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ORIGINAL ARTICLE

Leukotriene antagonism reduces the generation of endothelin-1 and interferon-γ and inhibits eosinophilic airway inflammation

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Abstract

The cysteinyl leukotrienes (cysLTs) and the peptide hormone endothelin (ET)-1 are potent bronchoconstrictor substances, and these mediators are also claimed to be implicated in the development of eosinophilic airway inflammation. In the present study, we have investigated the effect of the $cysLT_1$ receptor antagonist montelukast on the development of an eosinophilic airway inflammation 24 h after intratracheal Sephadex (SDX) provocation in rats. Furthermore, the effect of montelukast treatment on the generation of ET-1 and other pro-inflammatory mediators has been studied. The inflammatory response was significantly reduced in the animals receiving SDX + montelukast compared to animals receiving solely SDX, as evaluated by a decrease in bronchoalveolar lavage fluid total cell count (10.3 ± 1.2 *vs.* $18.5 \pm 1.8 \times 10^4$ ml^{-1}, $P < 0.001$), number of eosinophils (299.7 ± 43.8 *vs.* $577.6 \pm 46.6 \times 10^2$ ml^{-1}, $P < 0.001$), and lymphocytes (116.8 ± 20 *vs.* $222.0 \pm 34.8 \times 10^2$ ml^{-1}, $P < 0.05$), as well as the degree of tissue inflammation ($P < 0.05$). Montelukast also inhibited the increase in the concentration of the pro-inflammatory mediators ET-1 (28.5 ± 7.5 *vs.* $40.9 \pm 7.3 \times$ pg ml^{-1}, $P < 0.05$) and interferon (IFN)-γ (4.3 ± 2.2 *vs.* $15.6 \pm 8.7 \times$ pg ml^{-1}, $P < 0.05$), but not tumor necrosis factor-γ or interleukin-8. In summary, treatment with the $cysLT_1$ receptor antagonist montelukast reduced the inflammatory response during development of an eosinophilic airway inflammation, possibly by inhibiting the release of pro-inflammatory mediators like ET-1 and IFN-γ. © 2002 Elsevier Science Ltd. All rights reserved

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Keywords inflammation; eosinophils; asthma; endothelin; cytokines; montelukast

INTRODUCTION

The cysteinyl leukotrienes (cysLTs) ($LTC_4/D_4/E_4$) and the peptide hormone endothelin (ET)-1 are potent bronchoconstrictor substances (1–3). In addition to the bronchoconstrictor properties, these mediators seem to be implicated in the development of the eosinophilic airway inflammation (4–6), which is a key feature of asthmatic airways. CysLTs have been shown to facilitate the migration of eosinophilic granulocytes *in vitro* (7), and aerosolized cysLTs elicit influx of eosinophils into guinea pig airways *in vivo(4)*. Moreover, LTD_4 challenge has been reported to increase the percentage of eosinophils in sputum from asthmatic patients(8).

The mechanism by which the cysLTs promote influx of inflammatory cells has not been fully elucidated, but both a direct chemotactic effect and recruitment of inflammatory cells by promoting the generation of other chemotactic mediators are possible mechanisms. During the development of an experimental eosinophilic airway inflammation, Namovic *et al*. found that the influx of eosinophils into the airways was preceded by an increase in the level of cysLTs (9). In comparable studies, also employing the model of Sephadex (SDX) provocation in rats, we have found a similar pattern with regard to ET-1 (10). The rapid generation of both cysLTs and ET-1 is consistent with a hypothesis that these mediators may play important roles in the initiation of an eosinophilic airway

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inflammation. *In vitro* studies have indicated that cysLTs might in fact stimulate the generation of the pro-inflammatory mediator ET-1 (11). Based on these findings, and studies indicating an anti-inflammatory effect of $cysLT_1$-inhibitors on eosinophilic airway inflammations (12,13), we hypothesized that $cysLT_1$ receptor antagonists may inhibit the generation of the pro-inflammatory mediator ET-1. We have previously shown that inhibition of ET-1 activity reduces the inflammatory response in an experimental eosinophilic airway inflammation (5).

