EFFECT OF AN INHALED THROMBOXANE MIMETIC (U46619) ON *IN VIVO* PULMONARY RESISTANCE AND AIRWAY HYPERRESPONSIVENESS IN DOGS

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SUMMARY

1. We investigated the role of thromboxane A_2 in the airway hyperresponsiveness that follows the inhalation of ozone in dogs by examining the responses to an inhaled thromboxane analogue (U46619).

2. Measurements of pulmonary resistance were made in anaesthetized dogs; the concentration of inhaled agonist causing an increase of 5 cmH_2O l^{-1} s was calculated (provocative concentration). The effect of inhaled U46619 was studied on *in vivo* canine airway resistance, on airway responsiveness and on airways made hyperresponsive following the inhalation of ozone.

3. Inhaled thromboxane is a potent constrictor of the canine airway. The mean provocative concentration was 2.13×10^{-4} M, compared to acetylcholine which was 3.23×10^{-2} M.

4. Inhaled thromboxane did not result in the development of airway hyperresponsiveness to acetylcholine. Following U46619 inhalation the mean provocative concentration to acetylcholine was 3.92×10^{-2} M.

5. Canine airway was not hyperresponsive to inhaled thromboxane following the inhalation of ozone. This was not due to an inhibition of acetylcholinesterase as the dogs were hyperresponsive to carbachol (a muscarinic agonist not degraded by endplate cholinesterase).

6. These experiments do not support a role for thromboxane in the development of airway hyperresponsiveness following the inhalation of ozone in dogs.

INTRODUCTION

Airway hyperresponsiveness is a physiological hallmark of patients with asthma (Boushey, Holtzmann, Sheller & Nadel, 1980). It consists both of an increased sensitivity of the airways to a variety of constrictor agonists, as indicated by a smaller concentration of the agonist needed to initiate the bronchoconstrictor response, as well as a greater maximal response to the agonist. Humans (Golden, Nadel & Boushey, 1978) and dogs (Lee, Bleecker & Nadel, 1977) develop airway hyperresponsiveness following the inhalation of ozone. This effect is reversible and short lived, is maximal at 1 h and resolves by 1 week following the inhalation of ozone (Holtzmann, Fabbri, Skoogh, O'Byrne, Walters, Aizawa & Nadel, 1983).

Ozone-induced hyperresponsiveness in dogs is dependent upon the influx of neutrophils into the airway lumen (O'Byrne, Walters, Gold, Aizawa, Fabbri, Alpert, Nadel & Holtzmann, 1984). Pretreating dogs with either the cyclo-oxygenase inhibitor indomethacin (O'Byrne, Walters, Aizawa, Fabbri, Holtzmann & Nadel, 1984), or the thromboxane synthetase inhibitor OKY 046, prevents airway hyperresponsiveness induced by ozone (Aizawa, Chung, Leikauf, Uecki, Bethel, O'Byrne, Hirose & Nadel, 1985). In addition, pretreatment of dogs with OKY 046 prevents airway hyperresponsiveness after inhaled allergen (Chung, Aizawa, Becker, Frick, Gold & Nadel, 1986), leukotriene B$_4$ (O'Byrne, Leikauf, Aizawa, Bethel, Uecki, Holtzmann & Nadel, 1985) and platelet-activating factor (Chung, Aizawa, Leikauf, Uecki, Evans & Nadel, 1986). These results have been interpreted as indicating that thromboxane A$_2$ is a mediator involved in the pathogenesis of airway hyperresponsiveness induced by these stimuli in dogs. OKY 046 has also been reported to reduce acetylcholine airway hyperresponsiveness in asthmatic patients (Fujimura, Sasaki, Nakatsumi, Takahashi, Hifumi, Taga, Mifune, Tanaka & Matsuda, 1986), implicating thromboxane in the pathogenesis of airway hyper-responsiveness in asthmatic subjects.

