Effect of alkali treatment on physiological activity of cotton condensed tannin

M A Rousselle, M H Elissalde, L N Domelsmith

Abstract
Cotton dusts contain condensed tannins and endotoxins, which are suspected of contributing to the development of acute and chronic biological responses in some cotton textile mill workers. Condensed tannin extracted from cotton dust was coated on to cellulose powder, and the tannin coated powder was treated with a an alkali solvent system previously developed to reduce the endotoxin content and pulmonary toxicity of cotton dust. Physiological activities of the dusts and powders were compared by assaying the production of the arachidonic acid metabolites prostaglandin F2α (PGF2α), thromboxane A2 (TxA2) (the precursor to thromboxane B2 (TxB2)), leukotriene C4 (LTC4), and prostaglandin E2 (PGE2) by guinea pig pulmonary cells obtained by lung lavage. Cotton dust stimulated the pulmonary cells to produce a total of 29 pg metabolites per 10⁶ cells. Production of metabolites by cells stimulated with tannin coated cellulose powder was reduced to 8.3 pg/10⁶ cells. Alkali treatment of the tannin coated cellulose powder resulted in a further decrease in its ability to stimulate the cells, producing 3.5 pg metabolites per 10⁶ cells. The ability of the dusts and powders to stimulate production of metabolites of arachidonic acid by pulmonary cells from guinea pigs was highly correlated with tannin content of the materials, but not with endotoxin content as measured by the Limulus amoebocyte lysate (LAL) assay.

The development of acute and chronic biological responses to cotton dust by some textile mill workers is thought to result from long term exposure to a non-lint component in dust generated during processing of cotton fibres.¹ Non-lint components include parts of the cotton plant (bract, leaf, etc), inorganic material, fungi, and bacteria.² Endotoxins (lipopolysaccharides) produced by Gram negative bacteria that occur naturally on cotton plants are suspected of contributing to development of the biological responses to cotton dust.³⁴ We previously reported that treatment of a cotton dust designated DB 11/82 with 95% ethanol containing 0.2% sodium hydroxide reduced the endotoxin content of the dust by 90%.⁵ The University of Pittsburgh Graduate School of Public Health (UPGSPH) showed in their guinea pig model for byssinosis that inhalation of cotton dust resulted in increased respiratory frequency and decreased tidal volume. They also reported that cotton dust DB 11/82 treated with 0.2% sodium hydroxide solution consistently had reduced pulmonary toxicity in the guinea pig model, as shown by diminished changes in respiratory frequency and tidal volume relative to untreated cotton dust.⁶ Although our goal is to reduce or eliminate the biological activity of endotoxins in cotton dusts, the possible effect of any treatment on other constituents in cotton dusts cannot be ignored.

Condensed tannins found in non-lint structures of the cotton plant (but not in mature lint)⁸ and other related polyphenolics have been cited as potential aetiological agents of some of the symptoms and respiratory changes associated with the responses to cotton dust.⁹¹⁰ Studies of the effects of inhalation of condensed polyphenols by human subjects have been conducted, but results were inconsistent. In one study, subjective symptoms were reported, but no changes in pulmonary function could be measured.¹¹ In another study neither subjective symptoms nor changes in pulmonary function were found.¹² Several research groups have shown that condensed tannins have physiological activity in a variety of in vitro systems¹³⁻¹⁸ and in in vivo studies of hamsters and guinea pigs.¹９⁻₂⁰ Condensed tannins isolated from cotton plants (fig 1) are polyphenolic polymers (condensed proanthocyanidins); the monomer units are C₆⁻C₃⁻C₀ flavan-3-ols (procyanidin and prodelphinidin, with a terminal (+)-catechin unit).⁸ Under alkaline conditions condensed tannins are not stable and lability of the interflavanoid bond and the ether linkage in the pyran ring of condensed

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tannins lead to intramolecular condensations and rearrangements via a postulated quinone intermediate (fig 2).\textsuperscript{21}

Our solvent system for detoxification is strongly alkaline. To determine the effect of this system on condensed tannin in cotton, we extracted these from cotton dust, loaded the tannins on to cellulose powder, and then treated the tannin coated powder with the detoxification solvent system, a solution of 0.2\% sodium hydroxide in 95\% ethanol.

