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IL-17-induced cytokine release in human bronchial epithelial cells *in vitro*: role of mitogen-activated protein (MAP) kinases

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1 Recent data indicate that interleukin (IL)-17 may contribute to neutrophilic airway inflammation by inducing the release of neutrophil-mobilizing cytokines from airway cells. The aim of this study was to evaluate the role of mitogen activated protein kinases in IL-17 induced release of IL-8 and IL-6 in bronchial epithelial cells.

2 Transformed human bronchial epithelial cells (16HBE) were stimulated with either IL-17 or vehicle. Both groups were treated either with SB202190 (inhibitor of p38 MAP kinase), PD98059 (inhibitor of extracellular-signal-regulated kinase [ERK] pathway), Ro-31-7549 (protein kinase C [PKC] inhibitor), LY 294002 (a phosphatidylinositol 3-kinase [PI 3-kinase] inhibitor) or vehicle. IL-6 and IL-8 levels were measured in conditioned media by ELISA.

3 The IL-17-induced release of IL-6 and IL-8 was concentration-dependently inhibited by SB202190 and by PD98059 in bronchial epithelial cells without affecting cell proliferation or survival.

4 Ro-31-7549 and LY294002 had no significant effect on IL-17-induced IL-6 or IL-8 release in bronchial epithelial cells.

5 Taken together, these data indicate a role for p38 and ERK kinase pathways in IL-17-induced release of neutrophil-mobilizing cytokines in human bronchial epithelial cells. These mechanisms constitute potential pharmacotherapeutical targets for inhibition of the IL-17-mediated airway neutrophilia.

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Abbreviations: BSA, bovine serum albumine; DMSO, dimethyl sulphoxide; EDTA, ethylendiaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular regulated kinase; FCS, foetal calf serum; IL, interleukin; JNK, c-Jun amino-terminal kinase; LY 294002, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase/extracellular regulated kinase kinase; PBS, phosphate buffered saline; PD98059, 2′-Amino-3′-methoxyflavone; PI 3-kinase, phosphatidylinositol 3-kinase; SB202190, 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole; Ro-31-7549, 2-[1-(3-Aminopropyl)indol-3-yl]-3-(1-methylindol-3-yl) maleimide, acetate

Introduction

Airway neutrophilia is a characteristic of several chronic airway diseases such as chronic bronchitis, chronic obstructive pulmonary disease (COPD), bronchial asthma and cystic fibrosis (Balbi *et al*., 1997; Lamblin *et al*., 1998; Meyer & Zimmerman, 1993). By releasing proteolytic enzymes and free oxygen radicals, the increased number of neutrophils may contribute to the main features of these diseases such as airway obstruction, hypersecretion and remodelling (Schuster *et al*., 1992; Venaille *et al*., 1995; Weinberg & Hayes, 1982). As indicated in murine airways *in vivo*, the recruitment of neutrophils into the airways can be controlled by activated T-lymphocytes (Gavett *et al*., 1994; Nakajima *et al*., 1992). However, the precise mechanisms by which T-lymphocytes achieve this control are not fully understood.

Interleukin-17 is a recently characterized proinflammatory cytokine produced exclusively by activated T-lymphocytes (Yao *et al*., 1995). The IL-17 receptor (R) however, is widely distributed and is expressed in several tissues, including the lung, and on several cell types, including bronchial epithelial cells (Yao *et al*., 1997). We have recently shown that exogenous IL-17, given intratracheally, causes a selective increase of the neutrophil number paralleled by an increase in the activity of elastase and myeloperoxidase in murine airways (Hoshino *et al*., 2000; Laan *et al*., 1999). Hypothetically, IL-17 could thus link the activation of T-lymphocytes to the recruitment and activation of airway neutrophils. This is further supported by the recent observation that inhalation of endotoxin in mice causes increased IL-17 mRNA expression, followed by neutrophil airways (Larsson *et al*., 2000). In human bronchial cells *in vitro*, IL-17 induces the production of neutrophil-mobilizing cytokines such as IL-6 and IL-8 (Fossiez *et al*., 1996; Laan *et al*., 1999; Yao *et al*., 1995). Since these cytokines are known to modulate the recruitment, activation and survival of neutrophils (Bank *et al*., 1995; Richman-Eisenstat *et al*., 1993), the induced production of these cytokines in airway

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cells is likely to explain the effect of IL-17 on neutrophils in the airways. However, the intracellular signalling mechanisms mediating these effects of IL-17 in airway cells have not been elucidated.

