check.[60] Moody *et al*. revealed that the increase in FeNO is associated with house dust mite sensitivity in asymptomatic subjects.[61] Additionally, some atopic subjects without symptoms and airway hyperresponsiveness have airway eosinophilc inflammation.[62] These findings support the tight relationship between increase of FeNO levels and allergic airway inflammation.

NO AS A DIAGNOSTIC TOOL OF ASTHMA

Traditionally, asthma has been characterized by respiratory symptoms such as cough, wheeze, and dyspnea, reversible airflow limitation, and non-specific airway hyperresponsiveness.[63,64] These are evaluated by pulmonary function tests before and after inhalation of bronchodilators such as β-adrenergic receptor agonists, and bronchial challenge test with bronchoconstrictors such as histamine and acetylcholine.[64] For the airway inflammation, trans-bronchial biopsy (TBB) and brochoalveolar lavage (BAL) under the fi-

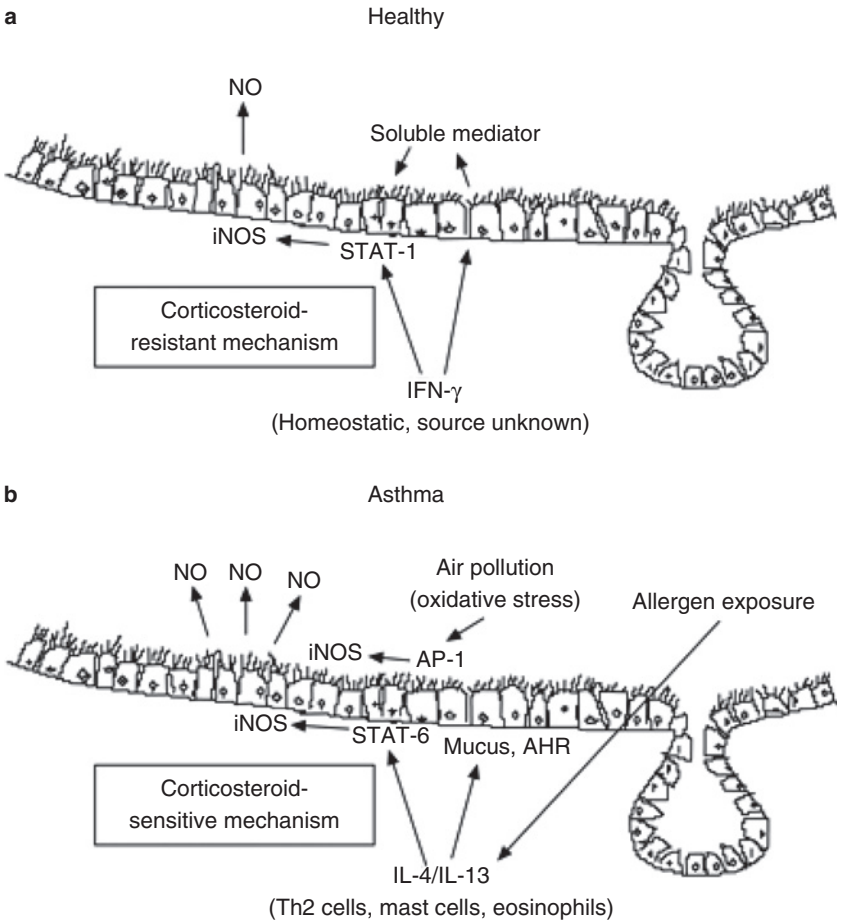

Fig. 2 A possible model of iNOS regulation of human airway. **a**) normal healthy airway, **b**) asthmatic airway (Adapted from reference 54).

beroptic bronchoscopic examination are applied. Recently, cell sorting of sputum induced by the inhalation of hypertonic saline has also been utilized. However, these methods are relatively invasive and sometimes induce asthma attacks. It is therefore difficult to apply widely in general clinical practice.