Activation of mammalian cells with a variety of inflammatory agents, including cotton dusts, can stimulate the cleavage of membrane associated phospholipid with the resultant metabolism of arachidonic acid into biologically active intracellular and intercellular chemical metabolites. These metabolites of arachidonic acid have a plethora of biological effects that influence arterial and venous blood flow, capillary permeability, tone of smooth muscle, and cell chemotaxis. Four bronchoactive metabolites are prostaglandin F\(_2\alpha\) (PGF\(_2\alpha\)), thromboxane A\(_2\) (TXA\(_2\)), the precursor to thromboxane B\(_2\) (TXB\(_2\)), leukotriene C\(_4\) (LTC\(_4\)), and prostaglandin E\(_2\) (PGE\(_2\)). Prostaglandin F\(_2\alpha\), TXA\(_2\), and LTC\(_4\) are potent bronchoconstrictor agents. In contrast to the bronchoconstrictors, PGE\(_2\) has bronchodilator effects in man.\textsuperscript{22}

In this paper we report data on the content of tannin and endotoxin in the dust before and after extraction, and of the tannin coated cellulose powder before and after alkaline treatment. The physiological activity of the cotton dusts and cellulose powders is compared by evaluating their ability to stimulate production of metabolites of arachidonic acid by guinea pig pulmonary cells obtained by lung lavage. This procedure has previously been applied in studies of lipopolysaccharide from Gram negative bacteria associated with cotton dust, and to cotton dust that was chemically treated by solvent systems designed to reduce the biological activity of endotoxins in the dust.\textsuperscript{23,24}

**Materials**

We obtained cotton dust designated DB 1/88 from Cotton Incorporated. Dust DB 1/88 was used as the source of condensed tannin and is the current control material for the UPGSPH inhalation studies using the guinea pig model. Microgranular cellulose powder (CC41) was obtained from Whatman BioSystems Ltd. Cyanidin chloride and delphinidin chloride standards were obtained from Sarsyntex (Merrignac, France). Cotton condensed tannin standard was a gift from Dr Bock Chan (United States Department of Agriculture, Agricultural Research Service, Western Region Research Center).

**Methods**

**PREPARATION AND ALKALI TREATMENT OF TANNIN-COATED CELLULOSE POWDER**

Details of the preparation of the tannin coated powder and its alkalai treatment have been previously described in detail\textsuperscript{25}; a brief summary is given here. Cotton dust DB 1/88 was extracted three times with a 1:1 mixture of anhydrous acetone and cyclohexane to remove lipids, waxes, pigments, and terpenoid aldehydes. The condensed tannin was then extracted from the dust with 70\% acetone; no clean up of the extract was attempted. Acetone was removed from

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*Figure 1* Structure of condensed tannin isolated from cotton squares (R = H, procyanidin; R = OH, pro-delphinidin).\textsuperscript{8}

*Figure 2* General structure of alkali-rearranged procyanidin condensed tannins (R = H or a continuation of the same type of structure).\textsuperscript{21}
the tannin solution on a rotary evaporator, cellulose powder was added to the remaining aqueous tannin solution, and the water was removed by freeze-drying. The tannin coated cellulose powder was treated with 95% ethanol (prepared from anhydrous ethanol and pyrogen free water) containing 0.2% sodium hydroxide for one hour at 65°C. The treated powder was neutralised with 95% ethanol containing 0.15% concentrated HCl, rinsed with 95% ethanol, and air dried.