The mitogen-activated protein (MAP) kinase family is central in mediating several changes in cell function such as cytokine expression, proliferation and apoptosis (Puddicombe & Davies, 2000; Robinson & Cobb, 1997). To date, three MAP kinase cascades have been identified and characterized from a molecular standpoint. These comprise the p38 MAP kinase, extracellular signal-regulated kinase (ERK) and c-Jun amino-terminal kinase (JNK). Once activated by their upstream regulators, the MAP kinases translocate to the cell nucleus where they modulate the activity of nuclear transcription factors and kinases which, in turn, cause the changes in cell function such as production of cytokines. Recently, specific cell permeable inhibitors have been developed for two of these MAP kinase pathways. SB203580 and analogues such as SB202190 specifically inhibit the p38 MAP kinase (Badger *et al*., 1996; Kramer *et al*., 1996) whereas PD98059 inhibits the upstream activator of ERK kinases, MEKs, resulting in a blockade of ERK mediated signalling (Alessi *et al*., 1995). Although there is no specific inhibitor available for the JNK MAP kinase pathway, two major upstream activators of JNK, the protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI 3-kinase), can be inhibited by specific inhibitors such as Ro-31-7549 and LY 294002, respectively, resulting in downstream inhibition of JNK-mediated signalling (Kawakami *et al*., 1998; Vlahos *et al*., 1995; Yano *et al*., 1995; Zhang *et al*., 1997).

The aim of this study was to characterize the involvement of MAP kinases in IL-17-induced release of two neutrophil-mobilizing cytokines, IL-6 and IL-8, in human bronchial epithelial cells, thereby identifying potential targets for pharmacotherapeutical inhibition of IL-17 mediated airway neutrophilia.

Methods

Materials

Transformed human bronchial epithelial (16HBE14$_{o-}$, abbreviated as 16HBE) cells (Cozens *et al*., 1994) were kindly donated by Associate Professor Dieter C. Gruenert (Gene Therapy Center, Cardiovascular Research Institute, Department of Laboratory Medicine, University of CA, U.S.A.) and were utilized because of their ability to respond to inflammatory stimuli similarly to the primary human bronchial epithelial cells (Massion *et al*., 1994). Trypsin-ethylendiaminetetraacetic acid (EDTA) solution, trypsin neutralizing solution, and HEPES buffered saline solution (HBSS) were used for subculture and were obtained from Clonetics (San Diego, DA, U.S.A.). Recombinant human IL-17 protein as well as human IL-6 and human IL-8 ELISA kits were obtained from R&D Systems Europe (Abington, U.K.). Penicillin-streptomycin, L-glutamine, amphotericin B and bovine serum albumin (BSA), were purchased from Sigma (St. Louis, MO, U.S.A.). Fibronectin (human) and collagen (bovine, type I) were obtained from Becton Dickinson Labware (Bedford, MA, U.S.A.), and MEM Earle-Eagle was from Life Technologies (Inchinnan, Scotland, U.K.).

Cell culture conditions

16HBE cells were grown in MEM Earle-Eagle medium with 10% of foetal calf serum (FCS), 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 5 mg ml^{-1} amphotericin B on collagen and fibronectin-coated dishes (Gruenert *et al*., 1990) using trypsin-EDTA solution, Trypsin neutralizing solution and HBSS for subculture. The cells were grown to confluence in 12-well plastic plates (Becton Dickinson, Mountain View, CA, U.S.A.) and at 18 h before the experiments, the concentration of FCS was reduced to 1% in order to minimize the basal (inherent) cytokine release. Before the addition of stimuli, the cells were washed twice with phosphate buffered saline (PBS) solution and placed in fresh medium with 1% of FCS. PBS supplemented with 0.1% BSA was used to deliver IL-17 and was also used as a vehicle. The utilized concentration of IL-17 was chosen based on previous concentration-response experiments, in which treatment with 100 ng ml^{-1} of IL-17 resulted in submaximal IL-8 release in 16HBE cells (Laan *et al*., 1999).

