SECTION 10

EPIDEMIOLOGY—DUSTS OF BIOLOGICAL ORIGIN

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DETECTION OF *n*-FORMYL-METHIONYL-LEUCYL-PHENYLALANINE (FMLP) IN COTTON DUST: BIOLOGICAL ACTIVITIES OF FMLP ASSOCIATED WITH PULMONARY RESPONSES TO COTTON DUST EXPOSURE*

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Abstract—Inhalation of cotton dust (20 mg m^{-3} for 6 h) by guinea pigs is marked by infiltration of granulocytes into the airways (11.7-fold) and airway closure (1.3-fold). In addition, aqueous extracts of cotton dust cause constriction of tracheal smooth muscle *in vitro*. Analysis of water or acetone extracts of cotton dust by HPLC reveals substantial quantities (mg g^{-1}) of both FMLP and oxidized FMLP. As with cotton dust extracts, FMLP is an agonist of guinea pig tracheal smooth muscle (EC_{50} = 91 nM FMLP). Both reduced and oxidized FMLP stimulate chemotaxis and respiratory burst activity in granulocytes. Furthermore, activated pulmonary granulocytes isolated from cotton dust-exposed guinea pigs induce contraction of trachealis muscle *in vitro*. Thus, FMLP may be involved in the pulmonary response to cotton dust exposure directly by causing airway constriction and indirectly by inducing inflammation characterized by granulocyte infiltration and activation.

INTRODUCTION

BYSSINOSIS is an occupational disease common to textile workers exposed to cotton, hemp or flax dusts. Byssinosis is characterized by the occurrence of chest tightness which is most dramatic on Mondays and declines as the week progresses (SCHILLING, 1956). This chest tightness is accompanied by increased airway resistance and decreased forced expiratory volume (MCKERROW *et al.*, 1958; MERCHANT *et al.*, 1974). Byssinosis is also associated with an inflammatory reaction characterized by infiltration of granulocytes into the airways (RYLANDER, 1990).

Animal models have been developed which mimic the human response to inhalation of cotton dust (ELLAKKANI *et al.*, 1984; CASTRANOVA *et al.*, 1987). Such models have demonstrated similar responsiveness to cotton dust extract (RYLANDER and NORDSTRAND, 1974) and endotoxin (FISCHER *et al.*, 1986). In human subjects, a strong correlation has been demonstrated between pulmonary function decline and the endotoxin content of cotton dusts (CASTELLAN *et al.*, 1987). Therefore, endotoxin has been proposed as a possible etiologic agent for byssinosis.

Recently, ROHRBACH (1991) reviewed evidence supporting tannin as a possible aetiological agent. *In vitro* effects of tannin include: decreasing the phagocytotic activity of alveolar macrophages; stimulating the release of neutrophil chemotactic agents by alveolar macrophages and airway epithelial cells; increasing arachidonic acid and interleukin-1 secretion from macrophages; and stimulating hydrogen peroxide

*This paper was included in Poster Session 7 and the discussion included in the summary presented in Section 12.

release from neutrophils. LAUQUE *et al.* (1988) have shown that inhalation of tannin results in pulmonary infiltration of neutrophils and the release of prostaglandin $F_{2\alpha}$ which could partially explain the inflammation and bronchoconstriction characteristic of cotton dust exposure.

AINSWORTH (1981) reported that a component of cotton dust extract distinct from endotoxin or tannin expressed chemotactic activity for neutrophils. They reported that this unidentified substance had a molecular weight between 200 and 2000, and proposed that it represented another possible aetiological agent for byssinosis.

In light of this possibility, the objective of the present study was to determine if the chemotactic peptide, *n*-formyl-methionyl-leucyl-phenylalanine (FMLP), was present in cotton dust and if so whether it exhibited pulmonary activities similar to those associated with cotton dust.

METHODS

Specific pathogen-free male English short hair guinea pigs (250–300 g) were obtained from Camm Research Laboratory Animals, Wayne, New Jersey. The bulk cotton dust sample used in this study was DB1/88 supplied by Cotton Incorporated, New York.