**ANALYTICAL METHODS**

A common assay for condensed tannin measures cyanidin and delphinidin produced by acid hydrolysis of the tannin. Because the product of alkali rearrangement of condensed tannin does not contain procyanidin or prodelphinidin structures, the acid hydrolysis assay can be used to quantify the effect of sodium hydroxide treatment on tannin from cotton dust. The presence of condensed tannin on the tannin coated cellulose powders was confirmed by thin layer chromatography (TLC) of the hydrolysates of the dusts and powders using cellulose TLC plates developed with Forestal reagent (acetic acid: HCl:water, 30:3:10). Cyanidin and delphinidin chlorides dissolved in butanol HCl mixture were chromatographed on the same plate as standards. For measurement of tannin content, cotton dust, cellulose powder, tannin-coated cellulose powder before and after alkali treatment, and condensed tannin isolated from cotton squares were hydrolysed in butanol HCl mixture (95:5). Condensed tannin content was quantified in triplicate by hydrolysis of the dusts and powders in the butanol/HCl reagent, based on the method of Bell and Stipanovic.

The resulting anthocyanidins (cyanidin and delphinidin) were quantified by measuring absorbance of the hydrolysates at around 550 nm. Purified condensed tannin isolated from cotton squares was used as a standard.

The endotoxin contents of the dust samples were determined in duplicate using the *Limulus* amoebocyte lysate (LAL) assay as previously described.

**ISOLATION AND STIMULATION OF GUINEA PIG PULMONARY CELLS**

For isolation of pulmonary cells, male English short haired guinea pigs weighing 300 g were anaesthetised with sodium pentobarbital and the lungs were lavaged with Earle's balanced salt solution without calcium or magnesium (pH 7.3, 20 mM HEPES). The suspension containing the lavaged cells was centrifuged at 500 g for 10 minutes at 4°C, and the cells were resuspended in RPMI-1640 tissue culture medium (pH 7.3, 20 mM HEPES, 10% fetal calf serum, RPMI + ) at a concentration of 5 x 10^5 cells per ml. Lavaged cells contained in 1 ml RPMI + were placed in each siliconised Leighton culture tube and incubated for 24 hours at 37°C in 5% CO2 air mixture. At the end of the incubation period the contents of each culture tube were washed twice with 5 ml of RPMI + and the unattached cells and supernatants were discarded after each wash. Immediately after the final wash, the cells were resuspended in 1 ml of RPMI + containing 1 mg of either cellulose powder, extracted or unextracted cotton dust, tannin coated cellulose powder, or the vehicle alone. The cells were incubated at 37°C in 5% CO2 air mixture and the reaction was stopped after one hour by cooling the suspension of cells in ice water for 10 minutes. The culture tubes were centrifuged at 1000 g for 30 minutes at 4°C and the supernatant solutions containing the arachidonic acid metabolites were collected and frozen at -80°C. The prostaglandins and leukotriene in the supernatants were quantified by radioimmunoassay with materials supplied in kits and procedures obtained from Dupont NEN.

**Results**

Thin layer chromatography of authentic samples of cyanidin and delphinidin and the hydrolysates of dust DB 1/88, extracted dust DB 1/88, and cellulose powder coated with the 70% acetone extract confirmed the presence of cyanidin and delphinidin in the hydrolysates of tannin coated cellulose powders. No cyanidin or delphinidin was detected in the hydrolysate of the control cellulose powder. Only weak traces of cyanidin and no delphinidin were seen in the hydrolysate of the tannin coated powder treated with alkali, despite application of this sample at a load several times that of the other samples.

The cotton dust DB 1/88 from which the tannin was extracted contained 4.51% tannin (table 1); after two successive extractions with 70% acetone, the tannin content of the dust was reduced to 1.32%. The tannin coated cellulose powder contained 2.07% tannin. Although this is less than half the tannin concentration found for dust DB 1/88, it is comparable to that of dust DB 11/82, the cotton dust used to develop the UPGSPH animal model for byssinosis (table 1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tannin (%) (SD)</th>
<th>Endotoxin (ng LPS/mg (SD))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton dust DB 1/88</td>
<td>4.51 (0.18)</td>
<td>758 (484)</td>
</tr>
<tr>
<td>Extracted dust DB 1/88</td>
<td>1.32 (0.10)</td>
<td>438 (23)</td>
</tr>
<tr>
<td>Tannin coated cellulose powder</td>
<td>2.07 (0.13)</td>
<td>0.095 (0.06)</td>
</tr>
<tr>
<td>Alkali treated tannin coated cellulose powder</td>
<td>0.59 (0.05)</td>
<td>0.03 (0.02)</td>
</tr>
<tr>
<td>Cotton dust DB 11/82</td>
<td>2.18 (0.08)</td>
<td>—</td>
</tr>
</tbody>
</table>
A mass balance accounted for 50% of the original tannin in the extracted dust (23%) and in crude tannin on the powder (27%); some tannin may have been lost by oxidation. Although we considered adding a compound such as ascorbic acid to reduce possible loss of tannin by this route, the potential interference of such a compound with planned inhalation studies using animal models prohibited this approach.