During the last years, a number of LT-inhibitors have been developed. In clinical practice, the $cysLT_1$ receptor antagonists have gained most interest. An inhibitory effect on bronchoconstriction in asthmatics has been documented (14–17). Evidence for an anti-inflammatory effect *in vivo* is, however, limited. To study the anti-inflammatory properties of the $cysLT_1$ receptor antagonist montelukast, we induced an eosinophilic airway inflammation in rats by intratracheal instillation of SDX. SDX is a dextran to which some rat strains are naturally sensitized, and bronchial or intratracheal provocation with these particles promotes an allergic eosinophilic inflammation (18). One group of animals were treated with montelukast prior to the bronchial challenge, while the control group received saline. The effect of montelukast treatment on the inflammatory response in bronchoalveolar lavage fluid (BALF) and lung tissue was studied. In addition, the level of ET-1 in the BALF was measured, as was the concentration of the pro-inflammatory mediators tumor necrosis factor (TNF)-α, interleukin (IL)-8 and interferon (IFN)-γ, as we previously have observed an increase of these cytokines following SDX provocation of rats (19).

METHODS

Experimental procedure

Male Wistar rats aged 12 weeks with an average weight of 320 g were used in the study. The experiments were approved by the Norwegian Ethics Committee for Animal Research, and performed according to the NIH guidelines for the use of experimental animals. SDX solution (5 mg ml^{-1}) (G-200 Superfine, PharmaciaUpjohn, Uppsala, Sweden) dissolved in phosphate-buffered saline (PBS) or PBS alone was given in a volume of 0.3 ml intratracheally to rats pretreated with either saline or montelukast (n = 12 in each group). A dose of 2 mg kg^{-1} body weight of the highly selective (20) $cysLT_1$ receptor antagonists montelukast (Merck Frosst, Canada Inc.) was chosen according to the manufacturer and administered orally 1 h prior to provocation, and repeated after 8 and 16 h. Pilot studies revealed no effect of montelukast treatment on the levels of ET-1, TNF-α, IL-8 and IFN-γ in rats receivng intratracheal PBS instead of SDX. Bronchoalveolar lavage was performed by instillation of 3+2+2 ml PBS in the right stem bronchus, distal to the upper lobe, and the procedure was repeated on the left side. The lavage fluid was collected into prechilled tubes containing EDTA and kept on ice until centrifuged at 800 g for 10 min. at 4°C.

Cell profile in BALF and tissue inflammation

Total cell recovery in BALF was counted in a Bürker hemocytometer, the profile of the inflammatory cells was evaluated on Diff-Quick® (Baxter Diagnostics AG, Düdingen, Switzerland)-stained slides, and at least 400 non-epithelial cells were determined. Lung and airway tissues were embedded in paraffin, and hematoxylin–eosin-stained histological sections (5 μm) were screened by two observers in a blinded manner. The inflammatory response was assessed in tissues from all animals. Twenty regions were examined and the inflammatory response graded as strong (score 3, $>$300 inflammatory cells), moderate (score 2, 75–300 inflammatory cells), weak (score 1, $<$75 inflammatory cells), or absent (score 0).

Biochemical analyses

ET-1 was determined using a radioimmuno-linked endothelin 1–21 specific [^{125}I] assay system (RPA 555) from Amersham Pharmacia Biotech International, Cardiff, U.K. Prior to ET-1 analysis, samples were extracted in duplicate from 2 ml BALF. The BALF was added to acetic acid on Sep-Pak C18 cartridges (Millipore Corp., Milford, MA, U.S.A.), which were prewashed with ethanol and acetic acid. The extract was freeze dried and dissolved in borate buffer. The ET-1 assay has a limit of detection equivalent to 1.6 pg ml^{-1}. For the samples with ET-1 concentrations below this limit, the values were set to 1.6 pg ml^{-1}. TNF-α was measured using a rat-specific sandwich enzyme-linked immunosorbent assay (ELISA) (Factor-Test-X®, Genzyme Corporation, Cambridge, MA, U.S.A.). Values below the detection limit of this assay system (10 pg ml^{-1}) were consequently set to 10 pg ml^{-1}. IFN-γ was analyzed using a rat specific ELISA (Cytoscreen®, BioSource International, Camarillo, CA, USA) and IL-8 (CINC/gro) was measured with Panatest® Rat IL-8 ELISA (Panapharm Laboratories, Kumamoto, Japan). All measurements were performed in duplicate, and values are given as means. For TNF-α, IL-8, and IFN-γ assays, the BALF was concentrated ten-fold prior to analysis. The BALF was concentrated using Minicon cells (Amion Division, W.R. Grace & Co., Beverly, MA, U.S.A.). Results are given as pg ml^{-1} unconcentrated BALF.