For inhalation exposure studies, 30 g of bulk cotton dust was placed in a generator and aerosolized with acoustical energy. Guinea pigs were exposed to 20 ± 1.5 mg cotton dust m^{-3} for 6 h. The count-median aerodynamic diameter of the aerosol was 1.5 ± 0.2 m. The dust generation and exposure system have been described in detail previously (FRAZER *et al.*, 1987).

Pulmonary inflammation was quantified as the number of granulocytes obtained by bronchoalveolar lavage with Ca^{2+}-, Mg^{2+}-free phosphate-buffered solution. Airway closure was determined by measuring *post mortem* pulmonary hyperinflation due to gas trapping (FRAZER *et al.*, 1989).

Soluble cotton dust components were extracted in either water or acetone. Water extracts were obtained by mixing 28.33 g of bulk cotton dust in 2 l. of water for 2 h at 22°C. The suspension was then centrifuged (3000 ***g*** for 15 min), filtered, lyophilized, reconstituted in 0.9% saline or distilled water and frozen. Acetone extracts were obtained by mixing 0.5 g of bulk cotton dust in 5 ml of acetone for 24 h. The suspension was filtered, lyophilized and reconstituted in distilled water.

Reduced and oxidized FMLP were analysed with an HPLC using a reverse-phase Bondapak C_{18} column and one of two mobile phases: (1) 50% methanol–50% 0.1 M acetic acid; or (2) 50% acetonitrile–50% 0.1 M acetic acid (VAN DYKE *et al.*, 1984). Oxidized FMLP was prepared by treating FMLP with hydrogen peroxide (0.75% final concentration) for 12 h at 22°C.

Airway contraction in response to agents was measured using isolated trachealis smooth muscle. Briefly, the trachea was removed from a guinea pig and muscle strips attached to a force–displacement transducer. The muscle strips were bathed in Krebs–Henlseleit solution at 37°C under optimum resting force (1–1.1 ***g***) for measurement of isometric contraction. Preparations were equilibrated for 1 h, a reference response to 120 mM KCl measured and then the test response determined.

Human peripheral granulocytes were obtained from venous blood and purified by dextran settling followed by centrifugal elutriation (JONES *et al.*, 1980). Superoxide

TABLE 1. PULMONARY RESPONSE TO INHALATION OF COTTON DUST*

Treatment	Granulocytes (cells per guinea pig)	Trapped gas (ml kg^{-1} lung)
Pre-exposure	$0.31 \pm 0.1 \times 10^7$	2.21 ± 0.29
0 h post-exposure	$1.45 \pm 0.4 \times 10^7$	5.00 ± 0.52
18 h post-exposure	$3.93 \pm 0.4 \times 10^7$	2.99 ± 0.59

*Values are means ± standard errors of measurements from six animals.

TABLE 2. RESPONSE OF TRACHEAL SMOOTH MUSCLE TO *in vitro* TREATMENT WITH COTTON DUST EXTRACT*

Cotton dust extract (mg ml^{-1})	% of response to 120 mM KCl
0.1	0
1	30 ± 9
3	62 ± 10

*Values are means ± standard errors of five experiments.

anion O_2^- production (nmoles per min per 10^6 cells) was measured spectrophotometrically by monitoring cytochrome C reduction at 550 nm. Hydrogen peroxide (H_2O_2) secretion (nmoles per min per 10^6 cells) was determined fluorometrically by measuring the degradation of scopoletin at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. Oxygen (O_2) consumption (nmoles per min per 10^6 cells) was measured with a Clark electrode. Chemotaxis was monitored by measuring cell migration (mm) using the leading front assay. Details of these assays have been described previously (VAN DYKE *et al.*, 1984).