The biological half-life of thromboxane is short, only 30 s, thus making it impossible to study in *in vivo* systems. Synthetic analogues of thromboxane A$_2$, such as the endoperoxide U46619 (9,11-dideoxy,11α,9α-epoxy-methanoprostaglandin F$_{2\alpha}$), mimic the biological effects of TxA$_2$ in a variety of tissues *in vitro* (Coleman, Humphrey, Kennedy, Levy & Lumley, 1981) and in animals *in vivo* (Aizawa *et al.* 1985) and has been the chemical entity used to determine the efficacy and potency of thromboxane A$_2$ receptor antagonists (Hall, Gillard, Guindon, Letts, Champion, Ethier, Evans, Ford-Hutchinson, Fortin, Jones, Lord, Morton, Rokach & Yoakim, 1987). Also, U46619 has been shown to increase the responsiveness of canine airway smooth muscle *in vitro* (Coleman *et al.* 1981). Canine tracheal smooth muscle show an increased response to electrical field stimulation but not to exogenous ACh in the presence of U46619 (Munoz, Shioya, Murphy, Primack, Dame, Sands & Leff, 1986; Serio & Daniel, 1988). Similar results have been demonstrated *in vivo* by measuring ACh responses in dogs on two occasions. On the first day the animals inhaled a sub-constrictor dose of U46619 in addition to ACh and on the second day a sub-constrictor dose of histamine. The animals inhaling the U46619 had significantly increased responses to ACh (Aizawa *et al.* 1985). These results support the hypothesis that thromboxane causes airway hyperresponsiveness to ACh. Recent studies in humans have also reported a role for thromboxane in airway hyperresponsiveness. In asthmatics, a thromboxane receptor antagonist has been reported to decrease airway responsiveness to inhaled methacholine (Fujimura, Sakamoto, Saito, Miyake & Matsuda, 1991). Asthmatics presenting to the emergency room with severe acute asthma have been reported to have increased urinary levels of thromboxane metabolites (Taylor, Ward, O'Shaughnessy, Dollery, Black, Barrow, Taylor & Fuller, 1991).

However, more recent studies of potent thromboxane receptor antagonists have shown no effect of these compounds on the airway hyperresponsiveness following inhalation of ozone in dogs (Jones, Lane & O'Byrne, 1990). The purpose of the present study was to examine further the effects of inhaled thromboxane on the function of the canine airway *in vivo*. We examined the effects of an inhaled

thromboxane analogue (U46619) on airway resistance, and on airway responsiveness to ACh. In addition to establishing whether inhalation of U46619 resulted in airway hyperresponsiveness, we also examined the effect of simultaneous inhalation of sub-maximal concentrations of U46619 and ACh. An experiment showing that thromboxane increases airway responsiveness to ACh would be consistent with the hypothesis that it is involved in ozone-induced airway hyperresponsiveness. Finally we examined the response of canine airway to inhaled U46619 following the inhalation of ozone. In order to rule out an effect of ozone on acetylcholinesterase we also measured airway responses to inhaled carbachol (a muscarinic antagonist not metabolized by acetylcholinesterase), before and after the inhalation of ozone.

METHODS

Physiological measurements

In all experiments random source mongrel dogs were used. The animals were treated according to the guidelines set out by the Canadian Council of Animal Care (C.C.A.C., 1980) and were approved by the ethics committee of McMaster University. The animals were anaesthetized with sodium pentabarbitone (30 mg/kg intravenously), and additional anaesthetic was given throughout the experiment when necessary (as determined by passive jaw muscle tone and eyelid reflex). The dogs were intubated (10 mm i.d. endotracheal tube) and ventilated (10 ml/kg tidal volume, 0·5 Hz, Harvard Apparatus 551, South Natick, MA, USA). Animals were ventilated for approximately 0·5 h to allow for stabilization and accurate measurement of baseline resistance. During this time an intravenous saline (0·9 % NaCl) infusion was started.