Statistical analysis

All values are expressed as means $\pm$ SEM. Statistical analyses were performed using scientific statistical software

(SigmaStat version 2.0, Jandel Scientific GmbH, Ekrath, Germany). The groups were compared using Kruskal–Wallis one way analysis of variance on ranks, followed by a multiple comparisons procedure *vs* controls using Dunn's method. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Cell profile in BALF and tissue inflammation

The SDX-induced increase in BALF total cell count was strongly inhibited by treatment with montelukast (10.3 ± 1.2 *vs* controls; $18.5 \pm 1.8 \times 10^4\,ml^{-1}$, $P < 0.001$) (Fig. 1(A). The numbers of eosinophils and lymphocytes were also significantly lower in the SDX + montelukast-treated animals than in the animals receiving solely SDX (eosinophils; 299.7 ± 43.8 *vs* $577.6 \pm 46.6 \times 10^2\,ml^{-1}$, $P < 0.001$, and lymphocytes; 116.8 ± 20 *vs.* $222.0 \pm 34.8 \times 10^2\,ml^{-1}$, $P < 0.05$) (Figs. 1(B) and (C). No significant inhibition by montelukast could be observed with regard to the number of neutrophils and macrophages (Figs. 1(D) and (E).

The histological evaluation of lung and airway sections following SDX-provocation of the animals revealed a pronounced inflammation peribronchially and surrounding the SDX particles, dominated by eosinophils, neutrophils and macrophages. Treatment with montelukast resulted in a moderate decrease in the extent of inflammation in the tissues (inflammatory score; 1.92 ± 0.08 *vs* 2.14 ± 0.05, $P < 0.05$) (Fig. 2).

Mediators in BALF

After 24 h, a significant increase in the concentration of both ET-1, IFN-γ TNF-α, and IL-8 was observed in the SDX-treated animals, compared to controls (Fig. 3). Treatment with montelukast prior to SDX provocation resulted in 30% decrease in the BALF level of ET-1 ($P < 0.05$) (Fig. 3(A)) and a 72% decrease in IFN-γ ($P < 0.05$) (Fig. 3(B)), compared to the animals receiving solely SDX (Fig. 3). The reduction in the concentration of TNF-α (30% and IL-8 (60%)) did not reach statistical significance (Figs 3(C) and (D)).

DISCUSSION

The SDX-induced inflammatory response was reduced by pretreatment of the animals with the $cysLT_1$ receptor blocker montelukast. We observed a decrease in BALF total cell count and in the number of eosinophils and lymphocytes, as well as a reduction in the degree of tissue inflammation. In addition, treatment with montelukast significantly inhibited the SDX-induced increase in the concentration of the pro-inflammatory mediators ET-1 and IFN-γ.

