

# Cotton Dust-mediated Lung Epithelial Injury

Garrison H. Ayars,\* Leonard C. Altman,\* Carol E. O'Neil,‡ Brian T. Butcher,‡ and Emil Y. Chi§

Departments of \*Medicine and §Pathology, University of Washington, Seattle, Washington 98195; and ‡Section of Clinical Immunology and Allergy, Department of Medicine, Tulane University Medical Center, New Orleans, Louisiana 70112

## Abstract

To determine if constituents of cotton plants might play a role in byssinosis by injuring pulmonary epithelium, we added extracts of cotton dust, green bract, and field-dried bract to human A549 and rat type II pneumocytes. Injury was measured as pneumocyte lysis and detachment, and inhibition of protein synthesis. Extracts of cotton dust and field-dried bract produced significant dose- and time-dependent lysis and detachment of both target cells, while green bract extract was less damaging. Extracts treated with polyvinylpyrrolidone to remove tannins produced significantly less injury. In contrast, purified 5,7,3',4'-tetrahydroxyflavan 3,4-diol (THF), a tannin in cotton dust and bract, caused substantial cell damage. Field-dried bract extract and THF also produced dose-dependent inhibition of pneumocyte protein synthesis. Endotoxin levels did not correlate with observed injury. THF added to rat tracheal explants caused epithelial disruption and desquamation, whereas endotoxin did not. Instillation of cotton dust and field-dried bract extract in rat lungs produced disruption of bronchial epithelium and smooth muscle constriction, while polyvinylpyrrolidone-treated cotton dust extract produced no injury. These findings suggest that extracts of cotton plants are toxic to alveolar, tracheal, and bronchial epithelium and that THF or other tannins may be the responsible agents.

## Introduction

Byssinosis is an occupational respiratory disease observed among workers exposed to airborne dusts produced in the processing of cotton and other vegetable products such as hemp, sisal, jute, and flax (1, 2). The clinical picture can present as two major and distinct syndromes, although there is presumably a continuum between the two. Firstly, an acute reaction may occur, which is often referred to as "Monday fever" or "cotton asthma" (3-5). Typically this occurs in workers on return to the dusty work environment on Mondays after being off work for the weekend. This acute reaction is characterized by dyspnea, chest tightness, and cough, and is associated with a decline in the forced expiratory volume in the first second. Secondly, a chronic bronchitis syndrome can develop, which is characterized by a cough, sputum production, and obstruction to airflow after long-term exposure to cotton or related dusts (6, 7). The possible etiologies of both of these syndromes have been extensively ex-

plored and multiple hypotheses have been put forth (1, 2, 8, 9), but a definite substance(s) causing byssinosis has yet to be identified. Existing evidence strongly suggests that the etiologic factor is a "water-extractable" substance in the bract, which is the leafy structure subtending the boll, rather than from the cotton fiber itself (3, 10). Although the mechanism by which bract may cause byssinosis remains uncertain, the cotton plant is known to contain greater than fifty biologically active substances, many with proven toxic properties (11-13). The purpose of the present study was to determine if aqueous extracts of cotton dust and bract contain a toxin(s) that might be directly injurious to the pulmonary epithelium. For this purpose, we developed models using monolayers of A549 and rat type II pneumocytes, rat tracheal explants and intact rat lungs as targets, and various aqueous extracts of cotton dust and bract, and chemical derivatives thereof as putative toxins. Our studies show that aqueous extracts of cotton dust and bract are directly toxic to pneumocytes and damage tracheal and bronchial epithelium, and that 5,7,3',4'-tetrahydroxyflavan 3,4-diol (THF),<sup>1</sup> a condensed tannin, may contribute to this injury. We hypothesize that the direct toxicity of this substance to pulmonary epithelial cells could be partially responsible for the chronic bronchitis observed in some patients with chronic cotton dust exposure.

## Methods

**Preparation of bract and dust extracts.** Aqueous extracts of green bract (GBE) (DES-056; Cotton Inc., Raleigh, NC, mechanically picked), field-dried bract (FDBE) (Acala SJ-4; Cotton Inc.), and cotton mill dust (CDE) (unsieved 100% West Texas dust, Cotton, Inc.) were prepared using a modification of the standard technique of Cotton, Inc. Briefly, 1 g of either cotton mill dust or bract was suspended in 25 ml endotoxin-free saline, stirred for 1 h at 4°C, and the mixture was then centrifuged at 4°C for 10 min at 10,000 g. The supernatant was passed through a 0.45- $\mu$ m filter and then lyophilized and stored at room temperature. Dry weights of the extracts were 6 mg CDE, 14 mg GBE, and 7 mg/g FDBE of starting material, respectively. Avrin, a microcrystalline cellulose, was used as a control.

**Chemical treatment of dust and bract extracts and measurement of tannin levels.** In some experiments, aqueous extracts of cotton dust and bract were treated with polyvinylpyrrolidone (PVP) to remove tannins, which are known astringent chemicals found in cotton dust and bract (14). In brief, after the extracts had been sterilely filtered, 1% wt/vol PVP was added; the mixture was stirred for 4 h at 4°C and centrifuged at 10,000 g to remove particulates. The supernatant was refiltered through a 0.45- $\mu$ m filter and lyophilized. For other studies, a terpenoid aldehyde- and tannin-depleted extract of CDE was used. This extract, kindly provided by Dr. A. A. Bell of the National Cotton Pathology Laboratory, Cotton Station, TX, was prepared as previously described (13). Briefly, 100-mg samples of cotton dust were extracted three successive times in

Address reprint requests to Dr. Altman, Department of Medicine, RM-13, University of Washington, Seattle, WA 98195.

Received for publication 28 May 1985 and in revised form 30 June 1986.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/86/12/1579/10 \$1.00

Volume 78, December 1986, 1579-1588

1. *Abbreviations used in this paper:* AM, alveolar macrophages; CDE, cotton dust extract; FDBE, field-dried bract extract; GBE, green bract extract; NBCS, newborn calf serum; PVP, polyvinylpyrrolidone; THF, 5,7,3',4'-tetrahydroxyflavan 3,4-diol.

a mixture of 9.9 ml of acetone/cyclohexane (50:50). While the dust was stirring in this solvent, 0.1 ml of water was added to increase the efficacy of terpenoid extraction. After this procedure, the mixture was centrifuged and the aqueous phase discarded. To more effectively remove tannins (and flavanol glycosides), this preparation was treated with three successive additions of 9.9-ml vol of 70% acetone. The tannin concentrations in various extracts were determined using this same method of extracting tannins and then processing the extract as follows: 0.2 ml of extract was placed in 9.8 ml of a mixture of 19 parts *n*-butanol:1 part concentrated HCl in glass-stoppered test tubes and heated at 98°C for 2 h. After cooling, the absorbance of the solution was read at 550 nm. Tannin concentrations were calculated based on:  $E_{550}^{1\%} = 240$ . Thus, tannin % = absorbance  $\times 5,000/240$  (13).

**Preparation of THF.** THF was a gift from Dr. Jonathan Edwards, Medical Research Council, Pneumoconiosis Unit, Llandough, Penarth, South Glamorgan, Wales, and was prepared as previously described (15).

**Endotoxin studies.** In certain experiments, lipopolysaccharide from *Escherichia coli* 026:B6 (Difco Laboratories, Detroit, MI) dissolved in RPMI 1640 was added to pneumocyte monolayers and tracheal explants as described below to determine if endotoxin was directly toxic to these targets. In addition, all dust extracts and derivatives used in the injury studies were assayed for endotoxin using a standardized *Limulus* amoebocyte lysate technique (16). Results are expressed in nanograms per milliliter.