Pulmonary granulocytes were obtained from guinea pigs 18 h after exposure to cotton dust. Briefly, cells were obtained by bronchoalveolar lavage with 10 aliquots (8 ml) of Ca^{2+}-, Mg^{2+}-free phosphate-buffered solution. Granulocytes were purified by centrifugal elutriation (JONES *et al.*, 1980). Preparations were approximately 90% pure as determined using an electronic cell counter equipped with a cell sizing attachment.

RESULTS

In response to inhalation of cotton dusts (20 mg m^{-3} for 6 h), both pulmonary inflammation and airway closure were noted (Table 1). Lavagable granulocytes increased 3.7-fold immediately following exposure and rise to an 11.7-fold increase 18 h post-exposure. In contrast, airway closure was most apparent immediately following cotton dust exposure (1.3-fold) and returned towards normal after 18 h.

Aqueous extract of bulk cotton dust exhibited agonist activity toward tracheal smooth muscle. Airway constriction was maximal at an extract concentration of 3 mg ml^{-1} (Table 2).

Analysis of cotton dust extracts by HPLC revealed peaks exhibiting retention times identical to those for reduced ($T_R = 16$ min) and oxidized ($T_R = 8.4$ min) FMLP standards (Fig. 1). Treatment of cotton dust extract or extract fractions collected from

peak (a) of the chromatogram with hydrogen peroxide resulted in ablation of peak (a), i.e. reduced FMLP and an increase in peak (b), i.e. oxidized FMLP. Quantification of reduced and oxidized FMLP in cotton dust extracts indicated that relatively high levels of this bacterially-derived chemotactic peptide could be found in cotton dust (Table 3).

TABLE 3. LEVELS OF REDUCED AND OXIDIZED FMLP IN COTTON DUST

Extraction	Reduced FMLP (mg g^{-1} dust)	Oxidized FMLP (mg g^{-1} dust)
Water	0.1	0.6
Acetone	3.2	6.0

As with cotton dust extract, addition of FMLP to isolated tracheal smooth muscle preparations induced contraction (Fig. 2). This contraction was transient occurring rapidly, peaking in approximately 3–5 min and then slowly returning toward baseline. The response was dose-dependent with an EC_{50} of 91 nM. In addition, pre-treatment with FMLP inhibited the response to subsequent treatment with FMLP but not with methacholine.

Reduced FMLP was also a potent stimulant of granulocytes, i.e. maximally increasing chemotaxis and respiratory burst activity at 10^{-7} and 10^{-6} M, respectively. Oxidized FMLP also activated granulocytes, although it was somewhat less potent than FMLP, with effects being significant at 10^{-6} M for both chemotaxis and the respiratory burst (Table 4).

Treatment of isolated tracheal smooth muscle from an unexposed guinea pig with pulmonary PMN obtained from a cotton dust-exposed guinea pig resulted in constriction (Fig. 2). Thus, the bronchoconstriction and granulocyte infiltration seen after cotton dust exposure (Table 1) may be causally related.

DISCUSSION

Data presented in this study indicate that cotton dust contains substantial levels of *n*-formyl-methionyl-leucyl-phenylalanine (FMLP) in both the reduced and oxidized form. Evidence supporting this statement is as follows: (1) high-pressure liquid chromatography yields peaks with retention times similar to those for reduced and oxidized FMLP standards; (2) material collected from the reduced FMLP peak and then oxidated with H_2O_2 migrates as oxidized FMLP; and (3) treatment of cotton dust extract with hydrogen peroxide results in ablation of the reduced FMLP peak and enhancement of the oxidized FMLP peak.