Because of the tight relation between FeNO and allergic airway inflammation and its non-invasiveness, attempts to use FeNO as a non-invasive tool for asthma diagnosis have been carried out in various clinical settings. Sato *et al*. examined 71 consecutive patients who visited out-patient clinics by complaining chronic cough continuing more than 3 weeks.[65] They examined FeNO, pulmonary function, serum IgE, methacholine airway responsiveness and induced sputum. FeNO is significantly higher in patients with asthma and cough variant asthma compared to other diseases including COPD and eosinophilic pneumonia without asthma, suggesting the usefulness of FeNO measurement in the diagnosis of asthma in patients with chronic cough. Cut-off value for FeNO for the diagnosis of asthma was 38.8 ppb with sensitivity of 79.2% and specificity of 91.3%. Similar results were also reported in patients with chronic cough by Chatkin *et al*., Fujimura *et al*., and Kowal *et al*..[66-68]

For the patients with non-specific respiratory symptoms and suspected to having asthma, Smith *et al*. examined FeNO and sputum eosinophils in addition to conventional peak expiratory flow and spirometric parameters before and after bronchodilator treatment.[69] They observed the overall superiority of FeNO measurements and induced sputum analysis in the diagnosis of asthma compared with conventional tests. Dupont *et al*. also reported the usefulness of FeNO in 160 asthmatic patients diagnosed by the presence of reversible airflow obstruction (delta FEV1 > 12%) and histamine airway hyperresponsiveness (PC20 < 8.0 mg/ml).[70] Their cut-off level of FeNO at expiratory flow rate of 200 ml/s (FeNO200) was 16.0 ppb with the sensitivity of 69.4% and specificity of 90.0%. Fortuna *et al*. also reported that the diagnostic accuracy of FeNO measurement was superior to that of the standard diagnostic spirometry in patients with symptoms suggestive of asthma.[71] Fukuhara *et al*. recently reported the results of their prospective validation

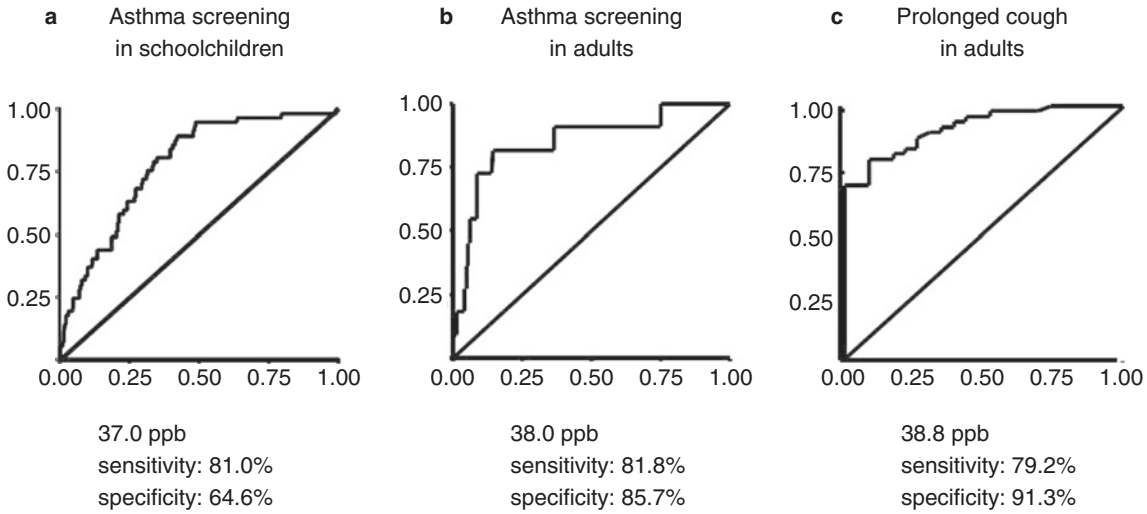

Fig. 3 The cut-off levels of fractional exhaled nitric oxide (FeNO) for diagnosing asthma obtained from 3 independent studies. **a**) for 277 school children, **b**) for 280 adult volunteers, **c**) for 71 patients with chronic cough (Adapted from reference 42).

study of asthma screening criteria based on subjective symptoms and FeNO at expiratory flow rate of 50 ml/sec ($FeNO_{50}$); i) recurrent cough, wheezeing, or dyspnea; ii) $FeNO_{50}$ > 40 ppb, iii) exclusion of other lung diseases.[42] A cut-off value of 40 ppb was determined by the results of their 3 previous independent studies on school children, normal adults, and patients with prolonged cough (Fig. 3).[59,60,65] When compared to conventional asthma diagnostic criteria based on GINA and JGL guidelines,[63,64] FeNO based criteria showed good sensitivity, specificity, and a concordance rate (k) (78.6%, 89.5%, and 0.62, respectively). However, 9 of 42 patients were misdiagnosed as not having asthma by FeNO based criteria, and 7 of these 9 patients were non-atopic according to their IgE levels. From these results, they suggested that FeNO could be used as a tool for the non-invasive accurate diagnosis of asthma, particularly in atopic patients in daily clinical practice.