**Preparation of pneumocyte target cells.** A549 pneumocytes obtained from the American Type Tissue Culture Collection, Rockville, MD, were grown and serially passed in Waymouth's MB 752/1 medium containing 3% newborn calf serum (NBCS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.25% sodium bicarbonate, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Gibco, Grand Island, NY) at 37°C in humidified air with 5% CO<sub>2</sub>.

Modifications of the techniques of Dobbs et al. (17), Fisher et al. (18), and Mason et al. (19) were used to obtain rat type II cells. Briefly, pathogen-free Sprague-Dawley rats were killed by intraperitoneal injection of phenobarbital (5 mg/100 g body wt). The lungs were lavaged in situ five times with 15 ml saline to remove alveolar macrophages (AMs). The pulmonary artery was then perfused using a phosphate-buffered salt solution containing 5.6 mM glucose, 1.3 mM MgSO<sub>4</sub>, and 1.9 mM CaCl<sub>2</sub>, at a pH of 7.4 (solution A). Lungs were removed and infused with 8–15 ml of elastase at a concentration of 40 orcein U/ml in solution A to inflate them to total capacity, and submerged in normal saline at 37°C for 40 min; total lung capacity was maintained by adding elastase solution as necessary. The lungs were minced to 1–2-mm<sup>3</sup> pieces, suspended in 5 ml NBCS, and sufficient solution A was added to make the solution total 20 ml. Cell fragments were poured into a sterile flask, shaken for 2 min at 37°C, and filtered sequentially through 100- and 20- $\mu$ m nylon mesh (Tetco, Inc., Monterey Park, CA). Filtered cells were washed with solution A containing 3 mg/100 ml deoxyribonuclease, centrifuged once at 550 g for 10 min, then resuspended in Waymouth's medium with 10% NBCS at  $2.5 \times 10^6$  cells/ml. The cells were placed in 75-cm<sup>2</sup> polystyrene flasks for 2 h at 37°C, and the pneumocyte-rich supernatant was removed (AMs selectively adhere to plastic) and placed in fresh plastic tissue culture dishes for overnight incubation. The next morning, the cells were washed with ethylenedinitrilotetraacetic acid, which preferentially removes residual AMs, and trypsinized for 10 min to detach the rat type II cells. Finally, the cells were washed and placed in microtiter plates after being labeled with <sup>51</sup>Cr as described below.

Purity of the type II cells was determined by both phosphine 3R fluorescent microscopy (20) and Papanicolaou staining (17). A mean purity of 93.0 $\pm$ 3.0% by fluorescence staining and >95% by the Papanicolaou technique was achieved. Rat type II pneumocyte viability was >95% by trypan blue exclusion. In addition, on selected preparations of rat type II pneumocytes, transmission electron micrographs confirmed the purity of these cells.

**Rat tracheal explant preparation and injury assays.** Rat tracheal explants were prepared from pathogen-free Sprague-Dawley rats as previously described (21). In brief, animals were killed, their tracheas removed surgically, and adherent connective tissue dissected away. The

extracted tracheas were then pinned, cut longitudinally, and sectioned transversely with a razor blade apparatus into 2.0  $\times$  6.0-mm uniform-sized explants. The explants were immediately placed in microtiter wells containing THF or endotoxin dissolved or suspended at various concentrations in 300  $\mu$ l RPMI 1640 media (Gibco). After 4 or 24 h of incubation, the explants were removed from the wells and the remaining fluids with desquamated cells were collected by aspiration. To harvest residual desquamated cells, the wells were washed twice with RPMI 1640 media, a third time with RPMI containing 0.5% trypsin, and finally with RPMI 1640 with 3% NBCS to neutralize the added trypsin. The aspirate and wash fluids were pooled and the desquamated cells were then counted visually. All determinations were performed in quadruplicate. In selected experiments, the explants were fixed with glutaraldehyde, dehydrated in graded alcohols, embedded in paraffin, and stained with hematoxylin and eosin. The tissue specimens were studied in a blinded fashion and photographed with an Olympus Vanox microscope.

**Pneumocyte lysis and detachment assays.** A549 pneumocytes and rat type II pneumocytes were plated in Falcon Microtest II plates at  $4 \times 10^4$  cells per well in 100  $\mu$ l Waymouth's medium containing 3% NBCS. After 18 to 24 h, the number of adherent cells from day to day ranged from 2 to  $5 \times 10^4$  per well for the A549 cells, and 3 to  $6 \times 10^4$  per well for the rat II pneumocytes. In each individual experiment, however, identical numbers of pneumocytes were present in each well. Sodium [<sup>51</sup>Cr]chromate (1 mCi/ml in saline, New England Nuclear, Boston, MA) was added at the time of plating at a concentration of 20  $\mu$ Ci/ml. After overnight incubation, cell monolayers were washed four times with RPMI 1640, which was dispensed by pipette and removed by vacuum aspiration. After the final wash, either CDE, FDBE, GBE, or other chemical derivatives of cotton bract or dust dissolved or suspended in RPMI 1640 media were added to the pneumocyte targets in a total volume of 100  $\mu$ l/well. Concentrations of the effector systems varied, as did the duration of incubation depending on the assay conditions. After co-culture of targets and the putative effector toxins, the microtiter plates were centrifuged for 5 min at 400 g. Release of soluble <sup>51</sup>Cr, a measure of cell lysis, was determined by aspirating 50  $\mu$ l of supernatant from each well, transferring the aliquots to glass tubes, and counting the gamma emissions in a spectrophotometer (Packard Instruments, Inc., LaGrange, IL). In each experiment, maximum and control (spontaneous) release of <sup>51</sup>Cr was recorded as a mean of four replicates. Maximum release was determined from wells incubated in 1% wt/vol Triton X-100. Control release was determined from wells incubated with medium only, and was generally 10–25% of maximum release for both the A549 and rat type II cells after 24-h incubations. Cell lysis is expressed as percent <sup>51</sup>Cr release as follows: % lysis = [(cpm test – cpm control)/(cpm Triton – cpm control)]  $\times$  100.

After aspiration of the aliquots for determination of soluble <sup>51</sup>Cr-release, detachment was measured indirectly by counting the <sup>51</sup>Cr-activity in tightly adherent cells after washing off detached and loosely adherent cells. To achieve this, adherent cells were lysed twice with 200- $\mu$ l aliquots of 1% Triton X-100, and the <sup>51</sup>Cr-activity in the combined lysates counted. Determinations were performed in quadruplicate, and cell detachment expressed as a percentage as follows: % detachment = [(cpm retained in control – cpm retained in test)/(cpm retained in control)]  $\times$  100. In previous studies, we have shown this to be a valid method of counting detachment (22). The standard error of the mean of four replicates for both percent lysis and percent detachment was <10%.

**Inhibition of pneumocyte protein synthesis by FDBE and THF.** As another measure of pneumocyte injury, we examined inhibition by FDBE and THF of protein synthesis in A549 pneumocytes as assessed by inhibition of [<sup>3</sup>H]leucine uptake using standard techniques (23). In brief, FDBE or THF were dissolved or suspended in RPMI 1640 media and added to A549 pneumocytes at varying concentrations. Coincident with the addition of these effector toxins, we added 1  $\mu$ Ci of [<sup>3</sup>H]leucine (specific activity, 55 Ci/mM, ICN Radioisotope, Irvine, CA) to each microtiter well. After a 24-h interval, the cells were collected on glass filters using an automatic sample harvester, and radioisotope incorporation was determined in a Tricarb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). Determinations were performed in quadruplicate.

plicate and results expressed as percent change in [ $^3\text{H}$ ]leucine incorporation as follows: % change [ $^3\text{H}$ ]leucine incorporated = [(cpm control – cpm incorporated in presence of test substance/cpm control)]  $\times$  100.

