Effects by 8-bromo-cyclicAMP on basal and organic dust-induced release of interleukin-6 and interleukin-8 in A549 human airway epithelial cells

K. Burvall*, L. Palmberg† and K. Larsson†

*National Institute for Working Life, Stockholm, Sweden, †The National Institute of Environmental Medicine, Lung- and Allergy Research, Karolinska Institute, Stockholm, Sweden

Abstract Inhalation of organic dust from a swine-confinement building leads to an intense inflammatory reaction with an increased number of inflammatory cells and mediators in the upper and lower respiratory tract of previously unexposed subjects. *In vitro* the dust induces cytokine release from epithelial cells and alveolar macrophages. It is known that intracellular cyclic AMP (cAMP) contributes to the regulation of inflammatory responses. We therefore investigated whether 8-Bromo-cAMP, a cell membrane-permeable cAMP analogue, would influence release of the cytokines interleukin-6 (IL-6) and IL-8 in a human airway epithelial cell line, A549, exposed to a suspension of the organic dust, and to a supernatant prepared by centrifugation (at low *g*-force) of a suspension of dust. The large particulate matter was thus sedimented, leaving bacteria, whole and cell wall constituents in the supernatant. Cytokine release was measured with enzyme-linked immunosorbent assay (ELISA). The cytokine release induced by a supernatant was 23% (IL-6) and 27% (IL-8) of the release induced by a dust suspension. 8-Bromo-cAMP (1 mM) doubled basal IL-6 release and IL-6 release induced by a dust supernatant ($P < 0.01$), and increased IL-6 release induced by a dust suspension by 19% ($P < 0.05$). 8-Bromo-cAMP did not affect basal IL-8 release, partially inhibited (28%) the release of IL-8 induced by a dust suspension ($P < 0.01$), but increased IL-8 release induced by a dust supernatant by 13% ($P < 0.05$). In summary, expression of the cytokines IL-6 and IL-8 is differentially regulated by 8-Bromo-cAMP, both with regard to basal and dust-induced release. The results indicate that 8-Bromo-cAMP attenuated IL-8 release by affecting signaling transductions induced by the particulate fraction.

Available online at http://www.sciencedirect.com

Keywords airway epithelial cells; A549; 8-Bromo-cAMP; cytokine release; organic dust; particles.

INTRODUCTION

Inhalation of organic dust from a swine-confinement building induces a systemic reaction (fever, malaise, muscle pain) with increased bronchial responsiveness and intense airway inflammatory reaction with an increased number of inflammatory cells and mediators in previously unexposed subjects (1,2). *In vitro* the dust induces release of the cytokines interleukin-6 (IL-6) and IL-8 in normal human bronchial epithelial (NHBE) cells, in a human airway epithelial cell line (A549) and in human alveolar macrophages (3,4).

The dust represents a complex mixture of feed, fecal particles, dander from swine, bacteria (whole bacteria and cell wall components) and fungi. Lipopolysaccharide (LPS), a constituent of the cell wall of Gram-negative bacteria and peptidoglycan, the main cell wall constituent in Gram-positive bacteria (but also present in Gram-negative bacteria) have been suggested as inducers of the airway inflammation caused by exposure to organic dust (5,6). A single causative agent has not been identified, though.

A suppressive signal in inflammation and cytokine release is generally provided by the second messenger cyclic 3′-5′-adenosine monophosphate (cAMP) (7,8). The aim of the present study was to investigate whether 8-Bromo-cAMP, a cell membrane-permeable cAMP-analogue, would influence the release of IL-6 and IL-8 induced by organic dust in a human airway epithelial cell line, A549. IL-6, initially considered a pro-inflammatory cytokine, although it has been suggested that IL-6 may act as an anti-inflammatory cytokine (9,10), also plays an active role in bone metabolism, reproduction, arthritis,

Received 9 January 2002, accepted in revised form 27 May 2002
Correspondence should be addressed to: Dr Lena, Palmberg, MD, Ph D, The National Institute of Environmental Medicine, Lung- and Allergy Research, Karolinska Institute, SE-171 77 Stockholm, Sweden.
E-mail:lena.palmberg@imm.ki.se

neoplasia and aging (11). IL-8 is a member of the chemokine superfamily and attracts neutrophils, T-cells and basophils to sites of injury (12).

