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Endothelial Cell Cytotoxicity of Cotton Bracts Tannin and Aqueous Cotton Bracts Extract: Tannin is the Predominant Cytotoxin Present in Aqueous Cotton Bracts Extract

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Using an *in vitro* cytotoxicity assay based on the release of ^{51}Cr from cultured porcine thoracic aortic and pulmonary arterial endothelial cells, we have demonstrated that cotton bracts tannin is a potent endothelial cell cytotoxin. It produces dose-dependent lethal injury to both types of endothelial cells with the aortic cells being somewhat more sensitive to tannin-mediated injury than the pulmonary arterial cells. Cytotoxic injury to the cells was biphasic. During the first 3 hr of exposure to tannin, no lethal injury was detected. However, during this period, profound changes in morphology were observed suggesting sublethal injury to the cells preceded the ultimate toxic damage. Comparison of the cytotoxicity dose curves for aqueous bracts extracts with those for tannin demonstrated that tannin was the major cytotoxin present in bracts.

Byssinosis is an occupational lung disease of textile workers that results from the action of some water-soluble compound(s) present in respirable cotton mill dust on the lungs (*1,2*). Workers with the disease experience an acute, reversible bronchoconstriction when exposed to the dusty mill environment. These workers also have a concomitant decrease in the number of circulating platelets and an influx of neutrophils into the airways (*3,4*). Most studies on the pathogenesis of the disease have been designed to identify the etiologic agents responsible for the bronchoconstriction, platelet activation and neutrophil recruitment characteristic of this disease.

Until recently, little emphasis has been placed on identifying agents that could alter the structural or functional integrity of the cells forming the blood: air barrier, i.e., airway epithelial cells and vascular endothelial cells. Recent studies by Cloutier and colleagues (*5–7*) have demonstrated that aqueous extracts of cotton bracts, the major botanical component of cotton mill dust (*8*), markedly alter both the functional capacity and intercellular permeability of tracheal epithelium *in vitro*. Obviously, compounds that increase epithelial intercellular permeability could significantly enhance the access of inhaled bioactive components in the dust to their target tissues. The studies described in this paper were designed to determine if similar effects were also observed with cultured vascular endothelial cells.

Methods and Materials

Preparation of Aqueous Cotton Bracts Extract and Purified Cotton Bracts Tannin

Aqueous extracts of cotton bracts were prepared as previously described (*9*) and stored as a desiccated, lyophilized powder at −20°C until use. The bracts used in this study were from Acala SJ-2 cotton grown in North Carolina in 1982 and harvested prior to senescence. This method of extraction yielded 455 mg of lyophilized cotton bracts extract (CBE) per gram of bracts. The tannin content of this CBE as determined by the spectrophotometric method of Bell and Stipanovic (*10*) was 52 mg tannin/gram CBE.

This Acala SJ-2 CBE was also the starting material for the isolation of purified cotton bracts tannin. The tannin was purified from the CBE by a previously described (*9*) modification of the method of Taylor (*11*). The tannin was stored at −20°C as a desiccated lyophilized powder. Immediately before use, stock solutions of tannin

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were prepared by dissolving the lyophilized tannin in serum-free Medium 199. These tannin solutions were then filter sterilized before use in the cellular toxicity experiments.

Isolation and Culture of Porcine Thoracic Aortic and Pulmonary Arterial Endothelial Cells

Porcine endothelial cells from the thoracic aorta and pulmonary artery were isolated by a brief treatment with collagenase as previously described (*12*). The cells obtained with this technique were washed free of enzyme with Earle's balanced salt solution, placed into fibronectin-coated 96 well Falcon tissue culture plates and cultured in Medium 199 supplemented with 10% fetal bovine serum, 100 U/mL penicillin G and 20 μg/mL gentamicin. After several days of culture, wells containing islands of cells with typical endothelial cell morphology were expanded to 35 mm well tissue culture plates. Cell lines with no evidence of smooth muscle or fibroblast contamination were harvested and stored in liquid nitrogen for subsequent experiments.