**Instillation of cotton extracts in vivo and histology.** Pathogen-free Sprague-Dawley rats were killed and CDE- and PVP-treated CDE at 2.5 mg/ml and FDBE at 1 mg/ml in RPMI 1640 media were immediately instilled intratracheally. Lung volumes were maintained for 4 h at total lung capacity with intermittent addition of more fluid. After this, the lungs were isolated and gently perfused at 10-cm height with 3% glutaraldehyde in cacodylate buffer. After a 4 h fixation, the left lung of each animal was sectioned longitudinally at similar locations. Lung tissue was dehydrated with graded alcohols and embedded in paraffin at 52°C. A series of 4.0- $\mu\text{m}$  sections was cut and stained with hematoxylin and eosin. Airways at similar locations were studied and photomicrographed with an Olympus Vanox microscope with 10 and 20 times lenses. Results were read in a blinded fashion.

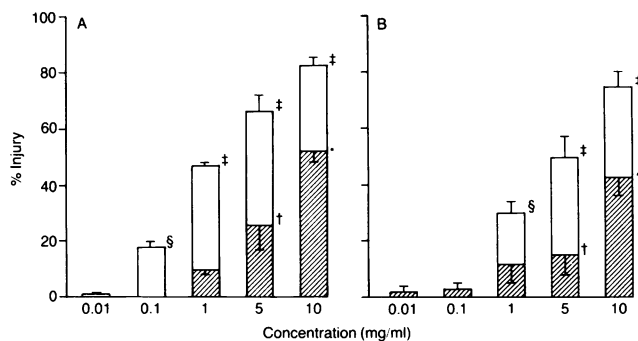
**Data analysis.** Student's two-tailed *t* test was used to test the significance of differences between group means and linear regression analysis was used to examine correlations between tannin concentrations and pneumocyte injury (24).

## Results

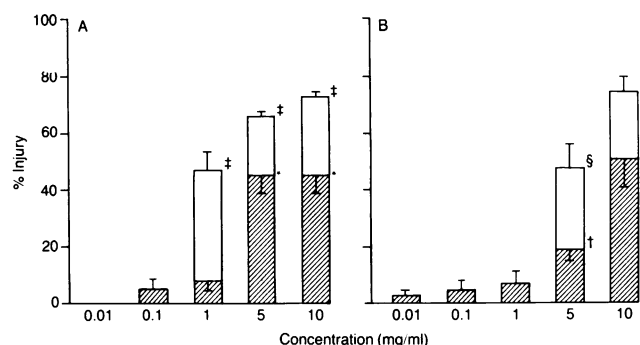
### Injurious effects of cotton dust and bracts on pneumocyte targets.

The addition of CDE to A549 and rat type II pneumocyte targets produced marked dose-dependent injury (Fig. 1, *A* and *B*). As shown, comparable injury was observed with both targets, and the proportions of lysis and detachment were similar. Specifically, significant injury was first observed for the A549 targets at a concentration of 0.1 mg/ml and increased to ~80% at 10 mg/ml. With rat type II targets, significant injury was first evident at 1 mg/ml and likewise plateaued at 80% at 10 mg/ml. When FDBE was used as the effector toxin, similar results were obtained (Fig. 2, *A* and *B*). In contrast, GBE caused significantly less lysis of A549 cells, no lysis of type II cells, and significantly less detachment of type II cells. (Fig. 3, *A* and *B*). Avrin, a microcrystalline cellulose used as a control, did not produce significant injury in concentrations up to 10 mg/ml (data not shown).

**Kinetics of cotton dust- and bract-induced pneumocyte injury.** Since the prior studies were all performed at 18 h, we next sought to determine the degree of injury induced at other time points using A549 cells as targets. As shown (Fig. 4, *A* and *B*), CDE and FDBE produced similar patterns of time- and dose-depen-



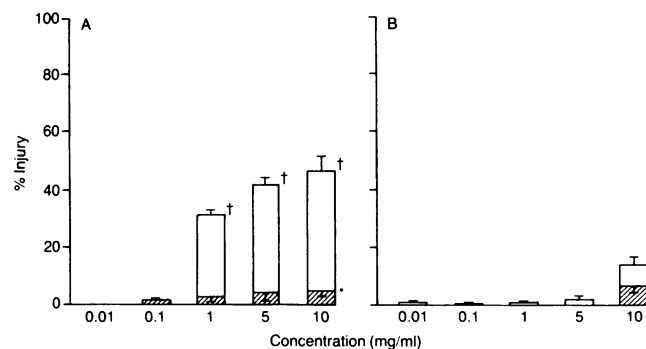
**Figure 1.** Lysis and detachment of (*A*) A549 and (*B*) rat type II pneumocytes produced by CDE. The concentration of CDE is plotted on the abscissa, and (□) percent detachment and (■) lysis are plotted on the ordinate. Results are expressed as the mean percent injury  $\pm$  SEM after an 18-h incubation. CDE caused dose-dependent lysis and detachment of both pneumocyte targets: \*  $P < 0.001$  and †  $P < 0.05$  compared with control lysis; ‡  $P < 0.001$  and §  $P < 0.05$  compared with control total injury (lysis plus detachment).



**Figure 2.** Lysis and detachment of (*A*) A549 and (*B*) rat type II pneumocytes produced by FDBE. The concentration of FDBE is plotted on the abscissa and (□) percent detachment and (■) lysis are plotted on the ordinate. Results are expressed as the mean injury  $\pm$  SEM after an 18-h incubation. FDBE caused dose-dependent lysis and detachment of both pneumocyte targets: \*  $P < 0.001$  and †  $P < 0.05$  compared with control lysis; ‡  $P < 0.001$  and §  $P < 0.05$  compared with control total injury (lysis plus detachment).

dent injury. Addition of CDE and FDBE at concentrations from 1.25 to 10 mg/ml produced only slight injury at 4 h but damage was readily detectable by 8 h with both extracts and increased linearly from 4 to 18 h. GBE again produced lesser amounts of injury at comparable time points and concentrations (Fig. 4 *C*) with essentially no significant lysis seen, even at 10 mg/ml.

**Effect of PVP treatment of cotton dusts and bracts on pneumocyte targets.** Since the above studies clearly showed that aqueous extracts of CDE and FDBE were capable of producing substantial injury to alveolar pneumocytes, we were next interested in determining what was the active injurious substance(s) in these extracts. It is known that cotton bract and dust contain high quantities of astringent tannins which act as natural pesticides for cotton plants, and we hypothesized that these chemicals might be the injurious agents. Therefore, we compared the toxicity of dust and bract extracts before and after PVP treatment, a technique known to remove tannins from these extracts (14). As shown in Table I, PVP-treated extracts of CDE, FDBE, and GBE produced significantly less injury to either A549 or rat type



**Figure 3.** Lysis and detachment of (*A*) A549 and (*B*) rat type II pneumocytes produced by GBE. The concentration of GBE is plotted on the abscissa and (□) percent detachment and (■) lysis are plotted on the ordinate. Results are expressed as the mean percent injury  $\pm$  SEM after an 18-h incubation. GBE caused significant detachment injury of A549 pneumocytes with minimal lysis, and insignificant damage to rat type II targets: \*  $P < 0.05$  compared with control lysis; †  $P < 0.001$  compared with control total injury (lysis plus detachment).