The A549 cells were exposed to a suspension of organic dust and to a supernatant, prepared by centrifugation of a dust suspension at a speed (low g-force) whereby particles to the size of eukaryotic cells were sedimented and bacteria (whole bacteria and cell wall constituents) were left in the supernatant. By this approach, we hoped to obtain information on the target for a possible suppressive effect by 8-Bromo-cAMP.

MATERIAL AND METHODS

Material

8-Bromoadenosine 3′:5′-cyclic monophosphate sodium (approx. 98%) was purchased from Sigma-Aldrich, Sweden. Settled organic dust was collected in a swine-confinement building approximately 1.20 m above the floor. The dust was prepared by vortexing a stock suspension (1 mg/ml) in Ham's F12 medium and on use diluting to 100 μg/ml in Ham's F12 [with 1% penicillin/streptomycin, without fetal bovine serum (FBS)]. To obtain a supernatant containing bacteria and bacterial cell wall constituents, the suspension was subjected to centrifugation at $150 \times g$ for 9 min in a Heraeus Omnifuge 2.ORS. The supernatant was removed by aspiration. The same preparation of dust was always used for comparisons between dust suspension/dust supernatant.

Cell culture

The A549 cells (ATCC Number: CCL-185), originally described as alveolar type II (13) were cultured in Ham's F12 medium containing 10% FBS, supplemented with 1% penicillin/streptomycin and 1% L-glutamine (Ham's F12 complete medium) in a 5% CO_2 atmosphere at 37°C.

Cytokine release

The A549 cells were seeded at 1.5×10^5 cells/well in six-well plates (Nunc, Roskilde, Denmark) and grown in 2 ml Ham's F12 complete medium for 2 days, when the medium was renewed. Cell cultivation was continued for an additional 20 h to 90–100% confluency. The cells were washed twice in Ham's F12 (without FBS) prior to exposure to organic dust suspension (100 μg/ml in Ham's F12 with penicillin/streptomycin, without FBS) or dust supernatant, with or without 8-Bromo-cAMP (1 mM) for 24 h. The concentration of dust (100 μg/ml) and exposure time are based on previous studies to establish optimal conditions. On no occasion was the aspirated medium turbid, an indication of bacterial growth. The medium was centrifuged at 1000 g for 10 min to pellet all particles. The supernatants were stored at −20°C until assayed. The cells were detached by exposure to trypsin/EDTA solution (0.05%/0.02% in calcium- and magnesium-free phosphate-buffered saline; Seromed Biochrom KG, Berlin, Germany) and counted in a hemocytometer. Each experiment was performed in triplicates.

IL-6 and IL-8 in the epithelial cell supernatants were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) (14). Commercially available antibody pairs were used. For IL-6 measurement, the monoclonal antihuman capture antibody was MAB206 (R & D Systems Europe, Abingdon, U.K.) and the detection biotinylated antihuman polyclonal antibody was BAF206. The recombinant human standard was 206-IL-010 (R & D Systems Europe, Abingdon, U.K.). For IL-8 measurement, the monoclonal antihuman capture antibody was MAB208 (R & D Systems Europe, Abingdon, U.K.) and the detection biotinylated antihuman polyclonal antibody was BAF208. The recombinant human standard was 208-IL-010 (R & D Systems Europe, Abingdon, U.K.). Absorbance was read at 450 and 650 nm with a Thermomax 250 reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The cytokines were measured and production expressed as pg/10^6 cells. The detection range of the IL-6 assay was 3–375 pg/ml and of the IL-8 assay 50–3200 pg/ml.

Statistics

Data are presented as mean ± SEM based on nine samples (three separate experiments in triplicate). Statistical analysis was performed by the use of Wilcoxon signed rank test. A P value of < 0.05 was considered significant.

RESULTS

Effects by 8-Bromo-cAMP on basal IL-6 and IL-8 release and cell number

Cultivation for 24 h with 8-Bromo-cAMP (1 mM) doubled basal IL-6 release ($P < 0.01$), but did not influence basal release of IL-8 ($P = 0.69$) (Fig. 1). The increased cAMP level during the 24-h incubation did not affect cell number, which was similar in the presence [1.19 (± 0.09) $\times 10^6$ cells] and absence [1.18 (± 0.10) $\times 10^6$ cells] of 8-Bromo-cAMP (1 mM).

