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Plant constituents of cotton dust and lung effects after inhalation

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ABSTRACT: Experiments were performed to assess pulmonary reactions after inhalation of cotton dusts with different levels of tannins, terpenoid aldehydes and bacterial endotoxins. Guinea-pigs were exposed to cotton dust. Free lung cells were obtained by lavage 24 h later. A dose-response relationship was found between the number of neutrophils and the amount of endotoxin in the dust. No influence of terpenoid aldehyde or tannin levels could be detected. Cotton mill workers were exposed to dust from glanded and glandless cottons in an experimental cardroom. The average decreases in forced expiratory volume in one second (FEV_1) over the workday after carding the two cottons were the same, although levels of dust, tannin or terpenoid aldehydes were different. The level of airborne endotoxin was, however, equal. The results support observations from other studies on the importance of endotoxin for the development of the acute reactions observed after cotton dust exposure.
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Persons exposed to cotton dust may experience a series of symptoms, some of which are most significant the first workday of the week and are collectively referred to as byssinosis [1]. A decrease in respiratory function, most commonly estimated as the forced expiratory volume in one second (FEV_1), occurs among workers as well as previously unexposed subjects. A subjective feeling of chest tightness may, likewise, occur among workers as well as among previously unexposed subjects, although higher exposure levels are required for the latter. After several years of exposure to high levels of dust, chronic bronchitis or symptoms of occupational asthma may develop. In experimental and epidemiological studies the change in respiratory function over the shift is often used as a criterion of the reaction to cotton dust.

Several studies have shown significant dose-response relationships between the amount of bacterial endotoxin in the dust and the decrease in pulmonary function over the workshift or feeling of chest tightness [2–6]. In spite of this, the role of different chemical agents derived from the cotton plant in the development of these effects cannot be ruled out. Such agents could also predispose the various cells involved in the reaction to respond to endotoxin in an augmented fashion.

During recent years several studies have dealt with tannins which are present in cotton dust. In a series of *in vitro* experiments, tannins prepared from cotton dust have been shown to contract smooth muscle, activate platelets and release chemotactic agents from alveolar macrophages (AM) [7–10]. On the other hand, extracts from flax dust, which may also cause byssinosis, are able to contract smooth muscle and release 5-HT from human platelets *in vitro*, although no condensed tannins are present in flax [11]. Furthermore, tannins are poorly soluble in water.

In view of the controversy concerning the importance of components originating form the cotton plant in the development of reactions after inhalation, experiments were undertaken in which animals and humans were exposed by inhalation to two cotton dusts which contained different levels of plant derivatives, particularly tannins and terpenoid aldehydes.

Materials and methods

Animal exposure

In the animal experiments a standard cotton dust provided by Cotton Incorporated was used. Part of this dust was extracted (at the National Cotton Pathology Laboratory, College Station, Texas) twice with a 75:25 mixture of hexane and ethyl acetate followed by two extractions with 70:30 acetone:water in 0.1% ascorbic acid solution. This process removed all terpenoid aldehydes and about 60% of the tannins [12]. The remaining portion of tannins was bound to the particles. The amount of endotoxin on the dust was not influenced by the treatment.

Aerosols from the standard and treated dusts were generated using an apparatus consisting of a plexiglass plate with a groove into which the dust was placed. The plate rotates under a Venturi device, which sucks the dust out of the groove into an exposure chamber. The amount of dust in the chamber was controlled by

varying the size of the groove and the rotation speed. Guinea-pigs weighing 300–400 g were placed in the chamber for two hours.

Determinations of the amount of airborne dust in the exposure chamber were made by sucking samples of air through Millipore filters which were weighed. The amount of endotoxin on the filter was determined using the Limulus method as described previously [13].

Exposure effects

The animals were killed with an intraperitoneal injection of pentothal 24 h after the cessation of exposure, and a lung lavage was performed. Ten ml of saline was gently introduced into the trachea through a cannula and sucked back into the syringe again. The process was repeated ten times with the same fluid. About 6 ml of the washout fluid was retrieved. It has previously been shown that the number of cells per ml in the retrieved portion and that remaining in the lung is the same - the value in the washout fluid can thus be used to calculate the total number of cells in the airways [14].