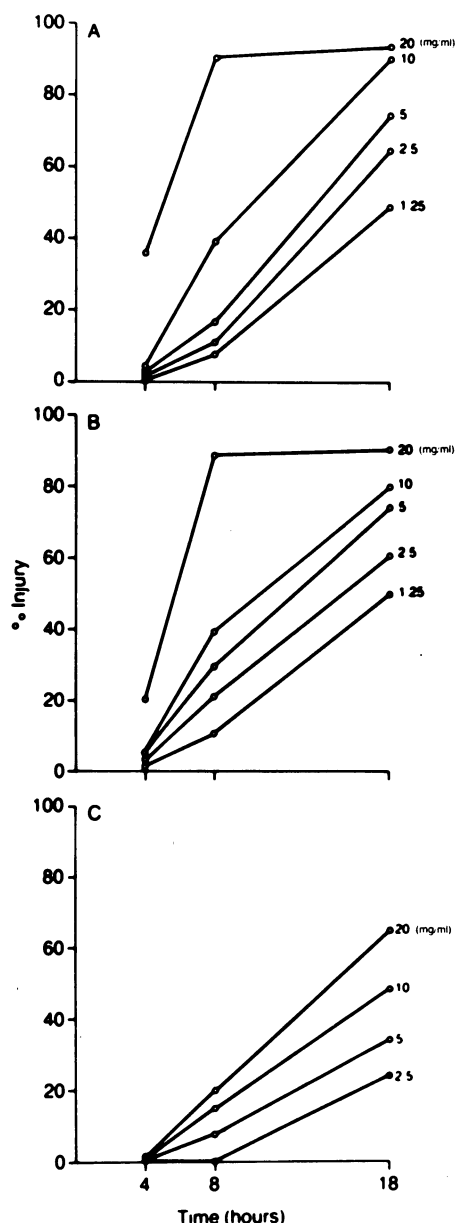


Figure 4. Time course and dose response of (A) CDE, (B) FDBE, and (C) GBE, mediated A549 pneumocyte injury. Time is plotted on the abscissa and the percent total injury (lysis plus detachment) is plotted on the ordinate. Results are expressed as the mean total injury  $\pm$  SEM of four separate studies. CDE and FDBE produced greater amounts of injury than GBE at comparable time points and concentrations.

II pneumocytes than comparable concentrations of non-PVP-treated extracts. These data suggest that the tannins in these dust and bract extracts may be the agents responsible for the observed injury. To support this observation, another method of organic extraction was used to remove tannins from CDE. As shown in Table II, similar results were obtained; specifically, the treated CDE caused less total injury to both pneumocyte targets and less lytic damage to A549 cells; however, the modest degree of lysis of rat type II cells was unchanged. These results further suggest that tannins may be the injurious agents in CDE.

**Tannin levels in the various extracts.** To determine if there was a direct correlation between tannin levels and the injury observed, we measured the concentration of tannins in both

untreated and chemically treated cotton extracts (Table III). As expected, avrin contained no tannins, and CDE and FDBE had measurable and similar tannin levels. Surprisingly, GBE contained a higher percentage of tannins than CDE and FDBE, although our aforementioned injury assays showed GBE to be the least injurious of the extracts tested. A possible explanation of this apparent paradox is presented in the Discussion section. PVP treatment of the extracts removed 67.7, 82.1, and 90.5% of the tannins from CDE, FDBE, and GBE, respectively, and as noted above these treated extracts produced markedly less pneumocyte injury than their native equivalents (Table I). In addition, using the organic extraction method of Bell and Stipanovic (13), 92.3% of the tannins were removed from CDE and this treated extract also had little injurious potential (Table II). In an effort to further examine the correlations between tannin concentrations and pneumocyte injury we reanalyzed the data in Figs. 1–3 and Tables I–III by linear regression analysis. Specifically, we calculated exact tannin concentrations using the percent tannin figures in Table III and the cotton extract concentrations indicated in Figs. 1–3 and Tables I and II. These values were then plotted against the total and lytic injury observed at each concentration. The *R* values, *F* ratios, and statistical significance of each of these correlations are presented in Table IV. As shown, there were significant correlations between tannin concentration and injury, both total and lytic with both target cells.

**Injurious effects of THF on pneumocyte targets.** To examine the role of specific tannins in pneumocyte injury, we examined the toxicity of purified THF against A549 and rat type II pneumocytes (Fig. 5, A and B). THF is a condensed tannin present in cotton dust and bract in large quantities. As shown, THF produced marked dose-dependent A549 and rat type II pneumocyte injury after an 18 h incubation. Measurable injury was observed at a concentration of 1.0  $\mu$ g/ml with the A549 targets, while the rat type II pneumocytes were slightly more resistant with injury first seen at 10  $\mu$ g/ml. Of note, the injury produced by THF was predominantly lysis.

**Inhibition of protein synthesis by FDBE and THF.** To measure the injurious potential of various cotton extracts by another

Table I. Effect of PVP Treatment on Cotton Bract and Dust Extract-mediated Pneumocyte Injury\*

Extract	Treatment	Targets (% total injury)	
		A549	Rat II
CDE	None	66.2 $\pm$ 6.2	50.1 $\pm$ 7.9
CDE	PVP	5.1 $\pm$ 1.9	10.1 $\pm$ 5.5
FDBE	None	66.2 $\pm$ 8.7	47.9 $\pm$ 10.9
FDBE	PVP	0.6 $\pm$ 0.6	2.2 $\pm$ 1.6
GBE	None	32.7 $\pm$ 2.0	3.2 $\pm$ 2.0
GBE	PVP	3.7 $\pm$ 3.1	—

CDE, CDE at 5 mg/ml; FDBE, FDBE at 5 mg/ml; GBE, GBE at 5 mg/ml; PVP, PVP-treated extracts (see Methods section for details).

\* Extracts were incubated with A549 and rat II pneumocytes overnight and total injury was measured (lysis plus detachment) as described in the text. The results are the mean  $\pm$  SEM of four separate studies.

‡ *P* < 0.001 for untreated vs. PVP treated.

§ *P* < 0.01 for untreated vs. PVP treated.

Table II. Injury Produced by Tannin- and Terpenoid Aldehyde-depleted CDE Compared with Untreated CDE\*

	Target		Rat II	
	A549			
	Lysis	Total injury	Lysis	Total injury
	%	%	%	%
Untreated CDE	25.0±10.3 (5)	66.8±7.6 (5)	13.5±6.2 (6)	50.0±7.9 (6)
Tannin- and terpenoid aldehyde-depleted CDE*	0.1±0.1 (4) }‡	0±0 (4) }§	14.1±5.3 (6)	5.8±2.5 (6) }§

\* CDE and tannin- and terpenoid aldehyde-depleted CDE, each at 5 mg/ml, were incubated with A549 and rat II pneumocytes overnight and lysis and total injury (lysis plus detachment) measured as described in the text. The results are the mean±SEM of *n* determinations (numbers in parentheses). CDE was organically extracted to remove tannins and terpenoid aldehydes as described in the text. ‡ *P* < 0.05 CDE vs. tannin- and terpenoid aldehyde-depleted CDE. § *P* < 0.01 CDE vs. tannin- and terpenoid aldehyde-depleted CDE.

method, we examined the inhibition of protein synthesis in A549 pneumocytes produced by selected cotton extracts. Using FDBE as representative of one of the crude extracts, we observed dose-dependent inhibition of [<sup>3</sup>H]leucine uptake with ~50% inhibition occurring at a concentration of 1.25 mg/ml and essentially complete inhibition seen at 5 mg/ml (Fig. 6 A). Purified THF produced even greater inhibition of protein synthesis, with a measurable effect seen at 63 µg/ml and nearly complete inhibition evident at 1.0 mg/ml (Fig. 6 B).

**CDE- and FDBE-mediated rat lung injury in vivo.** To determine if dust extracts were toxic to pulmonary epithelium in vivo, CDE at 2.5 mg/ml and FDBE at 1.0 mg/ml were instilled intratracheally in rats. These extracts produced disruption of the bronchial epithelium with destruction of the basal cell layer and marked constriction of bronchial smooth muscle (Fig. 7, A–C). In contrast, a PVP-treated CDE at 2.5 mg/ml produced no significant injury (Fig. 7 D), similar to the findings when intratracheally instilled media alone was used as a control (not shown).