Cytotoxicity Assay

The release of ^{51}Cr from ^{51}Cr-loaded endothelial cells was used as a measure of lethal cell injury. To load the cells with ^{51}Cr, confluent endothelial cell monolayers in 12 well Co-Star tissue culture plates were incubated with ^{51}Cr sodium chromate in Medium 199 supplemented with 10% fetal bovine serum for 2 hr at 37°C in a fully humidified atmosphere of 5% CO_2:95% air. The cells were then washed with Earle's balanced salt solution to remove any excess ^{51}Cr.

Test solutions were then placed on the cells at a volume of 1 mL/well and the cells were incubated at 37°C in a fully humidified atmosphere of 5% CO_2:95% air for the specified times (6 hr unless otherwise indicated). At the end of incubation, 0.5 mL of the 1 mL volume in each well was removed and the quantity of ^{51}Cr determined in a gamma counter. All test solutions were assayed in quadruplicate wells. With each set of assays, quadruplicate wells were incubated in parallel with serum-free Media 199 to determine spontaneous ^{51}Cr release. Total ^{51}Cr available for release was determined by lysis of quadruplicate wells of ^{51}Cr loaded cells with 1% NP-40 in Earle's balanced salt solution. Percent ^{51}Cr release was

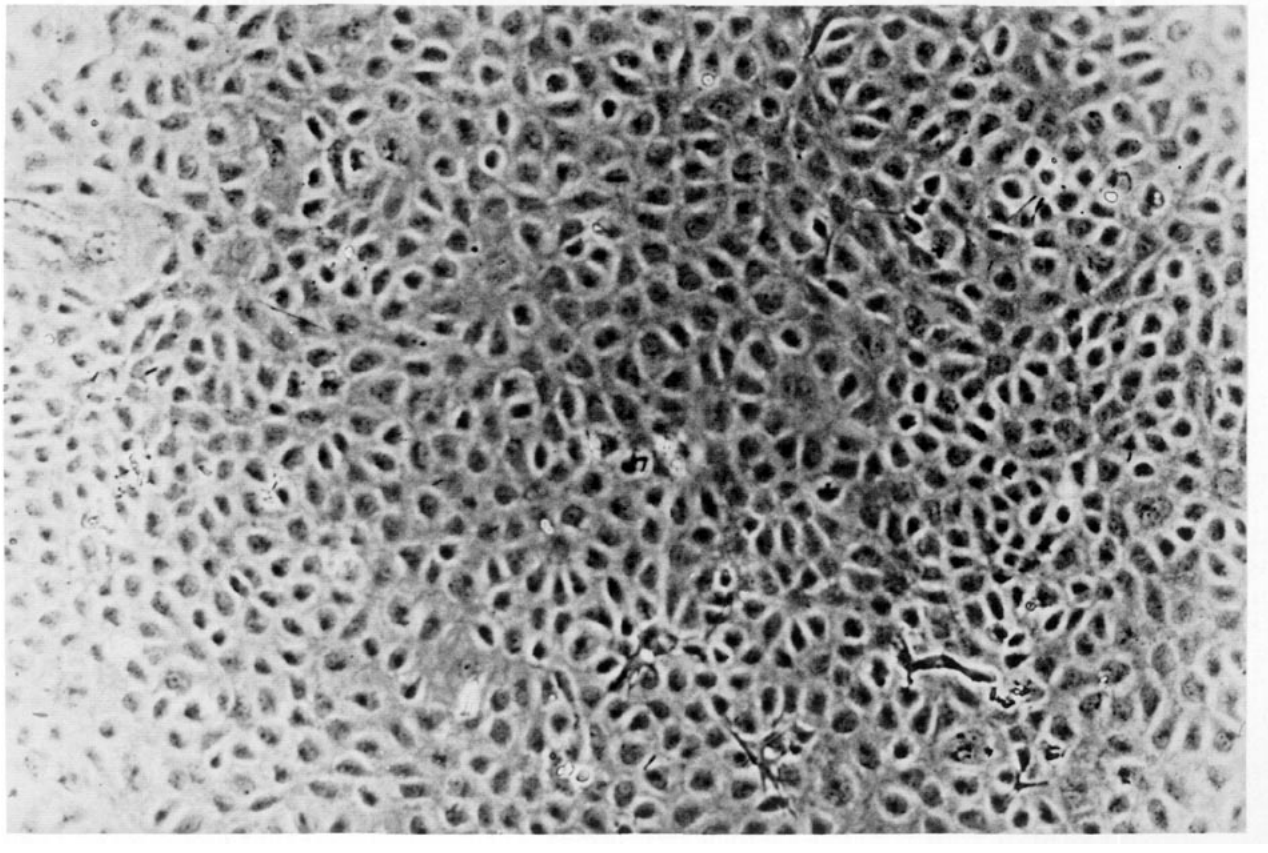

FIGURE 1. **Phase-contrast photomicrograph of control, confluent fourth passage porcine pulmonary artery endothelial cells. The cells maintain this morphology throughout a 6-hr incubation in serum-free Medium 199. Magnification = 125×.**

Table 1. Tannin dose–response curves for the release of ^{51}Cr from porcine aortic and pulmonary arterial endothelial cells.

	% ^{51}Cr release[a]	
Tannin, μg/mL	Aortic	Pulmonary arterial
2.5	0	4.5 ± 3.7
10.1	0	5.1 ± 3.6
25.2	6.2 ± 0.9	14.1 ± 13.4
37.8	47.5 ± 0.5	18.4 ± 14.5
50.4	60.7 ± 2.7	44.1 ± 11.6
101.0	58.3 ± 0.7	61.0 ± 3.5
252.0	53.3 ± 2.4	47.1 ± 12.5

[a] Values shown are means ± SEM ($N = 3$).

then calculated from the following equation:

$$\%\ ^{51}Cr\ \text{release} = \frac{\text{CPM (Test)} - \text{CPM (spontaneous)}}{\text{CPM (Total)} - \text{CPM (spontaneous)}}$$

Microscopy