The number of different free lung cells in the lavage fluid was counted in a Bürker chamber and the number of neutrophils was determined by centrifuging the cell suspension in a Cyto-centrifuge and staining with Giemsa's stain.

Lactate dehydrogenase (LDH) in the lavage fluid was determined immediately after the washing by using a kit from Sigma (Sigma Technical Bulletin No. 340 uv). LDH was expressed as units·ml^{-1} liberated per minute.

N-acetyl-ß-D-glucosaminidase (NAGase) and Cathepsin D were assayed by the methods of BARRETT and HEALTH [15]. Enzyme activities were expressed in µmol·ml^{-1} p-nitrophenol (NAGase) or units·ml^{-1} tyrosine (Cathepsin D) liberated per hour (all reagents from Sigma). Acid phosphatase was assayed by the method of Sigma (Sigma Technical Bulletin No. 104) and expressed in µmol·ml^{-1} liberated p-nitrophenol per hour. The sample of lavage was considered as representative to the total volume of liquid present in the lung and the values for cells and enzymes were expressed per ml and multiplied by ten to obtain value per lung.

Human exposures

The studies on humans were performed in an experimental cardroom measuring 6.4x5.3x2.8 m. A carding machine was situated in the middle of the room and cotton laps were processed. The dust levels could be varied by adjusting the ventilation.

Most cottons grown throughout the world have lysigenous pigment glands, which contain a large number of biologically active compounds including gossypol and volatile terpenes. Glandless cottons contain no pigment glands and the amount of glanded components is low or nonexistent.

In the experiments on humans, cotton from glanded and glandless cotton plants of the Stoneville variety (S213 and S209, respectively) grown in adjacent plots in Texas, USA, were used. The laps used for carding were coded and the code was not known when the experiments were performed. The dust exposure in the cardroom was determined as the average value from three vertical elutriators placed on different sides of the carding machine.

The filters were weighed to obtain the values for dust levels and the amount of bacterial endotoxin was determined using the Limulus amoebocyte assay [13].

In view of the previously described close relationship between levels of airborne endotoxin and decrease in lung function over the workshift, the exposures were adjusted to give airborne endotoxin values as similar as possible when the two kinds of cotton were carded.

Workers from a cotton mill, some of whom occasionally suffered from light chest tightness during work on Mondays, were recruited on a voluntary basis and studied on a Monday after an exposure-free week end. The subjects were sitting and walking in the experimental cardroom where cotton was being carded between 08.00 and 14.00 h. The experiment was carried out according to the principles of the Declaration of Helsinki. The study was approved by the Ethical Committee at the Medical Faculty.

A group of three workers was first exposed to glanded, and two weeks later to glandless cotton and again a month later to glanded cotton. Another group of four workers was first exposed to glandless cotton and at similar intervals as the previous group to glanded and glandless cotton.

As the reaction studied was of an acute nature and the workers were exposed to cotton dust in their normal work between experiments, all observations were treated as independent variables.

Before and after carding for six hours, the forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were determined using a Collins survey water spirometer with a microprocessor (Eagle one) according to the procedures recommended by the American Thoracic Society. The single, largest FEV_1 was expressed as a percentage of the predicted value referring to standard values for subjects with the same age and height. The differences in these FEV_1 and FVC percentages of predicted values before and after the workshift were calculated and the average change over the shift among all workers in a group exposed to the same kind of cotton was calculated (ΔFEV_1 % of predicted).

The subjects were also interviewed using a questionnaire including questions on the presence of cough, chest tightness, airways irritation and fever at the end of the exposure.

Statistical treatment

Differences between different groups in the animal and human experiments were evaluated for statistical difference using Student's t-test.

Results

Animal studies

The number of neutrophils in lungs of animals exposed to different kinds of cotton dust is reported in table 1. Exposure to the standard dust and to the dust with low levels of terpenoids and tannins caused an increase in the number of neutrophils in the airways. The dose-response relationship between the neutrophil response and the amount of airborne endotoxin in the different experiments is illustrated in figure 1. The dust with a low level of tannin and terpenoid aldehydes caused an invasion of neutrophils as could be predicted from its content of endotoxin.