Kinase inhibitors

A selective p38 inhibitor – SB202190 (4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole); a selective MEK inhibitor – PD 98059 (2′-Amino-3′-methoxyflavone); a selective protein kinase C (PKC) inhibitor – Ro-31-7549 (2-[1-(3-Aminopropyl)indol-3-yl]- 3-(1 -methylindol-3-yl)maleimide, acetate); and a selective phosphatidylinositol 3-kinase (PI 3 kinase) inhibitor – LY 294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) were all purchased from Calbiochem-Novabiochem, Nottingham, U.K.

All inhibitors were dissolved in dimethyl sulphoxide (DMSO) as recommended by the manufacturer and diluted further in PBS with 0.1% BSA before adding to the cell culture. In each experiment, the vehicle contained the same concentration of DMSO as was present in the treatment group containing the maximal concentration of DMSO. This maximal DMSO content did not exceed 0.05%.

Measurement of IL-6 and IL-8 protein

After 18 h of incubation, the conditioned media were removed from cells and frozen at the end of each experiment. Thawed samples were centrifuged (4000 r.p.m. for 10 min) to pellet cells and cell debris. The supernatants were analysed using ELISA kits for IL-8 and IL-6 according to the manufacturer's instructions.

Assessment of cell proliferation and viability

In order to detect whether the effect of SB202190 and PD98059 on cytokine release is due to an effect on cell proliferation or viability, 16HBE cells were grown to confluence in 12-well plastic plates and subsequently treated with SB202190, PD98059 or vehicle for 18 h. The cells were detached from 12-well plates thereafter, using Trypsin/

EDTA solution, and the cell number and viability detected in Burker chamber using Trypan blue staining (Campbell *et al*., 1993).

Data analysis

In order to evaluate the effect of inhibitors on IL-17 induced cytokine release but not on basal (inherent) cytokine release every concentration of inhibitor plus IL-17 treatment had a parallel vehicle (0.1% BSA in PBS) containing the same amount of respective inhibitor. The effect of inhibitor on IL-17 induced cytokine release was evaluated by subtracting the cytokine values after vehicle plus inhibitor treatment from the one containing IL-17 plus inhibitor.

For statistical analysis, the StatView® 4.01 software (Abacus concepts, Berkeley, CA, U.S.A.) was used. Only non-parametric tests were utilized. The Wilcoxon signed rank test was used for comparisons between vehicle and IL-17 treated cells, and the Spearman rank correlation for evaluating whether the IL-17 induced IL-6 or IL-8 release was dependent upon the concentration of certain inhibitors (Altman, 1997).

Results

IL-17 and cytokine release

At the concentration of 100 ng ml^{-1}, IL-17 significantly increased the release of IL-6 (vehicle: 306.4 ± 25.3 pg ml^{-1}, IL-17: 598.4 ± 47.9 pg ml^{-1}; Wilcoxon signed rank test: $P<0.001$, $n=17$) and IL-8 (vehicle: 716.0 ± 69.9 pg ml^{-1}, IL-17: 1668.8 ± 137.6 pg ml^{-1}; Wilcoxon signed rank test: $P<0.001$, $n=15$) in 16HBE cells as compared with vehicle.

p38 inhibition and IL-17-induced cytokine release

In the concentration range from $1 \times 10^{-8} - 1 \times 10^{-5}$ M the p38 MAP kinase inhibitor SB202190 concentration-dependently inhibited the IL-17 induced release of IL-6 and IL-8 in 16HBE cells (Figure 1). In contrast, SB202190 had no pronounced effect on 16HBE cell proliferation (vehicle: 0.86 ± 0.09 *vs* SB202190 1×10^{-5} M: $0.86 \pm 0.09 \times 10^{6}$ cells per well, $n=3$) or viability (vehicle: $94.7 \pm 0.6\%$ *vs* SB202190 1×10^{-5} M: $95.4 \pm 1.2\%$ viable, $n=3$).