Measurement of airway resistance. Lung mechanics were measured using an analogue pulmonary function computer (Hewlett–Packard 8816A, Palo Alto, CA, USA). Elastance was calculated according to the classical analysis of Von Neergaard & Wirz (1976). Resistance was calculated using a Mead & Whittenberger analysis (1953). Respiratory flow was measured from the pressure drop across a pneumotachograph (Fleisch no. 3, Instrumentation Associates, New York) attached to a differential pressure transducer (Hewlett–Packard 270, Waltham, MA, USA) and a pressure amplifier (Hewlett–Packard 8805C, Waltham, MA, USA) which was in turn connected to the respiratory analyser. The flow signal was calibrated with a rotameter (Rotameter Manufacturing Company, Croydon, UK) before every experiment. Pleural pressure was estimated from transpulmonary pressure. Oesophageal pressure was measured from a latex balloon positioned in the oesophagus (Mead, McIlroy, Selverstone & Kriete, 1955) connected to one port of a differential pressure transducer (Hewlett–Packard 267BC, Waltham, MA, USA) and a pressure amplifier (Hewlett–Packard 8805C). The other port of the differential pressure transducer was connected to the animal's side of the pneumotachograph. The pressure signal was calibrated using a water manometer before every experiment. The signals from both pressure amplifiers (flow and oesophageal pressure) were fed into the respiratory analyser which in turn calculated pulmonary resistance. The units of resistance are $cmH_2O\ l^{-1}\ s$. The resistance value was corrected for the resistance of the measurement system (approximately $2\ cmH_2O\ l^{-1}\ s$). Respiratory flow, transpulmonary pressure, resistance and dynamic compliance were recorded on paper by an 8-channel recorder (Hewlett–Packard 7758A, Waltham, MA, USA). Measurement of resistance are made from the paper record of the experiment.

Measurement of airway responsiveness. Airway responsiveness to acetylcholine, carbachol and U46619 were measured. The method used to determine airway responsiveness was the same for all agonists.

Following baseline resistance measurements and the resistance measurements made after an inhalation of saline, the dogs inhaled a range of concentrations of acetylcholine aerosols. The concentration range was in doubling doses from $4·3 \times 10^{-4}$ M (0·07 mg/ml) to $2·2 \times 10^{-1}$ M (40·0 mg/ml). The stock solutions of acetylcholine was prepared on the day of the experiment, by dilution of acetylcholine chloride in normal sterile saline (0·9 % NaCl); these solutions were stored on ice throughout the experiments. Aerosols were generated by a Bennett/twin nebulizer (Puritan Bennett, Los Angeles, CA, USA) at a flow rate of 8 l/min of dry medical air. The nebulized output was 0·196 ml/min with particles of an aerodynamic mass median diameter of 2·5 μm (geometric standard deviation 2·3). Each dose period consisted of five forced inhalations over 30 s (3 s

inspiration, 3 s expiration). To ensure a constant lung volume history, each dose was preceded by inflation of the animal to a pressure of 30 cmH_2O (Lee, Dumont, Djokic, Menzel & Nadel, 1979). The challenge was discontinued when the airway resistance increased to more than 5 cmH_2O l^{-1} s above baseline values. Airway responsiveness was calculated by interpolation, as the dose of acetylcholine causing an increase in resistance of 5 cmH_2O l^{-1} s above baseline. The value of 5 cmH_2O l^{-1} s above baseline is an arbitrary value suggested by Holtzmann *et al.* (1983). The concentration of acetylcholine causing a change in resistance of 5 cmH_2O l^{-1} s above baseline was termed the acetylcholine provocative concentration.

The dilutions of carbachol were prepared in a fashion similar to acetylcholine. The concentration ranged from 4.3×10^{-4} M (0.07 mg/ml) to 2.2×10^{-1} M (40.0 mg/ml). U46619 was stored in ethanol at −70 °C at a concentration of 5.7×10^{-3} M (2 mg/ml). Dilutions of the stock solution were made in saline immediately before each inhalation challenge. The doubling concentrations ranged from 1.8×10^{-5} to 1.1×10^{-3} M (6.25 to 400 μg/ml).

Production and measurement of ozone

Ozone was generated by passing pure oxygen through a high-intensity electrical field in a device built by L. D. Pengelly, P.Eng, PhD, McMaster University, Hamilton, Canada. Ozone was diluted with dry room air using a glass-and-Teflon mixing circuit to achieve the desired concentration (3 p.p.m.) and administered via the endotracheal tube by allowing the dog to breathe spontaneously from a flow-by system. Ozone concentration was measured continuously using a photometric ozone analyser (Bendix 8002, Lewisburg, WA, USA) calibrated periodically with known concentrations of ozone by the Ontario Ministry of Health. An ozone concentration of 3 p.p.m. was inhaled for 30 min through the endotracheal tube.