Table 1. – Number of neutrophils x10^6 in lungs of animals 24 h after exposure to cotton dust.

Exposure	Dust mg·m^{-3}	Endotoxin µg·m^{-3}	Neutrophils x10^6
Unexposed•	–	–	0.2 (0.2)
Standard dust	5.0	1.3	2.3 (1.7)
Standard dust	42.6	2.2	21.9 (12.3)**
Tannins and terpenoids removed	27.3	1.9	14.9 (10.2)**

Mean from ten animals in each group with standard deviation in parenthesis. Difference from unexposed: **p<0.01; •: ani-

Table 2 reports the data from enzyme determinations in lung lavage fluid. Exposure to the standard dust as well as to the dust with a low amount of terpenoid aldehydes and tannin, caused an increase in the amount of LDH in the lavage fluid. The amounts of NAGase and acid phosphatase were also increased. There were no significant differences in enzyme levels in lavage fluid from animals exposed to the two different dusts.

Human studies

The content of endotoxin in the two different kinds of cotton was equal–1.6 µg·g^{-1} of fibre. The dust generated from the glandless cotton contained an average of 0.7 µg of endotoxin per mg dust. (SD=0.2), whereas the dust from the glanded cotton contained 0.4 µg per mg dust (SD=0.2). When the cottons were carded the dust levels ranged between 1.3 and 3.9 mg·m^{-3}. Airborne endotoxin levels ranged from 0.6–1.6 µg·m^{-3}.

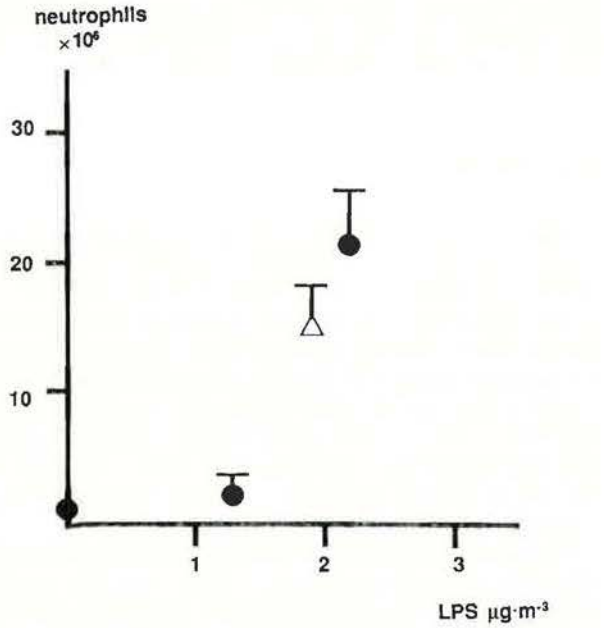

Fig. 1. – Amount of endotoxin (lipopolysaccharide, LPS) in different cotton dusts and number of neutrophils in lung lavage fluid 24 h after exposure. Filled circles: standard dust; open triangle: dust from which tannins and terpenoids were removed; bars: standard deviation; circle at origin: control value.

The amount of airborne endotoxin, when carding glandless cotton, was 1.0 µg·m^{-3} (SD=0.21) and the amount when carding glanded cotton was 1.2 µg·m^{-3} (SD=0.43). The experimental requirements had thus been met. Dust levels between the cottons were different, however; when glandless cotton was carded the dust level was 1.3 mg·m^{-3} (SD=0.1) whereas during the carding of glanded cotton, the level was 2.8 µg·m^{-3} (SD=1.0). This reflects the above mentioned difference in relative amount of endotoxin in the two dusts.

The spirometric measurements of the different subjects are reported in table 3. The changes in spirometric values induced by the exposure to cotton dust were small (-2.2% FEV_1 in both groups and -1.6 and -1.5% FVC, respectively). If the values obtained when a worker had

Table 2. – Enzymes in lung lavage fluid 24 h after exposure to different cotton dusts.