Cells were photographed at selected time intervals with a Zeiss IM 35 microscope equipped with phase contrast optics. These cells were then processed for scanning electron microscopy as previously described (*12*).

Results

Dose Dependence of Tannin Endothelial Cell Cytotoxicity

A 6-hr exposure to various tannin concentrations produced lethal cell injury as measured by ^{51}Cr release in both porcine aortic and pulmonary arterial endothelial cells. As illustrated in Table 1, the extent of lethal injury was dependent upon the tannin concentration. The cytotoxicity dose–response curves for endothelial cells from both anatomical locations were sigmoidal with a maximum ^{51}Cr release of 60 to 70% occurring at 50.4μg/mL and 101 μg/mL tannin for aortic and pulmonary arterial cells, respectively. This difference in the tannin dose required to produce maximal lethal injury was also reflected in the dose required to produce half maximal injury. On the basis of the EC_{50} values, aortic endothelial cells were more sensitive to tannin mediated damage than pulmonary arterial endothelial cells (39 ± 1 μg/mL vs. 50 ± 2 μg/mL; $p < 0.05$).

The sigmoidal shapes of the cytotoxicity curves suggest that tannin promotes injury to these cells via a saturable process, i.e., binding of tannin to a specific cellular protein or proteins is responsible for the ultimate lethal injury. Although these kinetic data are suggestive of a saturable process, final proof of this inference must await binding studies employing radiolabelled tannin. The differences in sensitivity of aortic and pulmonary arterial endothelial cells to tannin-mediated cytotoxicity also suggests that tannin acts via some specific interaction rather than by some nonspecific lytic mechanism. In addition, this observation suggests that endothelial cells from other anatomical locations within the lung may also vary in their sensitivity to tannin. Thus, if tannin-mediated endothelial cell cytotoxicity occurred *in vivo*, it should be dependent both upon the site of tannin deposition in the airways and the sensitivity of the vascular endothelial cells adjacent to this site.