IL-6 release induced by a dust suspension/dust supernatant

The dust suspension (100 μg/ml) induced a 21-fold increase ($P < 0.01$) and a dust supernatant a five-fold increase ($P < 0.01$) in IL-6 production compared with basal release (Fig. 1A). 8-Bromo-cAMP doubled the supernatant-induced IL-6 release ($P < 0.01$), whereas the enhancing effect on IL-6 release induced by dust suspension was less pronounced (+19%, $P < 0.05$) (Fig. 1A).

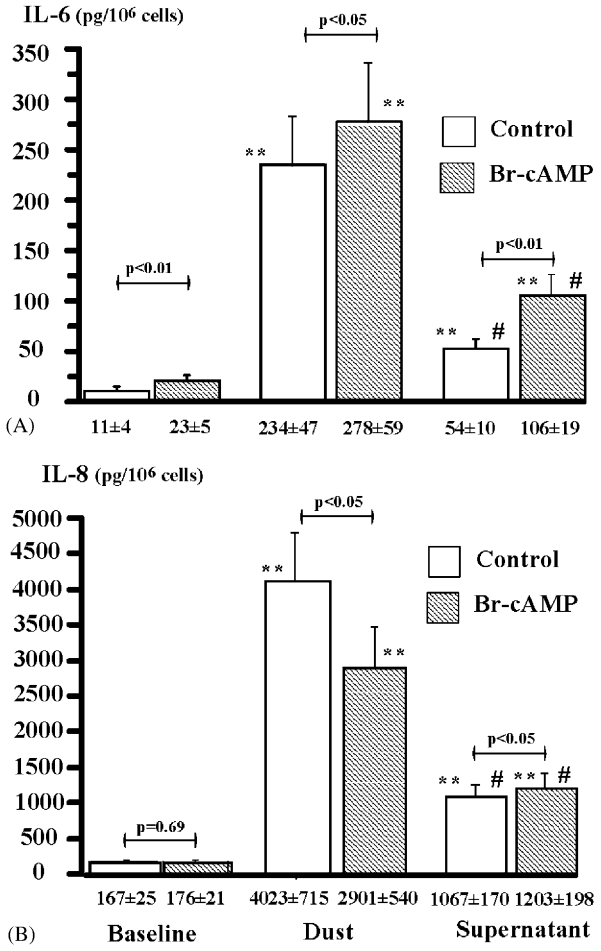

Fig. 1. The effect by 8-Bromo-cAMP on production of (A) IL-6 and (B) IL-8 in A549 cells; basal release and the enhanced production induced by exposure to a suspension of organic dust from a swine-confinement building and to a supernatant obtained by centrifugation of a dust suspension. The results (pg/10^6 cells) are expressed as means ± SEM of nine samples (three separate experiments in triplicate) and given below each bar. $^{**}P < 0.01$ compared with basal release, # $P < 0.01$ compared with dust.

IL-8 release induced by a dust suspension/ dust supernatant

Dust suspension (100 μg/ml) induced a 24-fold increase of IL-8 release in the absence and 16-fold increase in the presence of 8-Bromo-cAMP (1 mM) ($P < 0.01$ compared with basal release, Fig. 1B). The attenuation (28%) of dust-induced IL-8 release by 8-Bromo-cAMP was statistically significant ($P < 0.05$; Fig. 1B). In contrast, 8-Bromo-cAMP enhanced the IL-8 release induced by a dust supernatant by 13% ($P < 0.05$) (Fig. 1B).

DISCUSSION

In the present study, it was demonstrated that a cell membrane permeable cAMP analogue (8-Bromo-cAMP) stimulated basal IL-6 but not basal IL-8 release in a human airway epithelial cell line, A549. We have also shown that the cAMP analogue enhanced IL-6 release induced by a dust suspension but attenuated the IL-8 release induced by the same agent.

The use of cAMP enhancers have lead to somewhat diverging effects in *in vitro* studies on IL-8 release with human airway cell lines. In one human bronchial epithelial cell line, BEAS-2B, TNFα-stimulated IL-8 release was unaffected by pretreatment with forskolin, a direct activator of adenylyl cyclase in combination with rolipram, a specific inhibitor of cAMP-phosphodiesterase (15). In another human bronchial epithelial cell line (16HBE), basal and TNFα-stimulated IL-8 release was increased by β_2-adrenoceptor activation (leading to increased intracellular cAMP) and by the stable cAMP analogue, dibutyryl-cAMP (1 mM) (16). In the present study, a dual effect by 8-Bromo-cAMP was observed with regard to IL-8 release, since the cAMP-analogue enhanced IL-8 release induced by a dust supernatant, which is devoid of the particulate fraction, whereas a partial inhibition of IL-8 production was demonstrated when the A549 cells were exposed to a "complete" dust suspension, which also induced a several-fold increase in cytokine release compared with the supernatant.