Effects of the thromboxane analogue on airway responses to acetylcholine

Eleven random source dogs were anaesthetized, intubated and ventilated. An initial measurement of airway responsiveness to ACh was made. Forty-eight minutes later airway responses were performed to the thromboxane mimetic U46619. To increase the exposure to U46619 and to asses the repeatability of the airway response to this inhaled agonist, a second maximal dose of U46619 was given. Forty-eight minutes later airway responses to ACh were measured again.

On a separate occasion the dogs ($n = 6$) also inhaled a mixture of ACh and U46619. The concentration of each agonist inhaled was one doubling dose smaller than the maximal dose given in the earlier inhalation tests (i.e. the penultimate dose).

The effect of ozone-induced airway hyperresponsiveness on responses to inhaled U46619

Six dogs were studied on two separate occasions separated by at least 1 month. Airway responses to two different agonists (carbachol and U46619) were measured before and after ozone.

Drugs and chemicals

The drugs used were sodium pentobarbitone (MTC pharmaceuticals, Missisauga, Ontario), acetylcholine chloride (Sigma, St Louis, MO, USA), carbamylcholine (Sigma) and U46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$) (Sigma).

Statistics

Airway responsiveness is log normally distributed. Therefore the values for airway responsiveness do not have a Gaussian (or normal) distribution unless first log_{10} transformed (Fleming, Westfall, De La Lande & Jellett, 1972). Because of this, analysis of measurements of airway responsiveness was performed using log_{10}-transformed data and expressed as the geometric mean (antilog of the mean of the log data) and percentage standard error of the mean (%s.e.m.) (antilog of the s.e.m. of the log data). The changes in airway responsiveness within the groups were compared using a multiway ANOVA. Post-hoc comparisons (Multiple T and Dunnett) were performed where indicated by the ANOVA (SAS Institute Inc., 1988). Differences between groups were considered statistically significant at levels of $P < 0.05$ (Kleinbaum & Kupper, 1978; Snedecor & Cochran, 1980).

RESULTS

U46619 caused a concentration-dependent bronchoconstriction in all dogs ($n = 11$), and was significantly more potent than ACh (Fig. 1A). The molar provocative

concentration for U46619 was 2.13×10^{-4} (1.81) M (geometric mean (% S.E.M.)) and for acetylcholine was 3.23×10^{-2} (1.50) M ($P = 0.0002$) (Fig. 1*B*).

The ACh provocative concentration was not significantly different following inhalation of U46619, being 3.23×10^{-2} (1.50) M before and 3.92×10^{-2} (1.55) M after U46619 inhalation ($P > 0.05$) (Fig. 2).
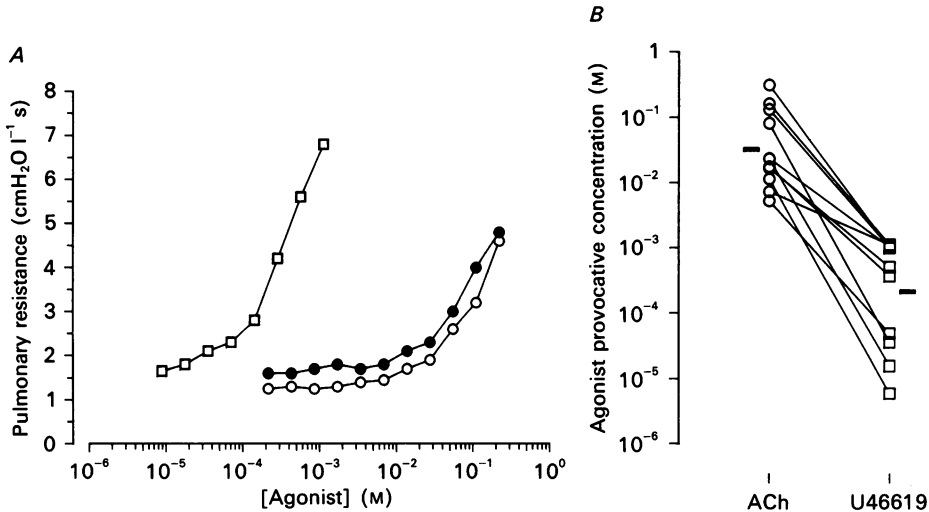

Fig. 1. *A*, results from a single dog showing the response of pulmonary resistance to increasing concentrations of inhaled ACh (○) and U46619 (□). Concentration is expressed in M to make a comparison between the two agonists. U46619 is a more potent bronchoconstrictor agent than ACh. Prior inhalation of U46619 does not alter the ACh dose–response curve (●). *B*, airway responsiveness to inhaled ACh (○) and U46619 (□) in all dogs. U46619 is a more potent bronchoconstrictor than ACh.