Treatment of the tannin coated cellulose powder with the ethanol sodium hydroxide solvent system reduced the assayable tannin content to 0.59%.

Table 1 also gives data from LAL assays for endotoxin content of the dusts and powders. Results are given as ng lipopolysaccharide (LPS) per mg dust or powder. Cotton dust is a heterogeneous material, and this heterogeneity is reflected in the variation in the LPS content reported in table 1 for dust DB 1/88, with a mean of 758 (SD 484) ng LPS per mg dust. After extraction of tannin, the dust residue contained 438 (SD 23) ng LPS per mg dust. The log difference in LPS content of less than one indicated that little endotoxin, if any, had been removed by the several extractions. Cellulose powder that had been coated with the extracted tannin contained 0.10 (SD 0.06) ng LPS per mg dust, also showing that 70% acetone did not extract intact endotoxin. Alkali treatment of the tannin coated powder reduced the already low concentration of endotoxin even further, to 0.03 (SD 0.02) ng LPS per mg dust.

Guinea pig pulmonary cells obtained by lung lavage were stimulated by cotton dust, extracted cotton dust, tannin coated cellulose powder, and alkali-treated tannin coated cellulose powder. Table 2 gives the data for the production of metabolites of arachidonic acid from the cells after stimulation by the vehicle alone (control) and by the dusts and powders.

Cotton DB 1/88 dust stimulated the production and release of PGE₂, PGF₂α, TXB₂, and LTC₄ from the pulmonary lavage cells of the guinea pig. Extraction of this sample of cotton dust with 70% acetone significantly reduced its capacity to stimulate production of PGE₂ by 68%, PGF₂α by 81%, TXB₂ by 89%, and LTC₄ by 82%. The tannin coated cellulose powder, and the extracted dust gave rise to a significantly lower production of the biologically active metabolites than did the untreated cotton dust DB 1/88. The activity of the tannin coated cellulose powder was, however, slightly greater than that of the extracted dust for PGF₂α, TXB₂, and LTC₄, and only slightly less than that of the extracted dust for PGE₂. Alkaline treatment of the tannin coated cellulose powder reduced the capacity of the powder to stimulate the pulmonary lavage cells to produce PGE₂ by 29%, PGF₂α by 65%, TXB₂ by 55%, and LTC₄ by 64% compared with the tannin coated cellulose powder.

The production and release of the four metabolites of arachidonic acid from the guinea pig lavage cells varied closely with the tannin concentrations in the samples, but not with endotoxin concentrations. Correlation coefficients (r) between the concentration of tannin in the various samples and the release of PGE₂ (r = 0.95), PGF₂α (r = 0.98), TXB₂ (r = 0.97), and LTC₄ (r = 0.98) were much higher than correlations between the endotoxin concentrations in the various samples and the release of PGE₂ (r = 0.65), PGF₂α (r = 0.57), TXB₂ (r = 0.50), and LTC₄ (r = 0.58).