Eosinophils and lymphocytes are the two cell lines most strongly associated with bronchial asthma (21). Therefore, it is interesting that the observed anti-inflammatory effect of montelukast seems to be related to a decrease in the number of both these cell types. With regard to eosinophils, Diamant *et al*. (17) did not observe an inhibitory effect of montelukast on the influx of eosinophils into the airways of asthmatic patients after allergen challenge, however, that study was not specifically designed or powered to evaluate sputum eosinophils. On the other hand, Pizzichini *et al*. (12) found a reduced percentage of eosinophils in induced sputum from asthma patients during montelukast treatment, and, in ovalbumin-sensitized rats, montelukast has also been shown to inhibit the influx of eosinophils into the airways(13). The current study also examined the effect of montelukast treatment on the number of BALF lymphocytes, since these cells also play an important role in the asthmatic airway inflammation (21). The inhibitory effect of montelukast on the influx of lymphocytes, in addition to a decrease in the number of eosinophils, is interesting. Using another $cysLT_1$ receptor antagonist, zafirlukast, similar findings of a reduced number of lymphocytes have been shown following segmental allergen challenge in asthmatics (22), but to our knowledge, reduction in the number of lymphocytes has not been reported after treatment with montelukast.

The mechanisms by which $cysLT_1$ receptor antagonists inhibit the influx of eosinophils and lymphocytes have not been elucidated. A direct chemotactic effect of cysLTs on these cells may explain the rapid influx of leukocytes into the airways (8,23). Thus, blocking $cysLT_1$ receptors would inhibit that mechanism. On the other hand, LTs might promote influx of inflammatory cells through the release of other pro-inflammatory mediators. One study could show a blunted TNF-α release in BALF from allergen-challenged asthmatics pretreated with zafirlukast (22), and *in vitro*, pranlukast inhibits the IL-4 and IL-5 response from isolated mononuclear cells (24). Finally, montelukast has recently been shown to reduce the number of IL-5 mRNA expressing cells after antigen stimulation in rats (13). The present study demonstrates that montelukast significantly inhibited the SDX-induced ET-1 release in BALF. This has previously not been reported. During the development of an SDX-induced airway inflammation, LTs in BALF are generated prior to the eosinophilic inflammatory response (19) and immediately before the generation of ET-1 (19). Since LTs seem to be generated before ET-1, and cysLTs have been shown to stimulate ET-1 release *in vitro* (11), it is possible that treatment with a $cysLT_1$ receptor antagonist might influence the generation of ET-1 and thereby reduce the chemotactic effect of this peptide *in vivo*. This hypothesis is concordant with the current results, since treatment with the $cysLT_1$ receptor antagonist montelukast inhibits the release of ET-1, as well as the influx of