For maximal induction of IL-8 synthesis and release, the activation of several signal transduction pathways, which cooperate to induce mRNA synthesis and suppress mRNA degradation, is required (17). The organic dust represents a complex activating agent and several signaling transduction pathways are consequently affected. The attenuation (28%), by 8-Bromo-cAMP, of the IL-8 production induced by a "complete" dust suspension therefore represents the net effect of an enhancement of the IL-8 release induced by agents in the supernatant and an inhibition of signaling transductions induced by agents in the particulate fraction. A complete inhibition of dust-induced IL-8 release by increased cAMP could therefore not be expected. Furthermore, with the concentration of dust used (100 μg/ml) not all cells were hit by a particle, which provides an additional explanation to why the inhibition was only partial in the present experimental set-up.

Although increased cAMP is generally thought to inhibit cytokine release, the secretion of IL-6 in response to organic dust was enhanced by 8-Bromo-cAMP in the A549 cells. Upregulated expression of IL-6, in response to elevated cAMP, has been reported for several cell types including human anterior pituitary cells and mesangial cells, osteoblasts and murine macrophages (18). The promoter for the IL-6 gene contains cAMP-responsive element (CRE) concensus motifs (19), which provides one explanation for the increase in IL-6 production by cAMP-enhancing agents.

In a study on alveolar type II cells isolated from rat fetuses, blockade of cAMP-phosphodiesterase activity was shown to attenuate IL-6 production induced by LPS at

10 μg/ml (20). However, results obtained with pure LPS at high concentrations are not comparable with those from this study on organic dust, since the LPS concentration in the dust suspension and dust supernatant is low, <0.5 ng/ml, with no major difference between the suspension and supernatant (unpublished results). Thus, it is unlikely that the effect by 8-Bromo-cAMP was exerted on LPS-induced cytokine release in the present study.

Furthermore, a previous dose–response study has shown that the A549 cells used in this laboratory are non-responders to LPS with regard to IL-6 release (4). Sensitivity to LPS at low doses is mediated by the Toll-like receptor (TLR) 4 (21,22), and a polymorphism at the TLR 4 locus has been shown to cause differences in response to LPS between and within mammalian species (23). Defects in the TLR 4 -gene of the A549 cells could, therefore, explain the absence of IL-6 production on exposure to LPS and the high dose of LPS (10–100 μg/ml) required to obtain an increased release of IL-8 (3). The LPS-induced production of IL-8 increased more in the absence than in the presence of serum (3), and was, therefore, more likely caused by unspecific activation of the A549 cells and not a result of receptor activation.

The discrepancies, between studies, in the effects on cytokine release by enhanced cAMP can be caused by several factors such as variations between species, between primary cells and cell lines, the mode by which intracellular cAMP is increased or different activating agents. All other parameters being the same in the present study, the varying effect by increased cAMP on IL-8 release induced by a dust suspension and a dust supernatant is probably due to variations in the signaling transduction induced by the particles in the dust suspension *vs*. the "particle-free" supernatant. The dust used in the study is settled dust, collected approximately 1.20 m above the floor, and therefore represents respirable dust.

In summary, production of the cytokines IL-6 and IL-8 was differentially regulated by 8-Bromo-cAMP with regard to dust-induced release. Thus, 8-Bromo-cAMP downregulated an inflammatory signal (IL-8) and at the same time upregulated an anti-inflammatory response mediated by IL-6. Attenuation of IL-8 release by 8-Bromo-cAMP administration was obtained only on activation with "complete" dust and not on activation with a supernatant, when IL-8 release was slightly increased. The target for inhibition by 8-Bromo-cAMP therefore appears to be the particulate fraction, which also was required for maximal induction of cytokine release from the A549 cells.

Acknowledgements

We thank Siw Siljerud for skilful technical assistance and Dr Britt-Marie Larsson for help with the endotoxin measurement.