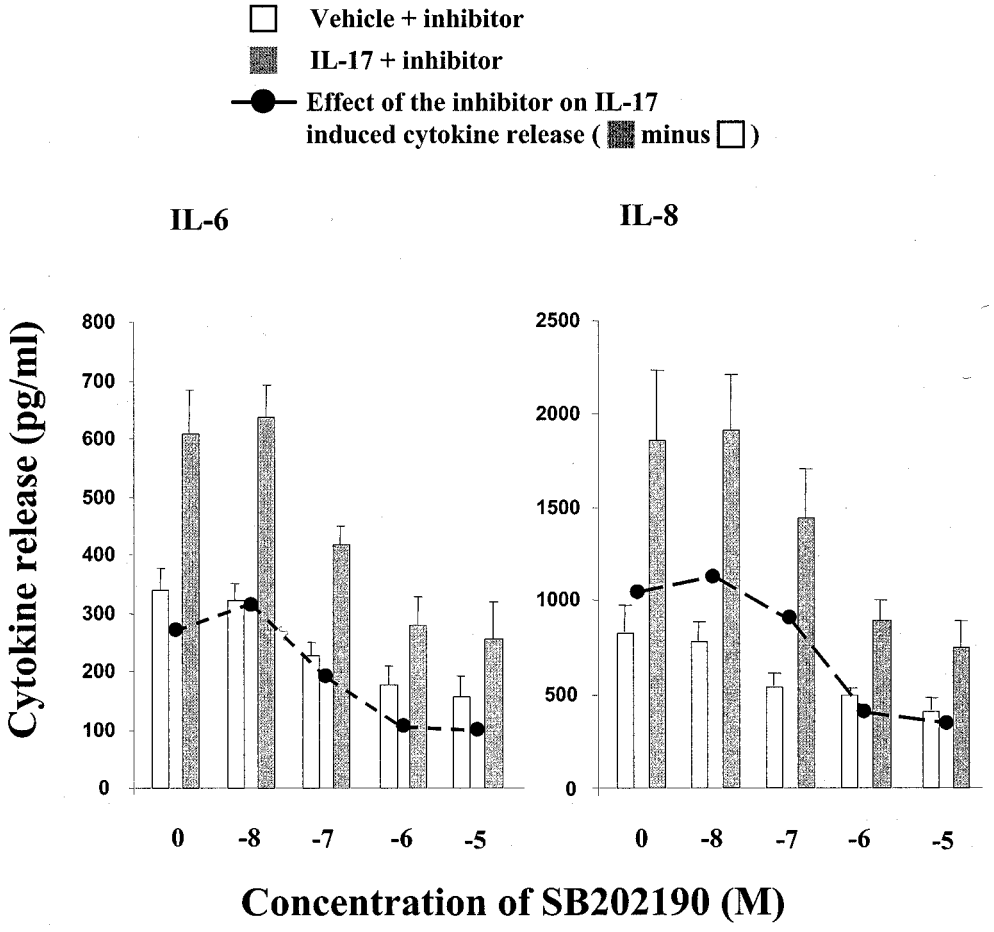

Figure 1 Effect of the p38 inhibitor, SB202190, on IL-17 induced release of IL-6 and IL-8 in transformed human bronchial epithelial (16HBE) cells. In the range from $1 \times 10^{-8} - 1 \times 10^{-5}$ M, SB202190 concentration dependently inhibited the IL-17 induced release of IL-6 (Spearman rank correlation: $r=0.8$, $P<0.01$) and IL-8 ($r=0.7$, $P<0.01$). Columns in the figure are mean with s.e.mean of four separate experiments.