NO AS A CONTROL TOOL OF ASTHMA

Understanding that the most basic event in asthma is airway inflammation and the tight correlation between FeNO and airway inflammation has motivated the application of FeNO as a monitoring tool for asthma control. In 2005, Smith *et al*. did a single-blind, placebo-controlled trial of adult asthmatics to examine the usefulness of FeNO measurements for the adjustment of ICS doses. With the FeNO based strategy, the maintenance doses of ICS were significantly reduced without compromising asthma control compared to those with an algorithm based on conventional guidelines.[72] In the same year, Pijnenburg *et al*. did a randomized controlled trial to examine the usefulness of FeNO for the titration of ICS in atopic children with asthma. They also found that FeNO improved airway hyperresponsiveness and inflammation without elevating the ICS doses.[73] Following these studies, several consecutive studies were conducted and controversial results were reported. For the adult asthmatics, Shaw *et al*. reported that a treatment strategy based on FeNO measurement did not result in a large reduction in asthma exacerbation or in the total amount of ICS therapy used over 12 months, compared with the current asthma guideline.[74] But when the results were pored over precisely, in the initial several months, the required dose of ICS was higher in the FeNO based group compared to the control group, the dose gradually declined and the final daily dose of ICS was significantly lower in the FeNO based group compared to the control group (average; 557 ug/day and 895 ug/day, respectively, $p < 0.028$). More recently, Powel *et al*. carried out a double-blinded, randomized controlled trial to examined the usefulness of asthma management in pregnancy guided by FeNO. They revealed that asthma exacerbations during pregnancy can be significantly reduce with a validated FeNO-based treatment algorithm.[75] For adolescents and young adults, Szefler *et al*. did the largest randomized controlled trial to date with 780 patients with asthma to examine the usefulness of FeNO-based asthma management in addition to guideline-based treatment. They concluded that the addition of FeNO as an indicator of asthma control resulted in higher doses of ICS, without clinically important improvements in symptomatic asthma control.[76] But the subgroup analyses of the patients with a higher number of positive skin tests or those with serum nonspecific IgE higher than 460 kU/L revealed that the FeNO monitoring group had significantly fewer maximum days with symptoms in 2 weeks than that of the control group (0.84 and 0.51, p

< 0.024 and $p < 0.007$, respectively). As shown in the previous section, FeNO is suggested to be a very useful tool to monitor airway inflammation in atopic subjects. From these facts, it is suggested that application of FeNO as a tool for asthma control might be limited to the patients with atopic asthma.

Another point that should be borne in mind is the fact that the period of previously introduced studies were only up to 12 months. Sont *et al*. compared the difference in histopathologic outcome of asthma when using airway hyperesponsiveness as an additional guide to long-term treatment. They demonstrated that the examined strategy group showed a greater reduction in thickness of the subepithelial reticular layer compared to the reference strategy group, suggesting a role for the monitoring of airway hyperresponsiveness or other surrogate makers of inflammation in preventing airway remodeling.[77] Long-term usefulness of FeNO as a monitoring tool for asthma control, whether it could be helpful in prevention of airway remodeling or in decreasing annual decline in FEV1, needs to be examined.

FUTURE DIRECTIONS

As noted above, FeNO is a very useful diagnostic tool and control monitoring maker of asthma. Usefulness of FeNO in asthma management is probably better than spirogram, induced sputum, and AHR test, because of its non-invasiveness, effort independency, measurement simplicity, and reproducibility. Although the FeNO analyzer has not been approved as a medical device, it will be widely used as a convenient clinical tool for asthma management in the near future in Japan.

The FeNO analyzer has been used as a clinical research tools and wide application of FeNO in the clinical setting revealed the issues that should be solved before its clinical application. Now, many researchers recognize that there is a minor population of subjects with very high levels of FeNO without respiratory symptoms, and that there are asthmatics with all asthmatic symptoms but with normal FeNO levels. The mechanisms behind these phenomena are unclear, meaning that the production mechanisms of NO in the airway and lung parenchyma have not been thoroughly clarified. Additional point is that, although the ATS/ERS guideline was established to standardize the FeNO measurements,[43] there are variations in FeNO values measured by different analyzers.[41,42] It also affects the determination FeNO cut-off levels for the diagnosis and control of asthma. In addition, to be used widely in the clinical practice, the development of handier, more accurate, less expensive measurement systems is required. Furthermore, not only more practical studies but also more basic studies will be warranted.

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