### Discussion

According to Bell,28 mill dusts contain tannins in concentrations ranging from 6–10% depending on particle size with peak concentrations occurring in particles in the range of 38–150 μm. He states that even the lowest concentration of tannins found in dusts (2%, or 20 000 ppm) is sufficient to cause many biological activities, and cites research demonstrating biological activity in a variety of cell systems at concentrations of tannin below 1000 ppm (0.1%), and even below 50 ppm. Rohrbach et al have found that tannin concentrations of 25–50 ppm change the function of alveolar macrophages in vitro.44 At these concentrations, the macrophage loses its ability to phagocytose particulate material. At higher concentrations, tannin triggers the release of arachidonic acid from membrane phospholipids. Russell and Rohrbach found noticeable endothelium dependent contraction of tissue from the pulmonary artery of rabbits at a tannin concentration of 2 ppm, with
maximal responses at 50 ppm.\textsuperscript{17} The tannin content (2.07\%\textsubscript{o}) of the tannin coated cellulose powder prepared in the current study is therefore at a concentration at which it is reasonable to expect biological activity.

The endotoxin concentrations in cotton dust DB 1/88 can be compared with other cotton dusts for which dose response relations have been obtained for change in forced expiratory volume in one second (FEV\textsubscript{1}) in human volunteers compared with quantity of cotton dust generated in a cotton card room during exposure to the dust.\textsuperscript{29} Castellan et al reported the slopes of the linear dose response regressions of percent change in FEV\textsubscript{1} v dust content of the air (mg dust/m\textsuperscript{3} air) during the exposures for a series of cottons. Karol et al reported the endotoxin content of dusts generated from some of the same cottons.\textsuperscript{30} Slopes ranged from \(-2.3\) for a white strict cotton from the San Joaquin valley, to \(-45\) for strict low middling spotted Mississippi Delta cotton with 24 000 \(\mu g\) endotoxin per g dust. A slope of \(-9.5\) was found for a standard cotton with 239 \(\mu g\) endotoxin per g dust, and a slope of \(-7.1\) for a cotton with 400 \(\mu g\) endotoxin per g dust. The dose response relation for the standard cotton represented a decrease in FEV\textsubscript{1} of around \(4^\circ\text{o}\) for exposure to card generated dust at a concentration of around 0.47 mg dust per m\textsuperscript{3} air. The endotoxin concentration of dust DB 1/88 is higher than that of dust from the standard cotton, and DB 1/88 would therefore be expected to be active in inhalation studies on humans.

Cotton dust DB 1/88 has been evaluated in the UPGSPH animal model for byssinosis, and was found to be sufficiently potent for use in their continued studies on the aetiology of byssinosis. The dust produced an increased breathing frequency and decreased tidal volume compared with baseline values during monitoring immediately postexposure and 18 hour postexposure for animals breathing air or air containing 10\%\textsubscript{o} CO\textsubscript{2}.\textsuperscript{31} This response was typical of that found for other cotton dusts and indicates that dust DB 1/88 is biologically active in vivo.

Although the highest concentration of metabolites was produced by the material with the highest endotoxin content (dust DB 1/88 with 758 ng LPS/mg), production of the metabolites by the dusts and powders in this study did not correlate with endotoxin content overall. For instance, although the ability of the tannin coated powder to stimulate production of the metabolites was significantly greater than that found for the extracted dust for PGF\textsubscript{2\alpha}, TxB\textsubscript{2}, and LTC\textsubscript{4}, and about equal to that found for PGE\textsubscript{2}, the endotoxin content of the tannin coated powder (0-095 ng/mg) was significantly lower than that of the extracted dust (438 ng/mg). Cotton dust, however, stimulated significantly higher production of the metabolites than did the tannin coated powder indicating that tannin content alone cannot account for production of the metabolites.

Comparison of the pulmonary toxicity of the dusts and powders described in this report is currently under way with the University of Pittsburgh guinea pig model for byssinosis.\textsuperscript{3}

We thank Dr Bock Chan for the purified cotton condensed tannin used as a standard. Endotoxin analyses were performed by Dr Janet Fischer, University of North Carolina School of Medicine. We gratefully acknowledge the help of Mario Lucineo and Lisa Lawrence (United States Department of Agriculture, Agricultural Research Service, Southern Region Research Centre) in the tannin analyses.

This work was presented in part at the 13th Cotton Dust Research Conference, Beltwide Cotton Conferences, Nashville TN, January 5–6, 1989.

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Accepted 3 April 1990