MEK inhibition and IL-17-induced cytokine release

In the concentration range from $1\times10^{-7}-1\times10^{-5}$ M, the MEK inhibitor PD98059 concentration-dependently inhibited the IL-17 induced release of IL-6 and IL-8 in 16HBE cells (Figure 2). In contrast, PD98059 had no pronounced effect on 16HBE cell proliferation (vehicle: 0.86 ± 0.09 *vs* PD98059 1×10^{-5} M: $0.78\pm0.06\times10^{6}$ cells per well, $n=3$) or viability (vehicle: $94.7\pm0.6\%$ *vs* PD98059 1×10^{-5} M: $96.0\pm0.6\%$ viable, $n=3$).

PKC inhibition and IL-17-induced cytokine release

In the concentration range from $1\times10^{-8}-1\times10^{-5}$ M, the PKC inhibitor Ro-31-7549 had no effect on IL-17 induced IL-6 and IL-8 release in 16HBE cells (Figure 3).

PI 3-kinase inhibition and IL-17-induced cytokine release

In the concentration range from $1\times10^{-8}-1\times10^{-5}$ M, the PI 3-kinase inhibitor LY294002 had no effect on IL-17 induced IL-6 and IL-8 release (Figure 4).

Discussion

The present study examined the effect of kinase inhibitors on IL-17 induced release of neutrophil-mobilizing cytokines IL-6 and IL-8 in human bronchial epithelial cells. The results show that the specific p38 inhibitor SB202190 concentration dependently inhibits the release of IL-6 and IL-8, producing its maximal effect at a concentration of 1×10^{-6} M without affecting cell proliferation or viability. This indicates an involvement of the p38 signalling pathway in IL-17 induced release of neutrophil-mobilizing cytokines and is in line with a previous study on the effect of IL-17 in chondrocytes (Shalom-Barak *et al*., 1998). It also suggests that the IL-17-induced signalling in bronchial epithelial cells is similar to that in response to other stimuli such as heat or osmotic shock as well as to the cytokines IL-1β and TNF-α (De Cesaris *et al*., 1998; Matsumoto *et al*., 1998).

The present study also shows that the specific ERK pathway inhibitor PD98059, although less potent and efficient than SB202190, concentration-dependently inhibits the release of IL-6 as well as IL-8 in bronchial epithelial cells. Similarly to SB202190, PD98059 has no pronounced effect on