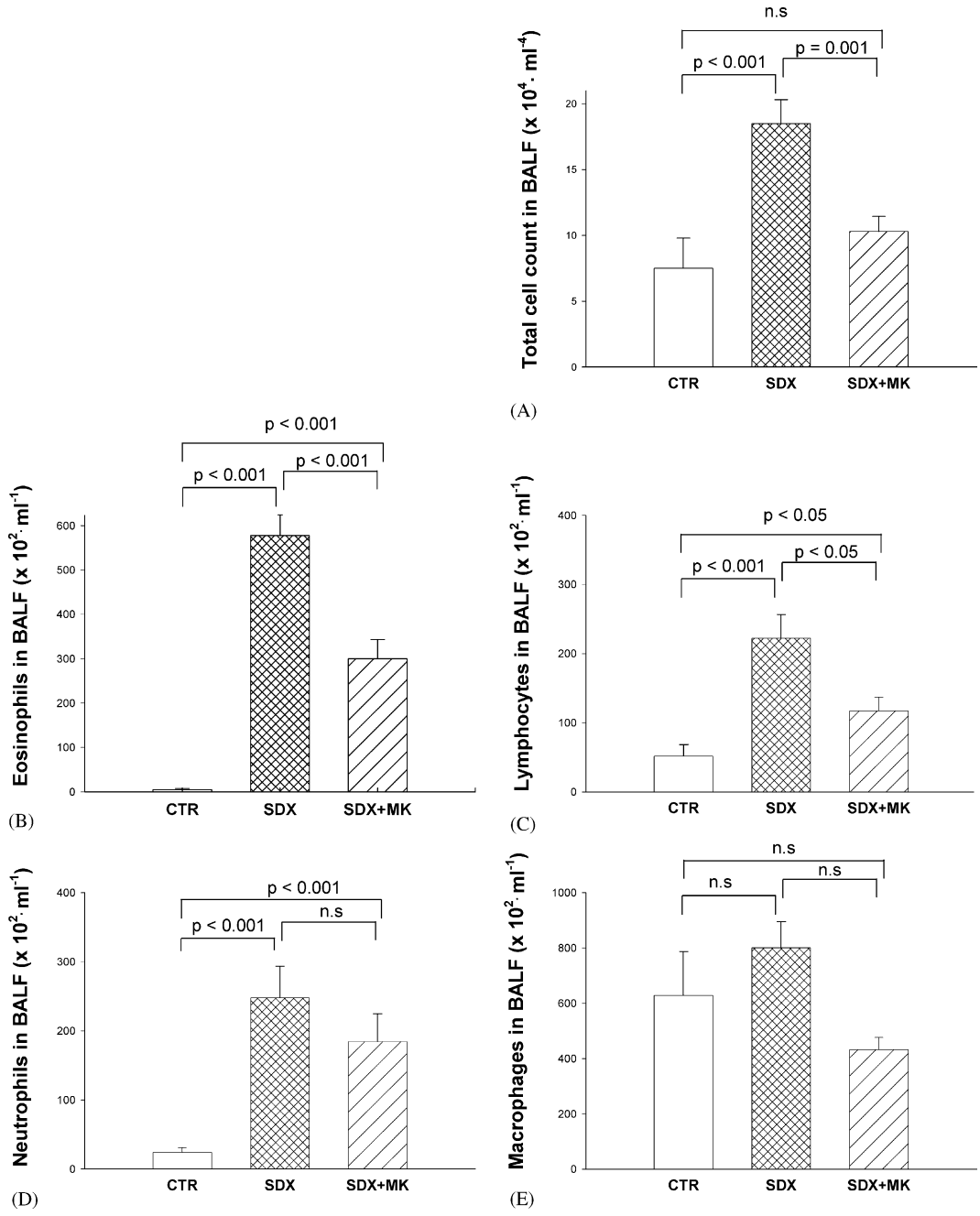

Fig. 1. Cell profile in BALF of rats evaluated 24 h after intratracheal provocation with saline (controls) or SDX, or SDX following montelukast treatment ($n = 12$ in each group). Values are given as means ± SEM.

eosinophils and lymphocytes. The pro-inflammatory peptide ET-1 is associated with the development of airway inflammation (5,6,10,25), and blocking of ET-receptors results in a decreased influx of both eosinophils and lymphocytes, a pattern similar to what is seen during treatment with montelukast. We do not believe that the reduced levels of ET-1 is a result of a reduced number of inflammatory cells after cysLT$_1$ receptor antagonism, since ET-1 is produced mainly in bronchial epithelial cells and in macrophages (10). In previous studies, as well, we have observed changes in ET-1 levels in BALF without alterations in the number of macrophages (5,10).

The IFN-γ response in the present study was also inhibited by the treatment with montelukast, possibly due to reduction of the number of lymphocytes, since IFN-γ is mainly produced by these cells (26). Another possibility is that the IFN-γ release is blunted due to decreased ET-1 concentration, since decreased levels of IFN-γ has been demonstrated after treatment with an ET receptor antagonist in the SDX model (19). Importantly, IFN-γ

upregulates monocyte and macrophage functions (27) and is also a potent activator of eosinophilic granulocytes (26). The concentration of TNF-α, which may be synthesised from a variety of cells including airway epithelial cells, macrophages, mast cells and eosinophils (28), was not significantly reduced by montelukast treatment in the current study, neither was the concentration of IL-8. However, there was a clear trend in the direction of reduced levels of these cytokines in BALF, as well.

In conclusion, treatment with the cysLT$_1$ receptor antagonist montelukast significantly reduced the inflammatory response in an SDX-induced airway inflammation in rats, as evaluated by a decrease in BALF total cell count, the number of eosinophils and lymphocytes, as well as the degree of tissue inflammation. The generation of ET-1 and IFN-γ in BALF was also reduced by montelukast treatment, indicating one possible mechanism for the anti-inflammatory effect of this cysLT$_1$ receptor antagonist.