REFERENCES

1. Larsson K, Eklund A, Hansson L-O, Isaksson B-M, Malmberg P. Swine dust causes intense airways inflammation in healthy subjects. *Am J Crit Care Med* 1994; **150:** 973–977.
2. Wang Z, Larsson K, Palmberg L, Malmberg P, Larsson P, Larsson L. Inhalation of swine dust induces cytokine release in the upper and lower airways. *Eur Respir J* 1997; **10:** 381–387.
3. Palmberg L, Larsson B-M, Malmberg P, Larsson K. Induction of IL-8 production in human alveolar macrophages and human bronchial epithelial cells in vitro by swine dust. *Thorax* 1998; **53:** 260–264.
4. Wang Z, Malmberg P, Ek A, Larsson K, Palmberg L. Swine dust induces cytokine secretion from human epithelial cells and alveolar macrophages. *Clin Exp Immunol* 1999; **115:** 6–12.
5. Sandström T, Bjermer L, Rylander R. Lipopolysaccharide (LPS) inhalation in healthy subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid. *Eur Respir J* 1992; **5:** 992–996.
6. Wang Z, Malmberg P, Larsson B-M, Larsson K, Larsson L, Saraf A. Exposure to bacteria in swine-house dust and acute inflammatory reactions in humans. *Am J Respir Crit Care Med* 1996; **154:** 1261–1266.
7. Zhang H, Kim Y, Govindarajan A, *et al*. Effect of adrenoceptors on endotoxin-induced cytokines and lipid peroxidation in lung explant. *Am J Respir Crit Care Med* 1999; **160:** 1703–1710.
8. Hallsworth M, Twort C, Lee T, Hirst S. β_2-adrenoceptor agonists inhibit release of eosinophil-activating cytokines from human airway smooth muscle cells. *Br J Pharmacol* 2001; **132:** 729–741.
9. Tilg H, Dinarello CA, Mier JW. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Viewpoint Immuno Today* 1997; **18:** 428–432.
10. Xing Z, Gauldie J, Cox G, *et al*. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* 1998; **101:** 311–320.
11. Keller TK, Wanagat J, Ershler W. Molecular and cellular biology of interleukin-6 and its receptor. *Front Biosci* 1996; **1:** 340–357.
12. Ben–Baruch A, Michiel DF, Oppenheim J. Signals and receptors involved in recruitment of inflammatory cells. *J Biol Chem* 1995; **270:** 11703–11706.
13. Leiber M, Smith B, Szakal A, Nelson-Rees W, Todaro G. A continous tumor cell line from human lung carcinoma with properties of type II alveolar epithelial cells. *Int J Cancer* 1976; **17:** 62–70.
14. Ek A, Larsson K, Siljerud S, Palmberg L. Fluticasone and budesonide inhibit cytokine release in human lung epithelial cells and alveolar macrophages. *Allergy* 1999; **54:** 691–699.
15. Fuhrmann M, Jahn H-U, Seybold J, *et al*. Identification and function of cyclic nucleotide phosphodiesterase isoenzymes in airway epithelial cells. *Am J Respir Cell Mol Biol* 1999; **20:** 292–302.
16. Linden A. Increased interleukin-8 release by beta-adrenoceptor activation in human transformed bronchial epithelial cells. *Br J Pharmacol* 1996; **119:** 402–406.
17. Holtmann H, Winzen R, Holland P, *et al*. Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *Mol Cell Biol* 1999; **19:** 6742–6753.
18. Zidek Z. Adenosine-cyclicAMP pathways and cytokine expression. *Eur Cytokine Network* 1999; **10:** 319–328.
19. Sitaraman S, Merlin D, Wang L, *et al*. Neutrophil-epithelial crosstalk at the interstitial lumenal surface mediated by reciprocal secretion of adenosin and IL-6. *J Clin Invest* 2001; **107:** 861–869.

20. Haddad JJ, Land S, Tarnow-Mordi W, *et al*. Immunopharmacological potential of selective phosphodiesterase inhibition. I. Differential regulation of lipopolysaccharide-mediated proinflammatory cytokine (interleukin-6 and tumor necrosis factor-α) biosynthesis in alveolar epithelial cells. *J Pharmacol Exp Ther* 2002; **300:** 559–566.
21. Lien E, Ingalls RR. Toll-like receptors. Crit Care Med 2002; **30:** S1–S11.
22. Hornef MW, Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J Exp Med* 2002; **195:** 559–570.
23. Smirnova I, Poltorak A, Chan E, McBride C, Beutler B. Phylogenetic variation and polymorphism at the Toll-like receptor 4 locus (TLR4). *Genome Biol* 2000; **1:** 1–10.