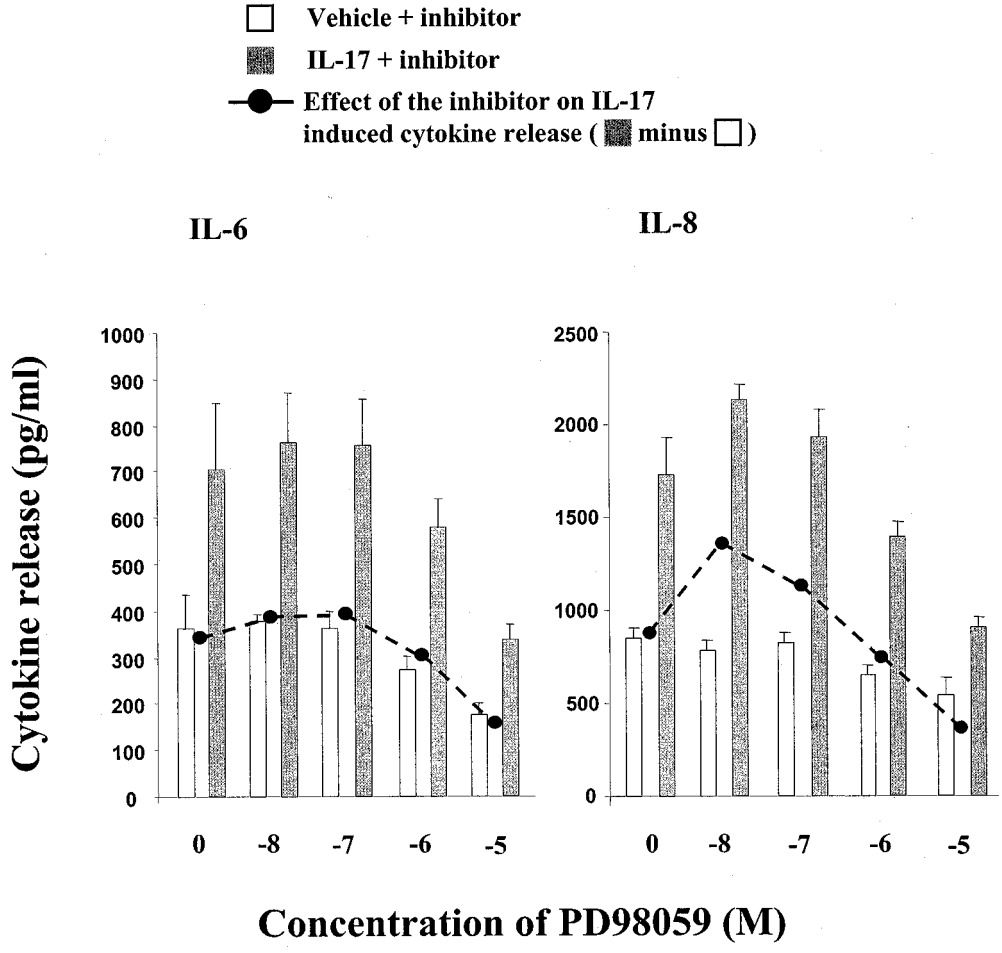

Figure 2 Effect of the MEK inhibitor, PD98059, on IL-17 induced release of IL-6 and IL-8 in 16HBE cells. In the range from $1\times10^{-7}-1\times10^{-5}$ M, PD98059 concentration-dependently inhibited the IL-17 induced release of IL-6 (Spearman rank correlation: $r=0.7$, $P<0.01$) and IL-8 ($r=0.8$, $P<0.01$). Columns in the figure are mean with s.e.mean of four separate experiments.