**Effect of THF on tracheal explants.** To further examine the toxicity of cotton dust constituents on ciliated airway epithelium, we prepared and cultured explants of rat tracheas. The addition of THF to these explants caused dose-dependent desquamation of epithelial cells (Table V). The effect was also time dependent, with fewer desquamated cells observed at 4 h compared with 24 h (data not shown). Furthermore, microscopic examination of tracheal explants exposed in this manner revealed frank desqua-

mation of the tracheal epithelium after 4 and 24 h of exposure (Fig. 8, A and B) with impressive and complete epithelial cell destruction at 24 h (Fig. 8 B). Tracheal explants cultured with media only had a normal appearance (Fig. 8 C).

**Endotoxin studies.** Finally, to examine whether the injury observed in our experiments was due to endotoxin in the cotton extracts, we measured endotoxin levels in the samples using the Limulus lysate method (Table VI). As can be seen, there was no correlation between the level of endotoxin and the injurious potential of the extract. Some of the highest endotoxin levels were present in “noninjurious” extracts, for instance, the terpenoid aldehyde and tannin-free extract. Similarly, the direct addition of lipopolysaccharide at concentrations up to 50 µg/ml, comparable with peak concentrations of endotoxin found in cotton dust (1), produced desquamation of only  $0.9 \times 10^6$  epithelial cells from tracheal explants comparable with the effect of media alone (Table V) and also failed to cause pneumocyte injury (data not shown). These findings suggest that endotoxin did not mediate the injury observed in our studies.

## Discussion

One of the most difficult problems associated with byssinosis research is the absence of agreement on the nature of the etiologic agent(s) responsible for causing the symptoms and pathologic changes of the disease in susceptible workers. It is known that the biologically active substance(s) is present in the bract of the cotton plant and is aqueously extractable (3, 10), but numerous

Table III. Percent Tannins in Cotton Extracts

Extract	% Tannin*
Avrin	0
CDE	0.625
FDBE	0.813
GBE	1.313
CDE · PVP	0.208
FDBE · PVP	0.146
GBE · PVP	0.125
TA & TF CDE	0.048

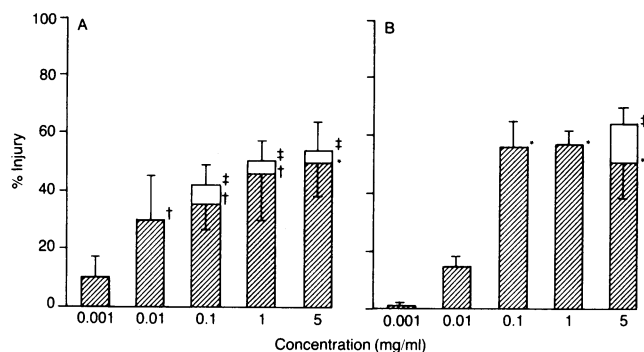
PVP, PVP-treated extracts; TA & TF, terpenoid aldehyde and tannin-free extracts.

\* See the Methods section for technique of measuring tannin levels.

Table IV. Statistical Correlations between Pneumocyte Injury and Tannin Concentrations\*

Condition	R value	F ratio	P
A549 targets/lytic injury	0.516	7.248	0.014
A549 targets/total injury	0.645	12.822	0.002
Rat II targets/lytic injury	0.601	9.603	0.007
Rat II targets/total injury	0.611	9.523	0.007

\* The tannin concentrations were calculated from the percent tannin figures shown in Table III and the cotton extract concentrations shown in Figs. 1–3 and Tables I and II. These values were plotted vs. total and lytic injury and linear regression analyses performed.



**Figure 5.** Lysis and detachment of (A) A549 and (B) rat type II pneumocytes produced by THF. The concentration of THF is plotted on the abscissa and (□) percent detachment and (▨) lysis are plotted on the ordinate. Results are expressed as the mean injury  $\pm$  SEM after an 18-h incubation. THF produced significant concentration-dependent lysis of both targets with minimal detachment; \*  $P < 0.001$  and †  $P < 0.05$  compared with control lysis; ‡  $P < 0.001$  compared with control total injury (lysis plus detachment).

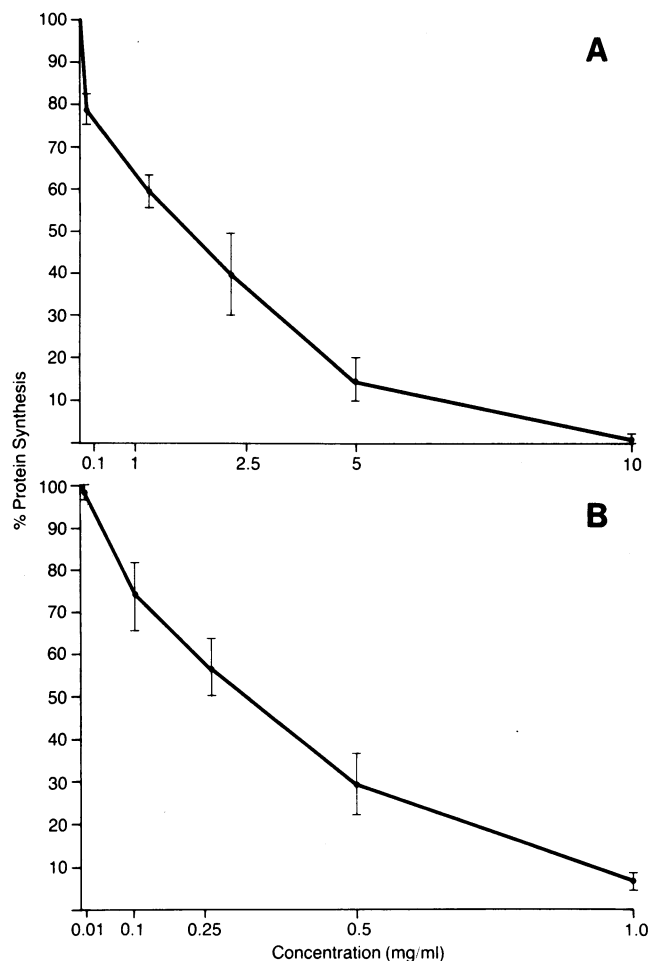
biologically active agents are present in this fraction, thus confusing the picture. For instance, histamine-releasing agents (25), direct smooth muscle contractors (26), endotoxins (27), bacterial proteolytic enzymes (28), chemotaxins (29), complement activators (30), and antigenic substances possibly responsible for immune complex formation (31, 32) have all been found in aqueous extracts of cotton dust and have been proposed to contribute to the pathogenesis of byssinosis. It is possible that several active materials may act synergistically or independently to cause the spectrum of disease seen in byssinosis, from acute dyspnea and bronchospasm to irreversible chronic obstructive lung disease with cough and sputum production, and that no single agent explains the whole picture. This hypothesis was suggested by Edwards, with particular reference to three distinct substances, namely an "aminopolysaccharide-protein complex," endotoxin, and THF, all of which are constituents of cotton dust (8). Specifically, Edwards speculated that the aminopolysaccharide-protein complex, which is capable of stimulating histamine release in vitro, might cause similar release in vivo, thus inducing bronchoconstriction, and that endotoxin could also produce pulmonary airway obstruction by an immunologic mechanism requiring previous sensitization. It also has been shown that tannins like THF are capable of acutely increasing capillary resistance, thus effecting pulmonary blood flow (33). Collectively, Edwards hypothesized that these reactions might produce the acute reaction known as "Monday fever."

To explain the chronic bronchitic syndrome seen in some patients after long-term cotton dust exposure, Edwards refers to data which shows that THF and aqueous extracts of cotton bract when instilled intratracheally in rats are capable of producing an inflammatory response and pulmonary transudation (8). Further, the aminopolysaccharide-protein complex from bract has similarly been reported to produce bronchial epithelial necrosis in human fetal lung (34). Other workers have shown that endotoxins can produce bronchitis when instilled intratracheally in animals (27). Based on these studies, Edwards hypothesized that prolonged exposure to any or all of these materials could cause a bronchitislike condition, as seen in chronic byssinosis (8).