Time Dependence of Tannin Mediated Endothelial Cell Cytotoxicity

Although a 6-hr exposure to tannin ultimately produces lethal cell injury, the development of lethal injury is biphasic as illustrated in Table 2. For both aortic and pulmonary arterial endothelial cells, little or no ^{51}Cr is released during the first 3 hr of exposure to a maximally cytotoxic dose of tannin. ^{51}Cr release is first detectable at 3 to 4 hr of exposure and then increases in a nearly linear fashion with time throughout the remainder of the exposure period.

Morphologic Changes Associated with Tannin Exposure

In addition to the already noted lethal cell injury, cotton bracts tannin also produced time-dependent changes in the morphology of the endothelial cells. As a point of

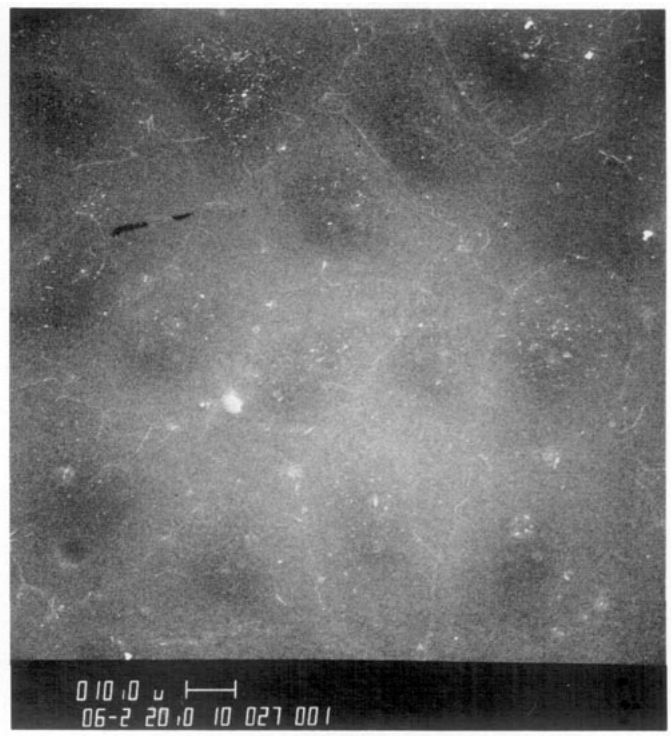

FIGURE 2. Scanning electron micrograph of the same control pulmonary artery endothelial cells shown in Figure 1. Magnification = 600×; bar = 10 μm.

Table 2. Time dependence for tannin-mediated cytotoxicity to aortic and pulmonary arterial endothelial cells.

Time, hr	% ^{51}Cr release[a]	
	Aortic[b]	Pulmonary arterial[c]
0.5	0	1 ± 1
1	0	0 ± 3
3	1 ± 1	3 ± 4
4	19 ± 1	24 ± 14
5	45 ± 2	42 ± 13
6	65 ± 3	56 ± 6

[a] Values shown are means ± SEM ($N = 3$)
[b] Exposure to 50 μg tannin/mL.
[c] Exposure to 101 μg tannin/mL.

comparison, a photomicrograph of confluent pulmonary artery endothelial cells cultured in serum-free Medium 199 is shown in Figure 1. A scanning electronmicrograph (SEM) of the same cells is shown in Figure 2. Both photomicrographs illustrate the normal "cobblestone" morphology of endothelial cells with tight, barely discernible intercellular junctions and prominent nuclear bulges.