FMLP is a chemotactic peptide of bacterial origin. Therefore, it would be expected to be found in cotton dust which is often contaminated with high levels of viable and non-viable bacteria. AINSWORTH *et al.* (1981) reported the presence of a low molecular weight (200–2000) substance in cotton dust extract which was chemotactic to neutrophils. The molecular weight of FMLP is 438 and data in Table 4 indicate that it is a potent chemoattractant for granulocytes. Data from the present study indicate that FMLP, like cotton dust extract, stimulates constriction of tracheal smooth muscle. BISHOP *et al.* (1986) have reported that a chemical analogue of FMLP, i.e. *n*-formyl-

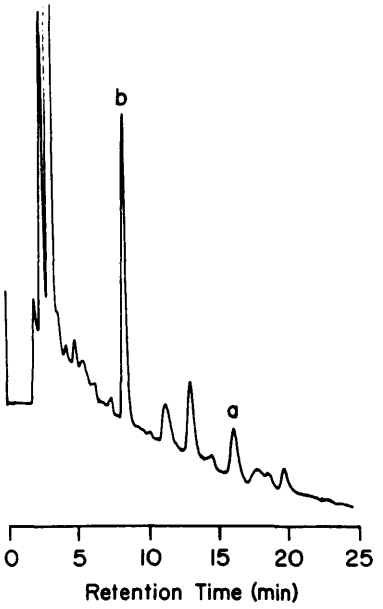

FIG. 1. Chromatogram of cotton dust extract using mobile phase 1, u.v. detection set at 254 nm and a flow rate of 1 ml min^{-1}. Peak (a) has a retention time ($T_R = 16$ min) identical to reduced FMLP. Peak (b) has a retention time ($T_R = 8.4$ min) identical to oxidized FMLP.

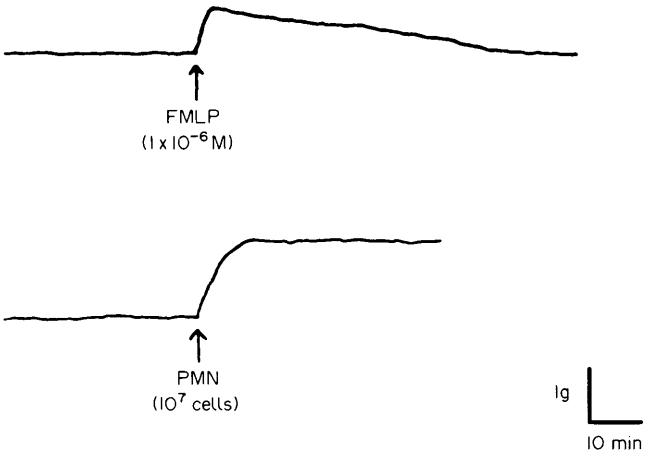

FIG. 2. The response of isolated tracheal smooth muscle to the addition of FMLP or granulocytes (PMN) isolated from cotton dust-exposed guinea pigs.

methionyl phenylalanine, caused an increase in airway resistance in rabbits and that this response was similar to that induced by cotton dust extract. BUCK *et al.* (1986) have reported that constrictive potency of cotton bract extract is not dependent on endotoxin. Further research found that the bronchoconstricting agent had a molecular weight of less than 500 and fluoresced at 430 nm (BUCK and WALL, 1988). Data from

TABLE 4. EFFECTS OF *in vitro* TREATMENT OF GRANULOCYTES WITH REDUCED OR OXIDIZED FMLP*

Treatment	Chemotaxis	O_2 consumption	O_2^- release	H_2O_2 secretion
Reduced FMLP	116	500	3840	6470
Oxidized FMLP	71	280	510	4760

*Values given are per cent increases above resting levels.

our laboratory indicate that FMLP exhibits a fluorescent peak at 436 nm. These data support the hypothesis that airway constriction following cotton dust exposure may be due in part to the presence of FMLP in the dust.

Data in Table 4 indicate that both reduced and oxidized FMLP stimulate granulocytes. Indeed, granulocyte infiltration is characteristic of the pulmonary reaction to cotton dust exposure (Table 1). It is of interest that the pulmonary granulocytes induce airway constriction *in vitro* (Fig. 2).

In summary, both bronchoconstriction and inflammation associated with cotton dust exposure may be due in part to the direct actions of FMLP on airway smooth muscle and on granulocytes. In addition, bronchoconstriction may also be an indirect response to FMLP-mediated activation of granulocytes.

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