Accordingly, we examined the direct toxicity of cotton bract and dust extracts in vivo and in vitro on pulmonary epithelial

cells to further characterize possible toxins in these preparations. Our in vitro studies showed that of CDE, FDBE, and THF were toxic to pneumocyte targets producing detachment and/or lysis, and that THF produced epithelial desquamation and destruction of tracheal explants. In vivo studies corroborated these in vitro findings; CDE and FDBE, when instilled intratracheally in rats, produced desquamation and destruction of bronchial epithelium.

Specifically, using pneumocyte targets we observed that the relative toxicities of CDE and FDBE were similar, in that both extracts produced comparable amounts of lysis and detachment at the same concentrations and time points. In contrast, GBE at comparable concentrations and time points was less toxic and produced little injury to rat type II targets, even after 18 h of incubation. After PVP treatment, all three extracts produced minimal pneumocyte injury, suggesting that tannins may be

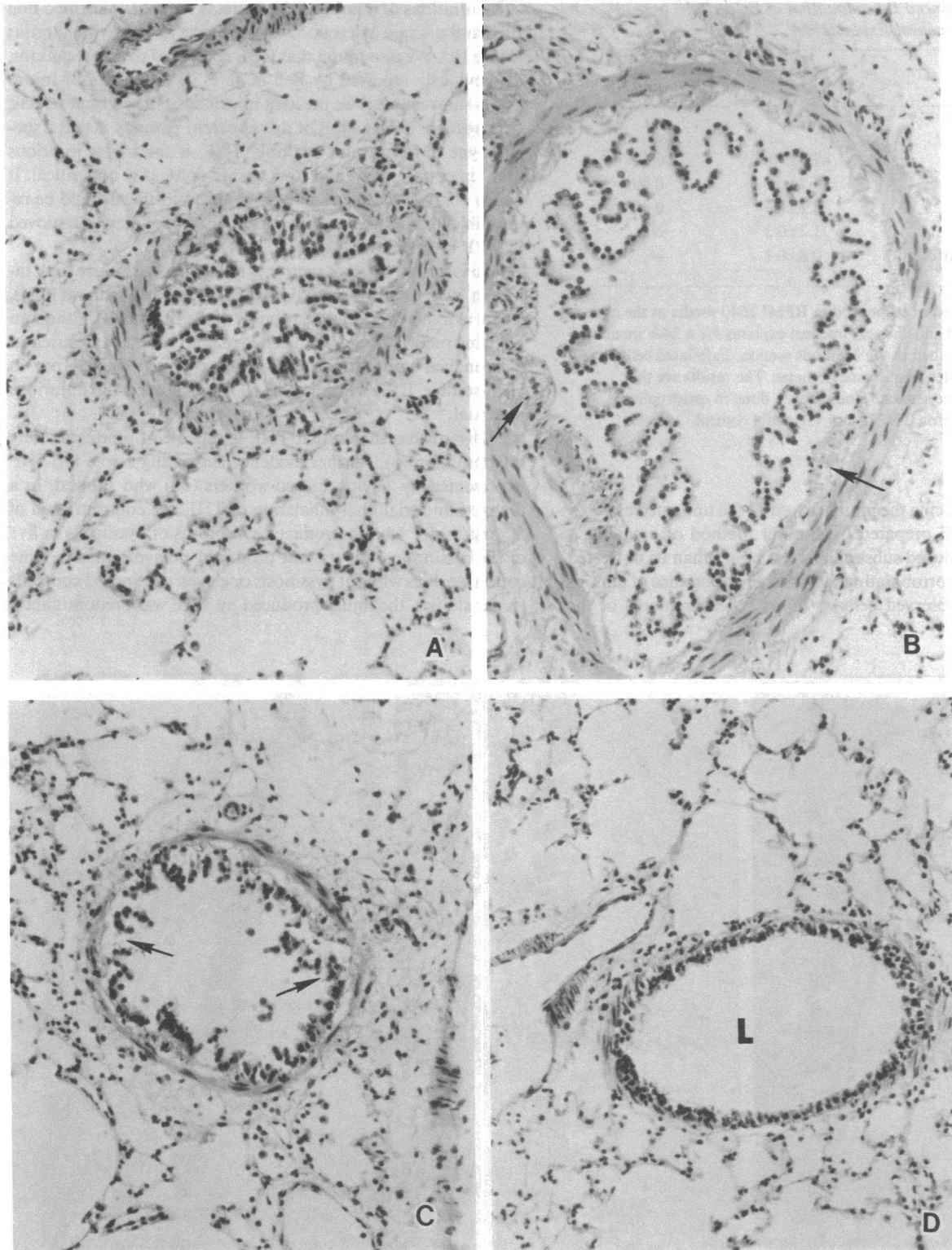


**Figure 6.** Inhibition of A549 pneumocyte protein synthesis by (A) FDBE and (B) THF. The concentrations used are on the abscissa and the percent protein synthesis on the ordinate. FDBE and THF were added to the A549 pneumocytes simultaneous with the addition of [ $^3$ H]leucine. The cultures were incubated for 24 h, and [ $^3$ H]leucine uptake by the pneumocytes was assayed as described in the Methods section. The results are expressed as follows:

$$\% \text{ change } [^3\text{H}] \text{leucine incorporated} = \left[ \frac{\text{cpm control} - \text{cpm incorporated in presence of test substance}}{\text{cpm control}} \right] \times 100.$$

The data are the mean  $\pm$  SEM of four studies.





**Figure 7.** Photomicrographs of lung sections from animals treated with (A and B) CDE, (C) FDBE, and (D) PVP-treated CDE. (A) Severely constricted bronchial airway with a highly convoluted epithelial layer. (B) Larger airway with folding and detachment of the epithelial layer and destruction of the basal cell layer (arrows). (C) Bronchial

airway with disruption of the epithelial cell layer. (D) Airway with no remarkable abnormalities in either the epithelial cell or smooth muscle layers. The appearance of the lumen (L) is normal. The appearance of bronchial airways from animals exposed to media only was identical to the airway shown in D.  $\times 200$ .

Table V. THF-induced Desquamation of Epithelial Cells from Rat Tracheal Explants\*

THF concentration mg/ml	Number of cells ( $\times 10^6$ )	P‡
1.0	47.0 $\pm$ 10.6	0.02
0.1	6.7 $\pm$ 0.7	0.001
0.01	3.2 $\pm$ 0.6	0.02
0.001	1.2 $\pm$ 0.2	NS
Media control	0.8 $\pm$ 0.1	—

\* THF was dissolved or suspended in RPMI 1640 media at the above concentrations and added to rat tracheal explants for a 24-h incubation period as described in the Methods section. Exfoliated cells were collected and counted in a hemocytometer. The results are the mean $\pm$ SEM of three separate studies, each done in quadruplicate.

‡ P values, comparison THF injury vs. media control.

involved in producing the injury caused by the untreated extracts. Tannin-free CDE prepared by another method of organic extraction also produced substantially less injury than its untreated equivalent, thus corroborating the possible importance of tannins in causing the observed cellular injury. Measurements of the

concentrations of tannins in the extracts we used confirmed that PVP and organic extraction were effective in removing tannins (Table III). We also found that GBE contained the most tannins, as previously reported by Bell et al. (13), although our injury assays showed this to be the least injurious of the cotton extracts. One possible explanation of this apparent paradox is that a specific type of tannin, for example THF, is the major injurious agent in cotton dusts, and total tannin content is not critical. It is also possible that another non-tannin substance could be responsible for epithelial cell injury and that it too may be removed by PVP or organic extraction.

To examine whether cotton extracts might produce lung injury in vivo, we instilled CDE and FDBE directly into rat lungs. These extracts produced disruption of the bronchial epithelium with destruction of the basal cell layer and marked constriction of bronchial smooth muscle. Again, as we observed in our in vitro studies, PVP treatment of CDE attenuated this injurious potential.