Within minutes of exposure of the cells to a dose of tannin that ultimately produces maximal lethal injury (101 μg/mL), this morphology dramatically changes. The intercellular junctions become highly demarcated and easily visible under light microscopy (Fig. 3). This demarcation is even more evident on SEM as shown in Figure 4. In addition, the nuclear bulge has become less apparent, suggesting that the cells had swollen. This altered morphology was maintained during the first 3 hr of exposure to tannin. Despite these profound morphologic changes, the cells remained viable during this period since no ^{51}Cr was released from the cells.

A second morphologic change detectable both by light (Fig. 5) and SEM (Fig. 6) occurred with the onset of ^{51}Cr release from the cells. The nuclei have become pycnotic and the cellular surface shows increased membrane folding and microvilli. This suggests that the cellular membranes have collapsed over the cytoskeleton and appears to signal the onset of lethal injury to the cells.

Comparison of the Cytotoxicity of Cotton Bracts Tannin and Aqueous Cotton Bracts Extract

Although purified tannin was cytotoxic for endothelial cells, it did not necessarily follow that aqueous bracts extracts would exhibit similar cytotoxicity even though these bracts contained high tannin levels. The bracts, for example, could contain other water-soluble compounds

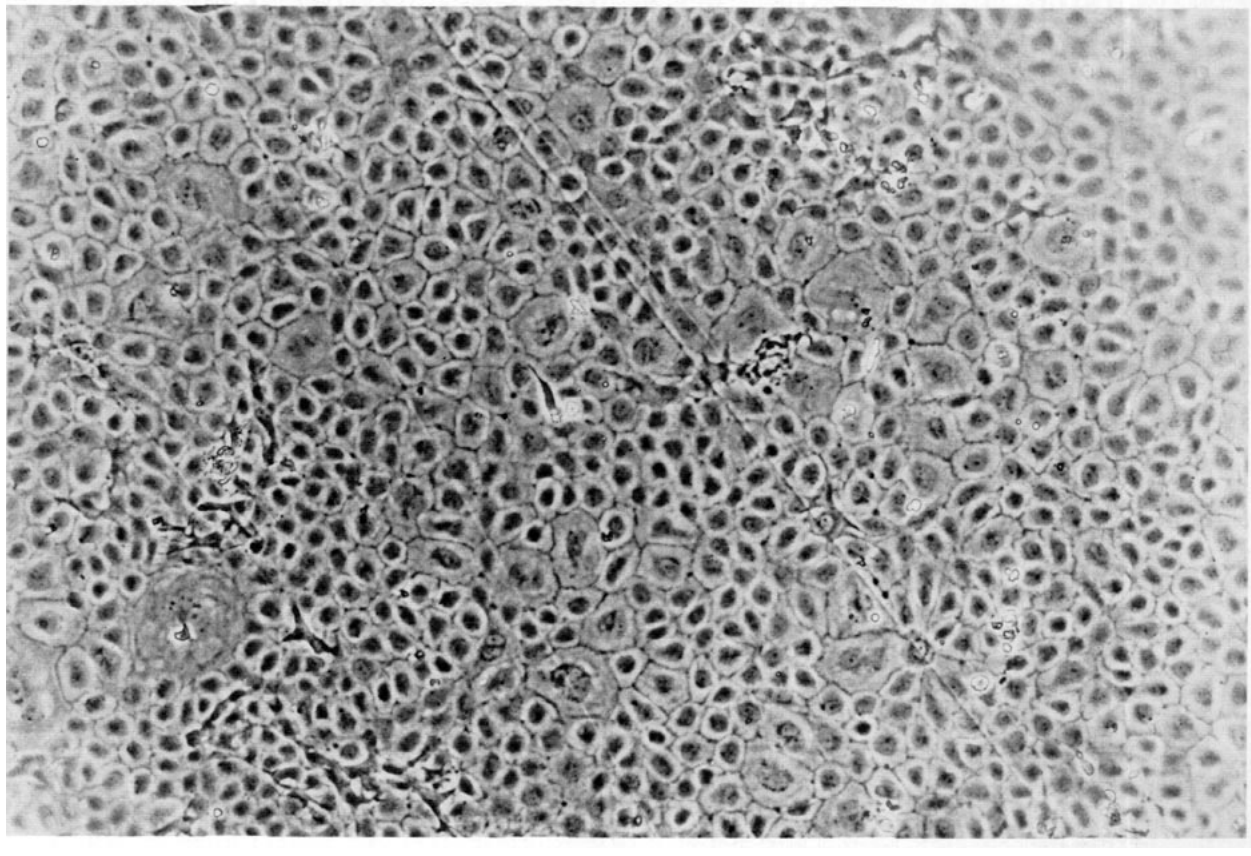