Mean baseline resistance for the first ACh challenge was 0.75 (0.09) cmH_2O l^{-1} s. This value was significantly increased in subsequent tests to 0.94 (0.10) cmH_2O l^{-1} s before U46619 and 1.06 (0.07) cmH_2O l^{-1} s before the 2nd ACh challenge. The second two challenges were not significantly different from each other. These differences, while statistically significant, are small in magnitude and have little significance physiologically.

When sub-maximal concentrations of ACh and U46619 were inhaled simultaneously there was no potentiation of ACh responses by the U46619. The resistance increase caused by inhalation of the two compounds together was 8.0 cmH_2O l^{-1} s (0.78). While this amount of constriction was significantly greater than that explained by the concentration of ACh alone (4.3 cmH_2O l^{-1} s (0.8)), it was not significantly different from the constriction caused by the concentration of U46619 alone (7.5 cmH_2O l^{-1} s (0.84)). These results also indicate that the constrictor effects of ACh and U46619 are not additive. It should be noted that these observations were made at levels of bronchoconstriction significantly below the maximum observed (12.5 cmH_2O l^{-1} s (2.1)) after U46619.

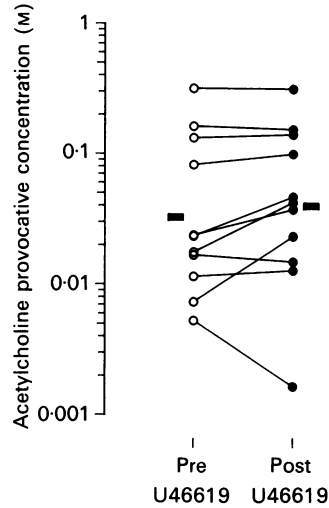Airway responses to inhaled thromboxane analogue (U46619) were not altered

Fig. 2. The acetylcholine provocative concentration before (○) and after (●) inhalation of U46619. Prior U46619 inhalation does not significantly alter the airway responsiveness to ACh.
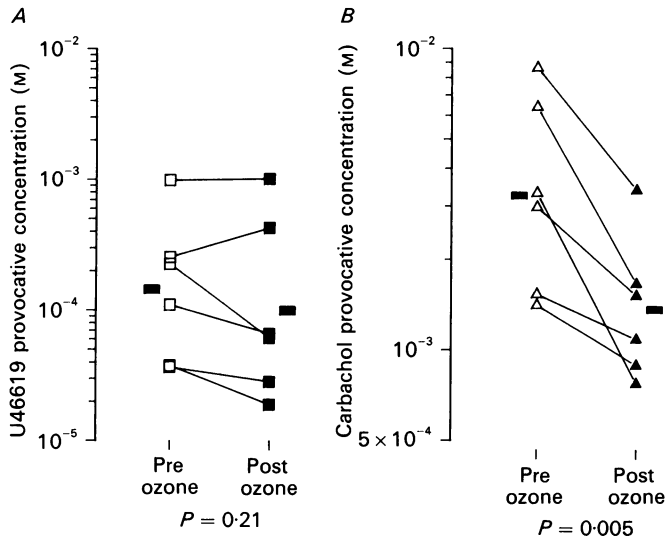

Fig. 3. *A*, airway responsiveness to inhaled U46619 before and after ozone. Ozone does not cause U46619 airway hyperresponsiveness. *B*, airway responsiveness to inhaled carbachol before and after ozone. Ozone does cause carbachol airway hyperresponsiveness.

following inhalation of ozone. The U46619 provocative concentration was $1\cdot44 \times 10^{-4}$ (1·67) M before ozone and $9\cdot93 \times 10^{-5}$ (1·89) M following ozone inhalation ($P = 0\cdot21$) (Fig. 3*A*).