Edwards has shown that THF is capable of producing lung inflammation (8). Further evidence suggesting a role for THF is presented by Taylor and co-workers (35) who showed, in a double-blind trial, that inhalation of THF at a concentration of 0.5 mg/ml for 15 min produced symptoms of byssinosis in five of six byssinotic patients, but produced no symptoms in nine cotton workers without byssinosis or eleven unexposed controls. In our studies, the injury produced by THF was predominantly

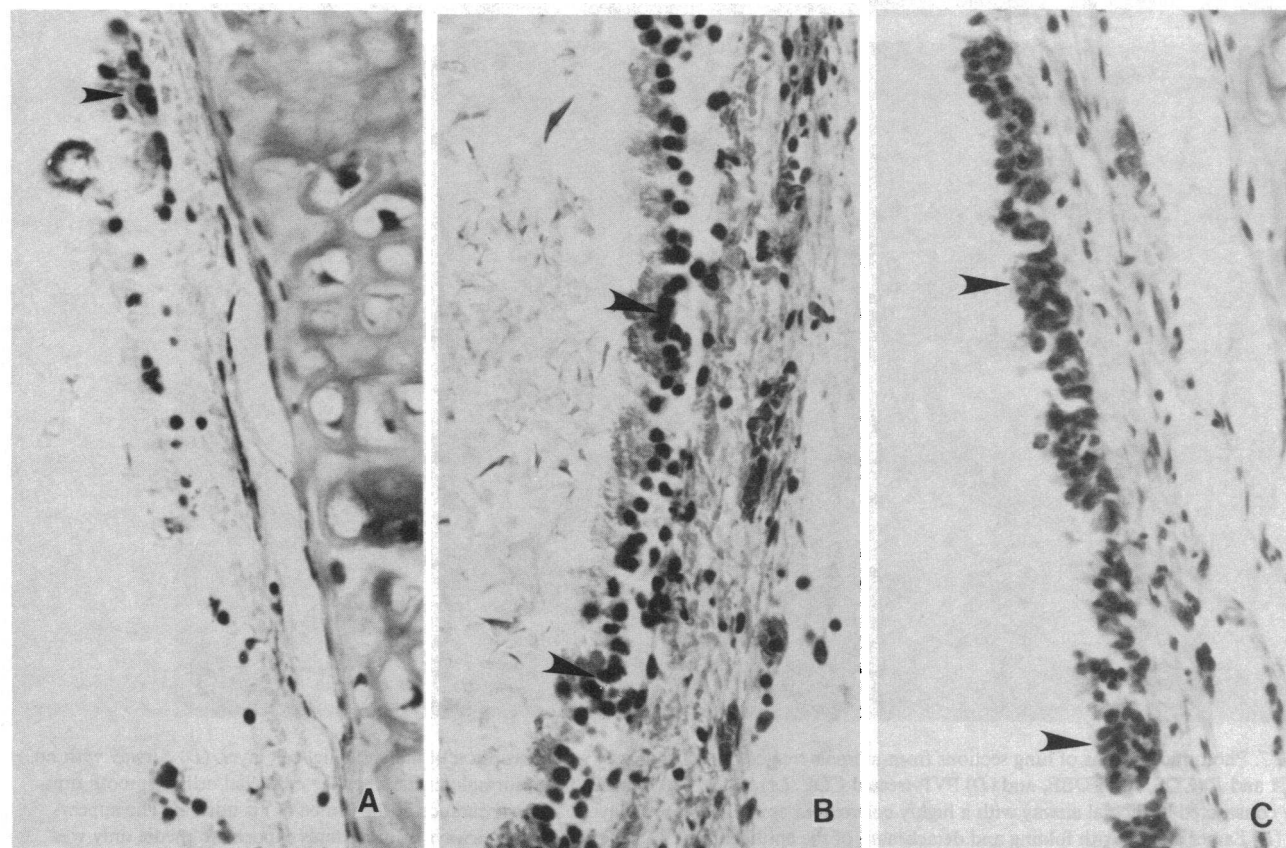


Figure 8. Photomicrographs of rat tracheal explants treated with THF at 1 mg/ml for (A) 4 h and (B) 24 h. After 4 h there is desquamation of the epithelial cell layer, but some viable cells are identifiable (arrow). After 24 h, the epithelial cell layer is completely destroyed and

only naked nuclei remain (arrows). In contrast, explants treated for 24 h with media alone (C) have an intact epithelium. Note the elongated shape of the intact epithelial cells (arrows).  $\times 250$ .



Table VI. Levels of Endotoxin in Cotton Extracts

		Endotoxin*
		ng/ml
Injurious		
1 CDE		360
2 FDBE		91
3 GBE		6
4 THF		45
Noninjurious		
1 CDE · PVP		510
2 FDBE · PVP		16
3 GBE · PVP		4
4 TA & TF CDE		9,500
5 AVRIN		1
6 MEDIA		<1

PVP, PVP-treated extracts; TA & TF, terpenoid aldehyde and tannin-free extracts.

\* Endotoxin levels were measured using the Limulus ameobocyte lysate method.

lysis of pneumocytes and desquamation and death of tracheal epithelial cells. Collectively, these reports show that THF is highly toxic to pulmonary epithelium and capable of reproducing the symptoms of byssinosis, which suggests that this agent may play a role in the pathogenesis of both the acute and chronic forms of this disease. Johnson and Hanson (36) recently reported that purified cotton bract tannins were cytotoxic in vitro to porcine endothelial cells at concentrations very similar to those used in our study. These results further document the marked toxicity of condensed tannins.

There is also data to suggest that endotoxin may play a role in byssinosis. For example, when endotoxin is instilled into rabbit lungs, it can produce a chronic bronchitis picture (27). Similarly, Castellan et al. have shown that the ability of cotton dust to evoke bronchospasm is highly correlated with the endotoxin levels in the samples (2). In our study, the endotoxin concentrations in the dust samples did not correlate with their ability to produce cellular injury (Table V). Also, we found that directly adding endotoxin to A549 and rat type II pneumocytes, and tracheal explants, even at concentrations exceeding those found in the most contaminated cotton dusts, produced very little pneumocyte or explant damage. These data suggest that endotoxin did not produce injury to lung epithelial cells in the systems we studied, but they do not refute the possible role of this agent in byssinosis by other mechanisms.

In summary, it is clear from our studies that cotton dust products are toxic to alveolar, bronchial, and tracheal epithelial cells. It is probable that conducting airway epithelial cells are particularly susceptible to cotton dust- and bract-induced injury because these cells are directly exposed to the influx of dust particles and function to trap particulates. Also, in chronic byssinosis, it is this part of the airway that is primarily involved, and we hypothesize that repeated exposure to respirable toxins in cotton dust and bract may lead to a chronic bronchitis syndrome similar to that observed in smokers. Finally, an important question to ask is whether the concentrations of cotton extracts and THF that we studied are clinically relevant. This can be approached by estimating the amount of cotton dust and hence

THF inhaled in the workplace (37). Assuming a 70-kg worker breathes 15 m<sup>3</sup> of air during an 8-h shift and that cotton dust levels range from 0.28 to 6.36 mg/m<sup>3</sup> of air (38), the inhaled dust burden could be between 4.2 and 95.4 mg in an 8-h shift. Assuming that 50% of the particles are in the respirable range (70% of particles have been shown to be <1.4 µm) (37), the pulmonary deposition could range from 2.1 to 47.7 mg/shift. Therefore, after years of exposure it is possible that many grams of cotton dust and THF could be deposited in the lungs, which suggests that the concentration ranges we studied are clinically relevant.