FIGURE 3. Phase-contrast photomicrograph of a monolayer of pulmonary artery endothelial cells 30 min after exposure to 101 μg/mL tannin in serum-free Media 199. Note the prominent intercellular junctions. Magnification = 125×.

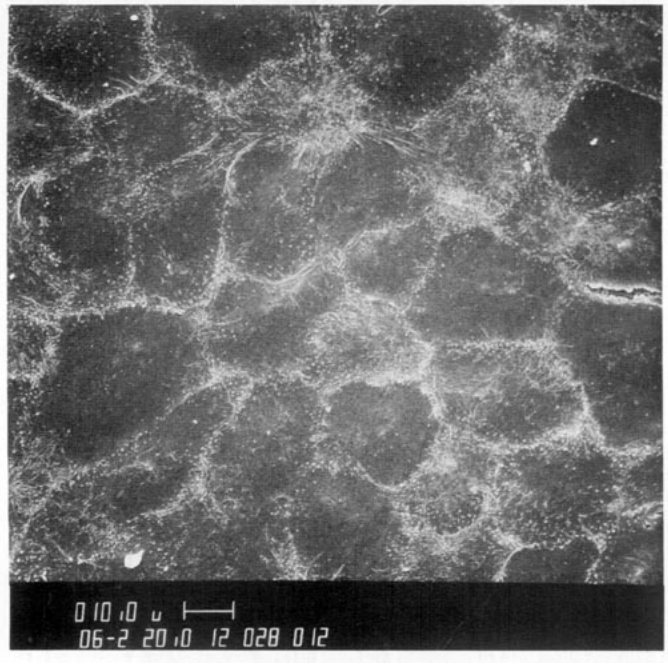

FIGURE 4. Scanning electron microscopic view of the same cells illustrated in Figure 3. Again the intercellular junctions are readily apparent. Magnification = 600×; bar = 10 μm.

that were even more cytotoxic than tannin or, conversely, compounds that diminished the cytotoxicity of the tannin.

To compare the relative cytotoxicity of cotton bracts tannin and aqueous extracts of cotton bracts from which the tannin was isolated, cytotoxic dose-dependent curves were obtained for tannin and bracts extract. To facilitate this comparison, the doses of bracts extract used were expressed in terms of their tannin concentration. As shown in Figure 7, the cytotoxicity dose curves for tannin and bracts extract were similar if not identical. Although the bracts curve was slightly less steep than that for tannin, both agents caused a maximal release of ^{51}Cr at approximately 100 μg/mL and both produced about the same maximal lethal injury, i.e., the release of 50 to 60% of the total ^{51}Cr. These results suggest that, despite the increased chemical complexity of the bracts extract, tannin is the major cytotoxin present in the bracts extract.