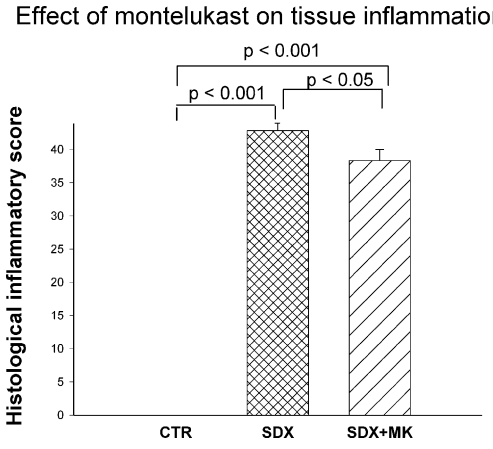

Fig. 2. Evaluation of airway tissue inflammation in rats evaluated 24 h after i.t. provocation with saline (controls) or SDX, or SDX following montelukast treatment.

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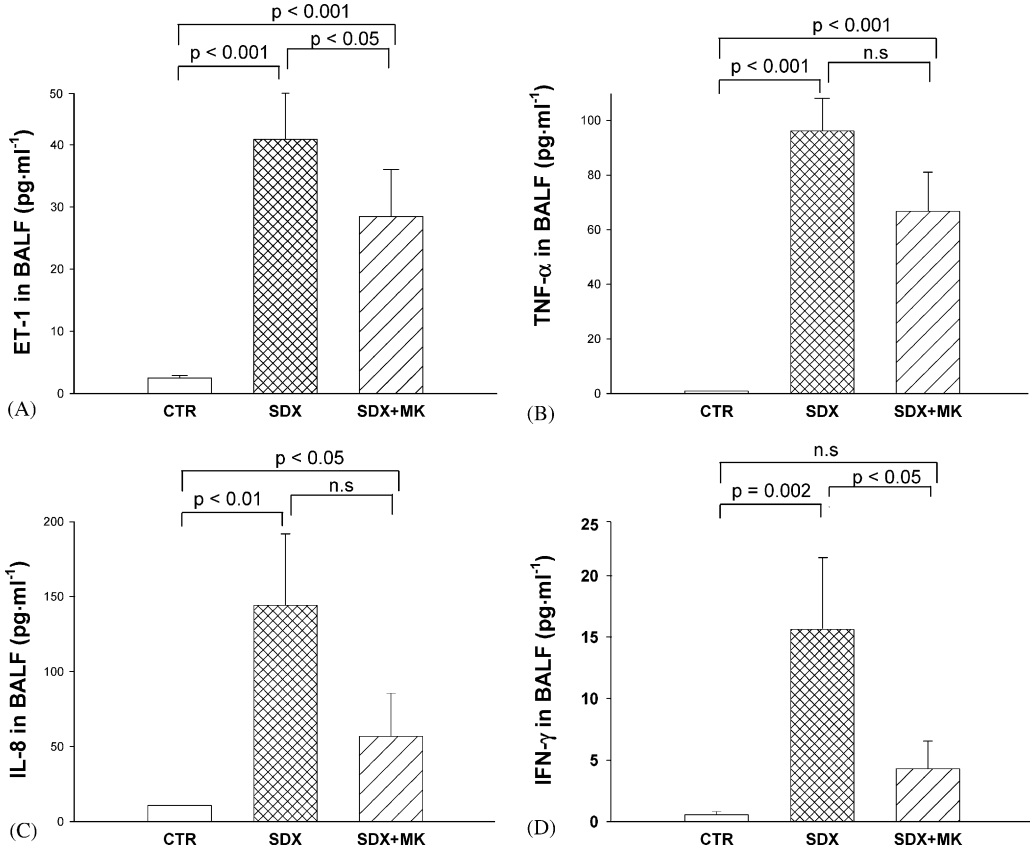

Fig. 3. Mediators in BALF of rats evaluated 24 h after intratracheal provocation with saline (controls) or SDX, or SDX following montelukast treatment (n = 12 in each group).Values are given as means ± SEM.

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