Airway responsiveness to carbamylcholine (CCh) was significantly increased following ozone inhalation. The CCh provocative concentration decreased from

3.25×10^{-3} (1·35) to 1.36×10^{-3} (1·25) M after ozone $(P = 0.0057)$. These results suggest that hyperresponsiveness following ozone inhalation is specific for muscarinic receptors (Fig. 3B), and that loss of acetylcholinesterase activity is unlikely to be a factor in the airway hyperresponsiveness that follows ozone inhalation.

<div align="center">DISCUSSION</div>

The results of the present study demonstrate that the thromboxane receptor agonist U46619 is a potent bronchoconstrictor of canine airway, but is not capable of increasing airway responsiveness to ACh. These observations do not support the hypothesis that thromboxane is the mediator responsible for ozone-induced cholinergic airway hyperresponsiveness.

Our results indicate that simultaneous inhalation of thromboxane and ACh does not increase the airway responsiveness to ACh; in fact, the two agonists do not even act additively. This observation conflicts with earlier evidence that thromboxane increased the response to ACh, in studies that measured ACh responses in conjunction with sub-constrictor doses (concentrations which caused no increase in resistance) of either U46619 or histamine (Aizawa *et al.* 1985). An important difference in our experiments is that higher concentrations of U46619, that were able to cause significant bronchoconstriction, were used. These results, taken together, suggest that if thromboxane is important in ozone-induced airway hyperresponsiveness and airway hyperresponsiveness in general, it must be present in the airway at the time of hyperresponsiveness and in concentrations that do not cause bronchoconstriction.

Evidence from an *in situ* preparation of dog trachealis suggests that cyclo-oxygenase inhibitors act on the postganglionic prejunctional nerve fibres (Bethel & McClure, 1990). These authors also reported an increase in contractile function following cyclo-oxygenase inhibition and interpreted this as indicating a more important role for inhibitory prostaglandins in airway responsiveness. In the cat the effect of intravenous U46619 on ventilation was abolished by vagal cooling, again indicating the importance of the parasympathetic nervous system in the effect of thromboxane (Shams & Scheid, 1990). However, the ganglionic blocker hex-amethonium, in doses which effectively blocked the effects of vagal stimulation, had no effect on the airway hyperresponsiveness following the inhalation of ozone in dogs (Jones, Lane, Manning & O'Byrne, 1987), ruling out a vagal pathway in the pathogenesis of airway hyperresponsiveness following ozone inhalation.

Another possible implication is that thromboxane may act through different receptor subtypes. This hypothesis could explain the ineffectiveness of specific thromboxane receptor antagonists in blocking ozone-induced hyperresponsiveness in dogs (Jones *et al.* 1990). However, the lack of effect of thromboxane receptor antagonists on ozone-induced airway hyperresponsiveness is also consistent with the hypothesis that thromboxane is not involved in the development of airway hyperresponsiveness.

Following ozone inhalation, canine airways do not become hyperresponsive to U46619, but they are hyperresponsive to CCh. The fact that many previous studies have demonstrated that ozone causes airway hyperresponsiveness to ACh but, in this study, to CCh and not to U46619 cannot be explained by an effect of ozone on

acetylcholinesterase. ACh and CCh both act on the muscarinic receptor but CCh is resistant to breakdown by acetylcholinesterase. By contrast U46619 acts on a non-muscarinic prostanoid receptor, classified as the TP receptor (Watson & Abbott, 1991). The differential effects of U46619, ACh and CCh suggest that the hyperresponsiveness following the inhalation of ozone may be a specific effect for muscarinic receptor activation, coupling or associated post-membrane mechanisms.

The studies presented in this paper further define the role of thromboxane A_2 in the pathogenesis of airway hyperresponsiveness. If thromboxane A_2 is to have an effect it must be present at concentrations that do not cause bronchoconstriction. At concentrations not causing smooth muscle contraction, U46619 still increases the response to electrical field stimulation, presumably by increasing the release of ACh prejunctionally. Thus, if thromboxane is to play a role in airway responsiveness it must be through a prejunctional pathway.

In summary, inhalation of U46619 does not result in the development of airway hyperresponsiveness. This observation suggests that thromboxane does not affect airway responses to ACh and as such might not play a role in the mechanism of airway hyperresponsiveness after ozone. These results imply that the mechanism of ozone-induced airway hyperresponsiveness lies within the pathway of muscarinic receptor activation and coupling.

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