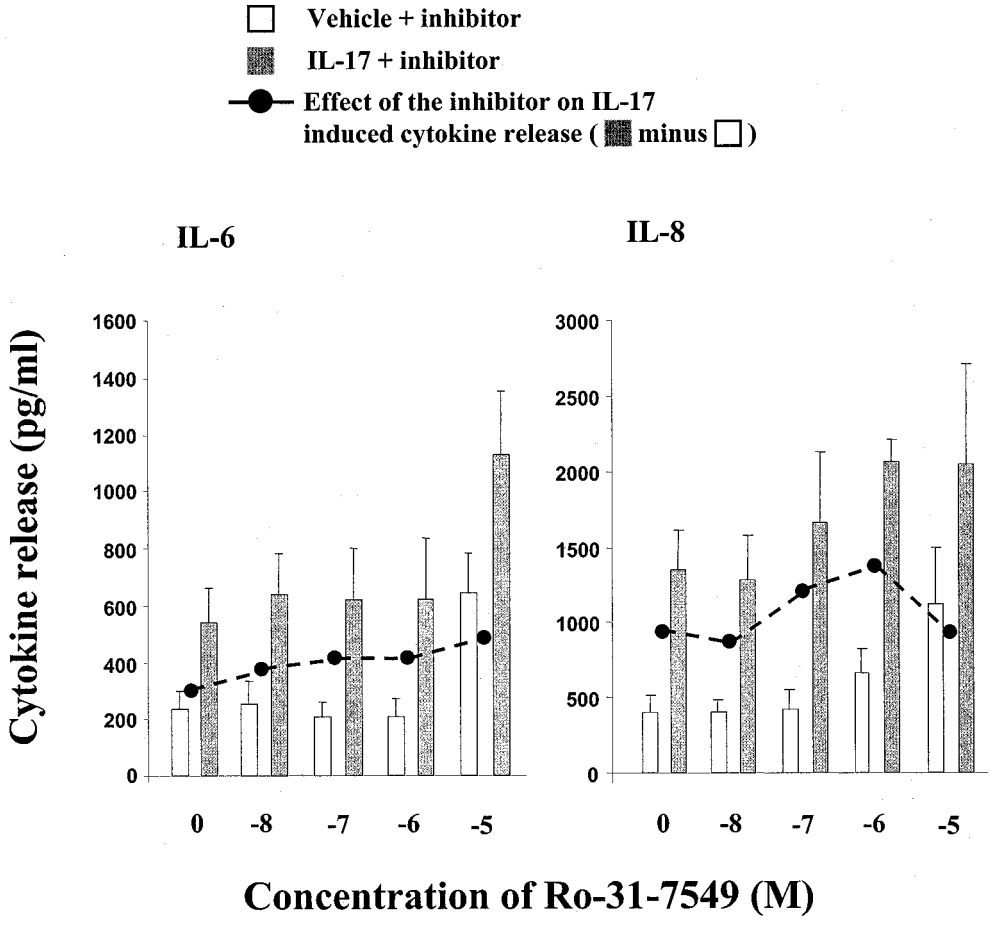

Figure 3 Effect of the PKC inhibitor, Ro-31-7549 on IL-17 induced release of IL-6 and IL-8 in 16HBE cells. In the range from 1×10^{-8}–1×10^{-5} M, Ro-31-7549 had no significant, concentration-dependent effect on IL-17 induced IL-6 or IL-8 release (Spearman rank correlation: $P>0.05$). Columns in the figure are mean with s.e.mean of 3–4 separate experiments.

cell proliferation or viability. Thus, the IL-17-induced release of IL-6 and IL-8 involves ERK, a mechanism known to be involved in various proliferation and differentiation responses as well as in the activation of important transcription factors such as Elk-1 (Alessi *et al*., 1995; Samet *et al*., 1998). Interestingly, it has been shown that IL-17 inhibits proliferation of intestinal epithelial cells (Awane *et al*., 1999). Whether this effect of IL-17 is ERK mediated and whether it involves the increased release of IL-6, a cytokine with capability to inhibit epithelial cell proliferation (Takizawa *et al*., 1993), needs further evaluation.

The PKC inhibitor, Ro-31-7549, and the PI 3-kinase inhibitor, LY 294002, had no substantial effect on IL-17 induced release of IL-6 or IL-8, suggesting that in bronchial epithelial cells, the PKC dependent or the PI 3-kinase dependent JNK are not involved in IL-17 mediated release of these cytokines. However, recent data suggests the possibility of PKC independent-IP 3-kinase independent JNK activation (Kawakami *et al*., 1998). Therefore, the involvement of JNK in IL-17 induced release of inflammatory cytokines in bronchial epithelial cells can not be excluded.

In addition to their inhibiting effect on IL-17 induced IL-6 and IL-8 release the MAP kinase inhibitors SB202190 and PD98059 also tended to reduce the basal release of these cytokines. Also, the maximal concentration of Ro-31-7549 tended to increase the basal release of IL-6 and IL-8 without modulating the IL-17 induced cytokine release. Although the precise mechanisms behind the basal release of cytokines in bronchial epithelial cells are not fully understood, it has been shown that the content of FCS as well as the disruption and isolation of cells may cause the activation of inducible genes under *in vitro* conditions (Aitken *et al*., 1996; Shibata *et al*., 1996). The modulating effect of inhibitors could thus be due to the inhibition of mechanisms triggered by *in vitro* conditions *per se*. *In situ*, however, virtually no IL-8 mRNA is detected in human airway epithelium without inflammatory stimulation (Inoue *et al*., 1994), suggesting that the basal release of cytokines is an *in vitro* artifact. Thus, the physiological relevance of the changes in the basal production of IL-6 and IL-8 is questionable. The exact mechanism as well as the biological significance of the effects of the inhibitors on basal cytokine release remain to be determined in future studies.