Dust exposure	LDH	NAGase	Acid Phosphatase	Cathepsin D
Unexposed°	63 (12)	2.7 (0.6)	20 (2.7)	0.33 (0.07)
Standard dust	221 (26)***	5.5 (0.5)***	73 (17)***	0.35 (0.11)
Tannins and terpenoids removed	182 (47)***	4.3 (12)**	59 (18)***	0.43 (0.06)

Mean from ten animals in each group with standard deviation in parenthesis. Exposure data for dusts, see table 1. Values for animals exposed to standard dust 5 mg·m^{-3} not available. Difference from unexposed: *** p<0.001, ** p<0.01; •: animals; LDH: lactate dehydrogenase; NAGase: N-acetyl-ß-D-glucosaminidase.

Table 3. – Subjective symptoms and pulmonary function changes after the shift among cotton cardroom workers exposed to dust from glanded and glandless cotton.

	Glanded							Glandless										
subject	KL	NW	HS	BL	AT	JS	ME	KL	NW	HS	KL	NW	HS	BL	AT	JS	ME	BL	AT	JS	ME
smoker		x				x	x		x			x				x	x			x	x
chest tightness	x		x	x				x	x		x	x		x				x			
dry cough	x			x	x	x				x				x	x	x	x	x		x	
Δ FEV_1	+1	-7	-2	+3	0	-4	-2	-1	-6	-4	-3	-6	-5	-2	+3	-4	-2	-1	0	-2	-2
Δ FVC	-2	-3	-2	-2	+1	-2	+3	-4	-4	-1	-3	-1	-2	-4	0	-4	0	-3	0	0	0

been exposed twice were excluded, a tendency for a greater decrease after exposure to the glandless cotton was found. On average, smokers had a larger decrease in FEV_1 than non-smokers (3.9 *vs* 0.9% $p<0.01$).

Four of the seven workers reported typical symptoms of chest tightness at the end of the work shift. Two persons had symptoms on all three test occasions, one on two out of three and one on one test occasion only. There was no relationship between the presence of chest tightness and the decrease in FEV_1; for the groups with and without chest tightness the average FEV_1 decrease was -1.9 and -2.4%, respectively. Also there was no relationship between smoking and chest tightness.

Discussion

A general reaction present after the exposure to cotton dust or its extracts is an invasion of neutrophils into the airways. This has been demonstrated for humans and animals. No such increase is found after exposure to inert dusts such as titanium oxide (TiO_2) or cellulose acetate [16].

The amount of endotoxin in airborne cotton dust and the number of neutrophils in the airways of guinea-pigs after exposure are dose-related. A close relationship has also been demonstrated in cotton workers between the FEV_1 decrease over the shift and the amount of airborne endotoxin [3] as well as between the number of blood neutrophils and the FEV_1 decrease after cotton dust exposure [17]. Furthermore, Yeung and Dy Buncio [18] demonstrated a relationship between blood neutrophils and the FEV_1 decrease among grain workers.

In the present experiments the increase in the number of neutrophils in airways of guinea-pigs 24 h after the exposure to the different dusts was related to the amount of airborne endotoxin. In this respect the results agree with the earlier observations where a positive dose-response relationship was found between the neutrophil increase after exposure to two different cotton dust extracts and their content of Gram-negative bacteria [19]. A reduction of the amount of tannins and terpenoid aldehydes from the standard dust did not influence the dose-response relationship between neutrophils and endotoxin in the dusts.

Exposure to the different dusts also caused an increase in the amount of enzymes with the exception of Cathepsin D in the lung lavage fluid. This is a typical response after the exposure to endotoxin and to cotton dust and has been reported previously [20]. Increased values have been found at 12, 24 and 48 h after exposure to airborne endotoxin. The reduction of terpenoid aldehydes and tannins did not influence the capacity of the dust to induce enzyme activity in the lung lavage fluid.