## Acknowledgments

We thank Joan Blackburn for typing this manuscript and Baxter Travenol Laboratories, Deerfield, IL for performing the endotoxin levels on the extracts.

Dr. O'Neil is the recipient of National Institutes of Health (NIH)-National Heart, Lung, and Blood Institute New Investigator Award HL 32621. This work was also supported by U. S. Public Health Service grants NGMS-GM24990 and DE02600 and by Specialized Centers of Research (SCOR)-NIH grant HL-15093.

## References

- O'Neil, C. E., B. T. Butcher, and J. E. Salvaggio. 1982. Mechanisms in byssinosis: a review. *Proc. ACS Symp. Cotton Dust: Controlling Occupational Health Hazard*, 189, Atlanta. 145-162.
- Castellan, R. M., S. A. Olenchok, J. L. Hankinson, P. D. Millner, J. B. Cocke, C. K. Bragg, H. H. Perkins, Jr., and R. R. Jacobs. 1984. Acute bronchoconstriction induced by cotton dust: dose-related responses to endotoxin and other dust factors. *Ann. Intern. Med.* 101:157-163.
- Buck, M. G., and A. Bouhuys. 1981. A purified extract from cotton bracts induces airway constriction in humans. *Chest*. 79:43S-49S.
- Merchant, J. A., L. C. Lumsden, and K. H. Kilburn. 1973. Dose response studies in cotton textile workers. *J. Occup. Med.* 15:222-230.
- McDermott, M., J. W. Skidmore, and J. Edwards. 1971. The acute physiologic, immunologic and pharmacologic effects of inhaled cotton dust in normal subjects. *Proc. Int. Conf. Respir. Dis. Textile Workers, 2nd, Spain*. 133-136.
- Butcher, B. T., C. E. O'Neil, and R. N. Jones. 1983. The respiratory effects of cotton dust. *Clin. Chest Med.* 4:63-70.
- Schachter, E. N., L. R. Maunder, and G. J. Beck. 1984. The pattern of lung function abnormalities in cotton textile workers. *Am. Rev. Respir. Dis.* 129:523-527.
- Edwards, J. 1981. Mechanisms of disease induction. *Chest*. 79:38S-42S.
- Noweir, M. H. 1981. Studies on the etiology of byssinosis. *Chest*. 79:62S-71S.
- Buck, M. G., and A. Bouhuys. 1980. Byssinosis: airway constrictor response to cotton bracts. *Lung*. 158:25-32.
- Davenport, A., and W. D. M. Paton. 1962. The pharmacologic activity of extracts of cotton dust. *Br. J. Ind. Med.* 19:19-32.
- Wakelyn, P. J., G. A. Greenblatt, D. F. Brown, and V. W. Tripp. 1976. Chemical properties of cotton dust. *Am. Ind. Hyg. Assoc. J.* 37:22-31.
- Bell, A. A., and R. D. Stipanovic. 1983. Biologically active compounds in cotton: an overview. *Proc. Cotton Dust Res. Conf., 7th, San Antonio*. 77-80.
- Loomis, W. E., and J. Battalle. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry*. 5:423-438.
- Edwards, J. H., and B. M. Jones. 1974. Immunology of byssinosis: a study of the reactions between the isolated byssinosis "antigen" and human immunoglobulins. *Ann. NY Acad. Sci.* 221:59-63.
- Fine, J. 1973. Limulus assay for endotoxin. *N. Engl. J. Med.* 289:484-485.

17. Dobbs, L. G., E. F. Geppert, M. C. Williams, R. D. Greenleaf, and R. J. Mason. 1980. Metabolic properties and ultrastructure of alveolar type II cells isolated with elastase. *Biochim. Biophys. Acta.* 618:510-523.
18. Fisher, A. B., L. Furia, and H. Berman. 1980. Metabolism of rat granular pneumocytes isolated in primary culture. *J. Appl. Physiol.* 49: 743-750.
19. Mason, R. J., M. C. Williams, R. D. Greenleaf, and J. A. Clements. 1977. Isolation and properties of type II alveolar cells from rat lung. *Am. Rev. Respir. Dis.* 115:1015-1026.
20. Kikkawa, Y., and K. Yoneda. 1974. The type II epithelial cell of the lung. I. Method of isolation. *Lab. Invest.* 30:76-86.
21. Lane, B. P., S. L. Miller, and E. J. Drummond. 1976. Use of tracheal organ cultures in toxicity testing. *Environ. Health Perspect.* 16: 89-98.
22. Ayars, G. H., L. C. Altman, H. Rosen, and T. Doyle. 1984. The injurious effect of neutrophils on pneumocytes in vitro. *Am. Rev. Respir. Dis.* 130:964-973.
23. Oppenheim, J. J., and B. Schechter. 1980. Lymphocyte transformation. In *Manual of Clinical Immunology*. N. R. Rose and H. Friedman, editors. American Society of Microbiology, Washington. 233-245.
24. Diem, K., and C. Lentner, editors. 1975. *Scientific Tables*. Geigy Pharmaceuticals, Basel. 810 pp.
25. Bouhuys, A., S. E. Lindell, and G. Lundin. 1960. Experimental studies on byssinosis. *Br. Med. J.* 2:324-326.
26. Mundie, T. G., M. Cordova-Salinas, V. J. Bray, and S. K. Ainsworth. 1983. Bioassays of smooth muscle contracting agents in cotton mill dust and bract extracts: arachidonic acid metabolites as possible mediators of the acute byssinotic reaction. *Environ. Res.* 32:62-71.
27. Cavagna, C., V. Foa, and E. C. Vigliani. 1969. Effects in man and rabbits of inhalation of cotton dust or extracts and purified endotoxins. *Br. J. Ind. Med.* 26:314-321.
28. Braun, D. C., L. D. Scheel, J. Tuma, and L. Parker. 1973. Physiological response to enzymes in cotton dust. *J. Occup. Med.* 15:241-244.
29. Ainsworth, S. R., and R. E. Neuman. 1981. Chemotaxis in cotton mill dust: possible etiologic agent(s) in byssinosis. *Am. Rev. Respir. Dis.* 124:280-284.
30. Mundie, T. G., R. J. Boackle, and S. K. Ainsworth. 1983. In vitro alternative and classical activation of complement by extracts of cotton mill dust: a possible mechanism of byssinosis. *Environ. Res.* 32: 47-56.
31. Edwards, J. H., and B. M. Jones. 1973. Pseudoimmune precipitation by the isolated byssinosis "antigen." *J. Immunol.* 110:498-501.
32. Kutz, S. A., M. S. Mentnech, S. A. Olenchock, and P. C. Major. 1980. Precipitation of serum proteins by extracts of cotton dust and stems. *Environ. Res.* 22:476-484.
33. Javillier, M., and J. Lavollay. 1946. Les substances agissant sur la resistance et la permeabilite des capillaries et la notion de vitamine. *P. Helv. Chim. Acta.* 29:1283-1289.
34. Edwards, J. H., E. Evans, P. H. Evans, P. G. Nicholls, and K. T. Rajan. 1975. Effect of a purified cotton dust extract on human lung in culture. *Proc. Br. Pulmonary Soc.* 394.
35. Taylor, G., A. Massoud, and F. Lucas. 1971. Studies on the aetiology of byssinosis. *Br. J. Ind. Med.* 28:143-151.
36. Johnson, C. M., and M. N. Hanson. 1985. Cotton bracts tannin is a cytotoxin for porcine endothelial cells. *Proc. Cotton Dust Res. Conf., 9th, San Antonio.* 130-133.
37. Rylander, R., and P. Morey. 1982. Airborne endotoxin in industries processing vegetable fibers. *Am. Ind. Hyg. Assoc. J.* 43:811-812.
38. Hammond, Y. Y., V. Dharmarajan, and H. Weill. 1981. Sampling of cotton dust for epidemiologic investigations. *Chest.* 79:108S-113S.