In conclusion, this study shows that the IL-17 induced release of IL-6 and IL-8 is inhibited by the specific MAP kinase inhibitors SB202190 and PD98059 in human bronchial epithelial cells, indicating a role for the p38 and ERK MAP kinase pathways in IL-17 induced release of neutrophil-

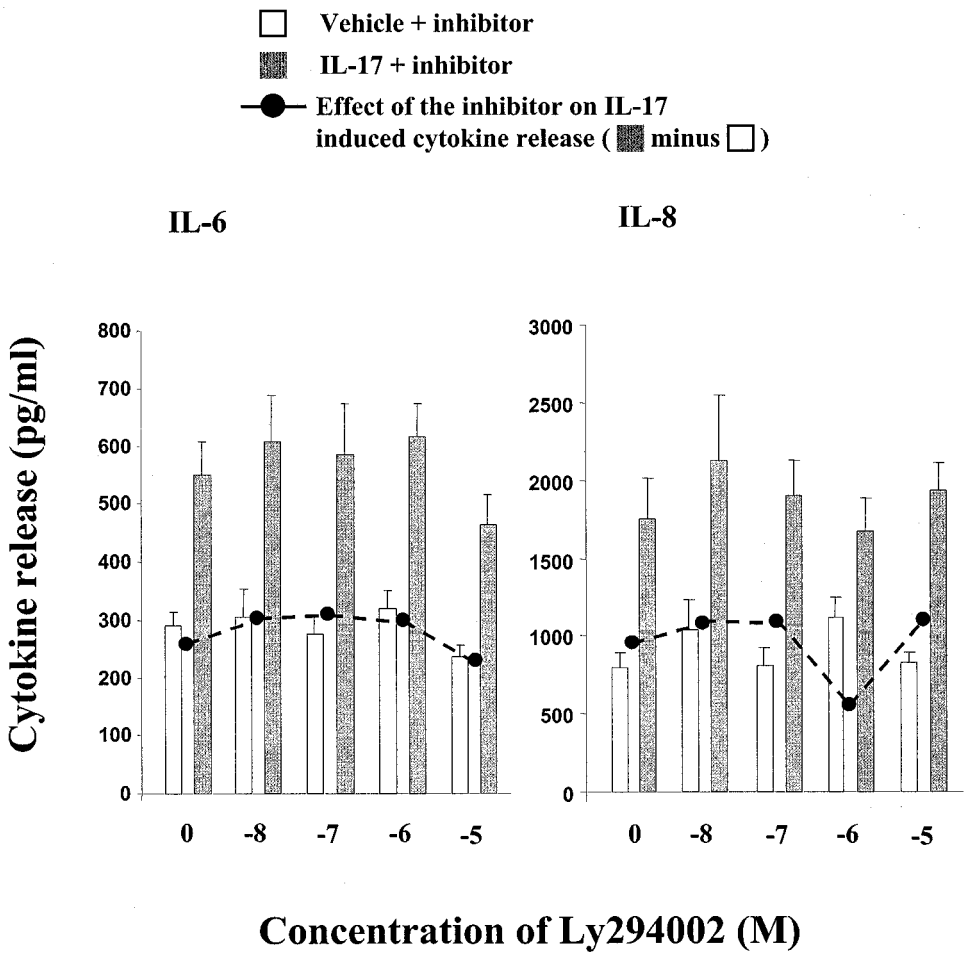

Figure 4 Effect of the PI 3-kinase inhibitor, LY294002, on IL-17 induced release of IL-6 and IL-8 in 16HBE cells. In the range from $1 \times 10^{-8} - 1 \times 10^{-5}$ M, LY294002 had no significant, concentration-dependent effect on IL-17 induced IL-6 or IL-8 in 16HBE cells (Spearman rank correlation: $P > 0.05$). Columns in the figure are mean with s.e.mean of 3–5 separate experiments.

mobilizing cytokines. These intracellular pathways constitute potential pharmacotherapeutical targets for inhibition IL-17-mediated airway neutrophilia.

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