This is contradictory to those earlier experiments where tannin has been found to be biologically active *in vitro*. However, exposure levels in these studies were very high ranging from 10–100 $\mu g \cdot ml^{-1}$ in the AM culture medium. This can be compared to the results from studies on the leucotactic activity of AM exposed *in vitro* [21]. At high levels of endotoxin (0.5–5 $\mu g \cdot ml^{-1}$), an inhibitory effect of the AM leucotactic activity could be demonstrated. To reproduce the stimulatory effect present *in vivo* [22], the concentration in the medium had to be ten-thousand fold lower, *i.e.* 0.0005 $\mu g \cdot ml^{-1}$. This is also the dose that corresponds to the AM cellular dose level in the lung during inhalation.

A difference in the relative amount of endotoxin was found between the dusts from the two types of cottons used in the experiment on humans. Such differences have previously been found between cottons grown in different geographical areas [23] which is related mainly to rain and frost [24]. The present data suggest that in addition to geographical location and climatic conditions, properties of the cotton plant itself may influence the number of Gram-negative organisms growing on the plant.

Endotoxin levels from the two samples of cotton were equal in the fibre material but different in the dust created by the carding. The latter difference reflects different physical properties of the fibres such as friability of the fibre and trash content, leading to different aerosolization characteristics.

The subjects used in the study were actively working in cotton mills and not selected for acute airway reactivity to cotton dust. The population is thus not similar to that which has been used in other experimental cardroom studies where a significant drop in FEV_1 after a pilot challenge was a prerequisite to selection [4, 6].

The magnitude of the changes in pulmonary function were small and without physiological significance. The decrease in FEV_1 over the workshift, as opposed to the slight increase that is normally present is, however, used

as the major criterion for cotton dust induced effects both by researchers and in legislation in the US. Although the decreases are not important after an acute exposure, further work must be undertaken to evaluate the significance of these effects for the later development of chronic effects. It may be that other effects such as bronchial hyperreativity are more important in this respect.

The changes are much smaller than those generally present in clinical pulmonary disease although subjective symptoms of discomfort were reported. This agrees with observations on airway restriction, which were felt by the subjects at around 0.6 cm $H_2O \cdot l^{-1} \cdot s^{-1}$, although this value is small compared to values present in clinical disease [25].

There was no difference in pulmonary function decrease after exposure to the two different kinds of cotton. This suggests that the difference in amount of components from cotton glands did not influence the decrease in FEV_1 over the workshift.

Workers exposed to dust from the glanded cotton had the same decrease in FEV_1 as workers carding glandless cotton, although they were exposed to almost twice as much dust which included components from glands.

The potentiating effect of smoking on the acute FEV_1 decrease after cotton dust exposure has been demonstrated previously [3, 26]. The absence of a relationship between the FEV_1 decrease and the subjective symptoms of chest tightness has also been found previously [3] suggesting a different pathogenesis for the two symptoms in the byssinosis syndrome.

In summary, results obtained from *in vivo* inhalation experiments on animals and humans have not supported the hypothesis that the cotton plant constituents which were studied here, are of importance for the development of the acute symptoms found in byssinosis syndrome. The results have further strengthened the role of endotoxin as the major component fur the acute response after cotton dust exposure.

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RÉSUMÉ: Nous avons expérimenté les réactions pulmonaires survenant après inhalation de poussière de coton comportant différents niveaux de tannin, d'aldéhyde terpénoïde et d'endotoxines bactériennes. Des cobayes ont été exposés aux poussières de coton. Les cellules pulmonaires libres ont été obtenues par lavage 24 h. après. Nous avons mis en évidence une relation dose-réponse entre le nombre de neutrophiles et la quantité d'endotoxines présentes dans la poussière. Nous n'avons pu détecter aucune influence de l'aldéhyde terpénoïde ni des niveaux de tannin. Des ouvriers des usines de coton ont été exposés à la poussière provenant de coton "avec et sans aglandules" dans une chambre de cardage expérimentale. Les diminutions moyennes du VEMS au cours de la journée de travail après cardage des deux cotons étaient les mêmes, quoique les niveaux de poussière de tannin ou d'aldéhyde terpénoïde soient différents. Par contre, le niveau d'endotoxine aériénne était le même. Ces résultats viennent confirmer les observations d'autres auteurs sur l'importance de l'endotoxine pour le développement des réactions aiguës observées après exposition à la poussière de coton.