Discussion

The condensed tannins are among the most prevalent water soluble compounds in cotton mill dust, accounting for up to 3.5% of the total water extractable material present in the dust (*10*). *In vitro*, purified cotton bract tannins exhibit a variety of biological activities that could contribute to the pathogenesis of byssinosis. Cotton mill

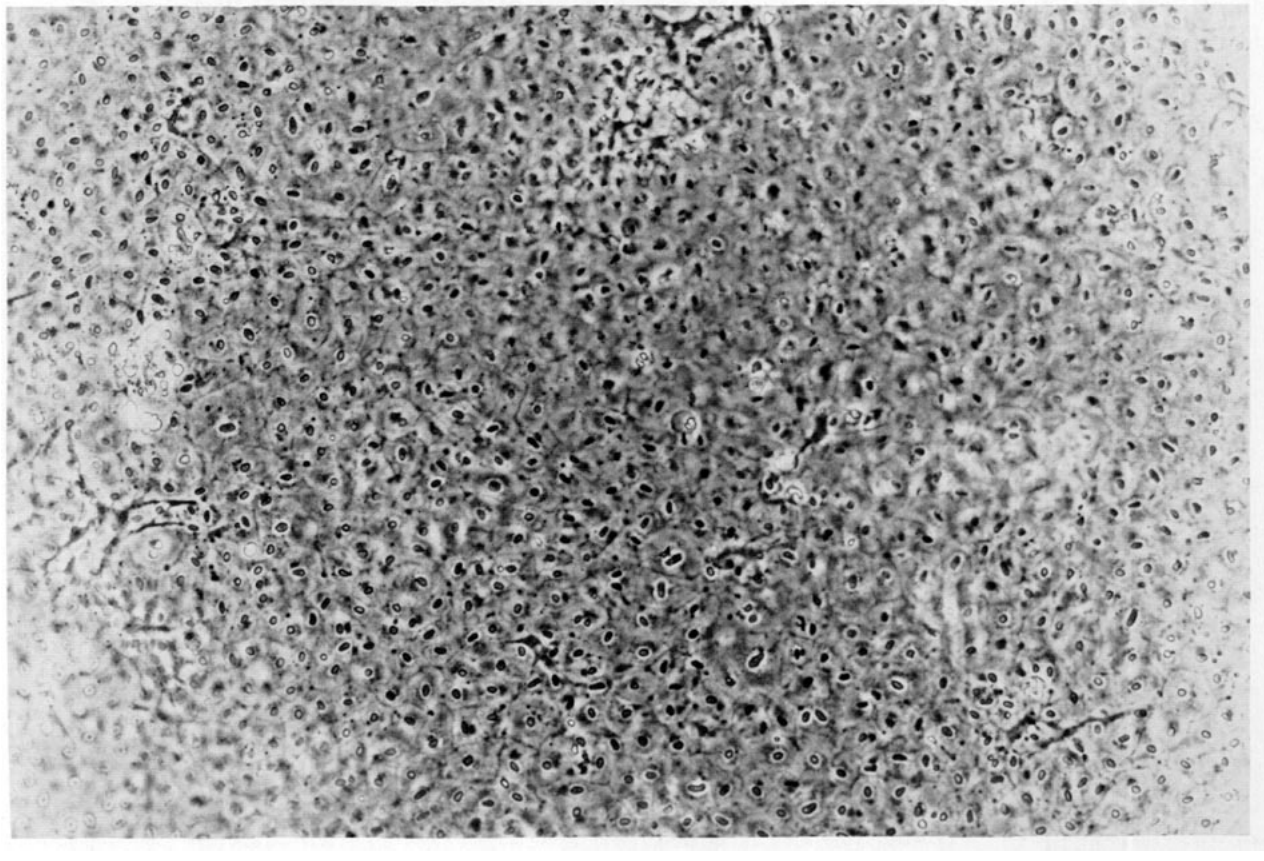

FIGURE 5. Phase-contrast photomicrograph of a monolayer of pulmonary artery endothelial cells 6 hr after exposure to a 101 μg/mL solution of tannin in serum-free Medium 199. The most striking change in morphology is the condensed pycnotic appearance of the nuclei. Magnification = 125×.

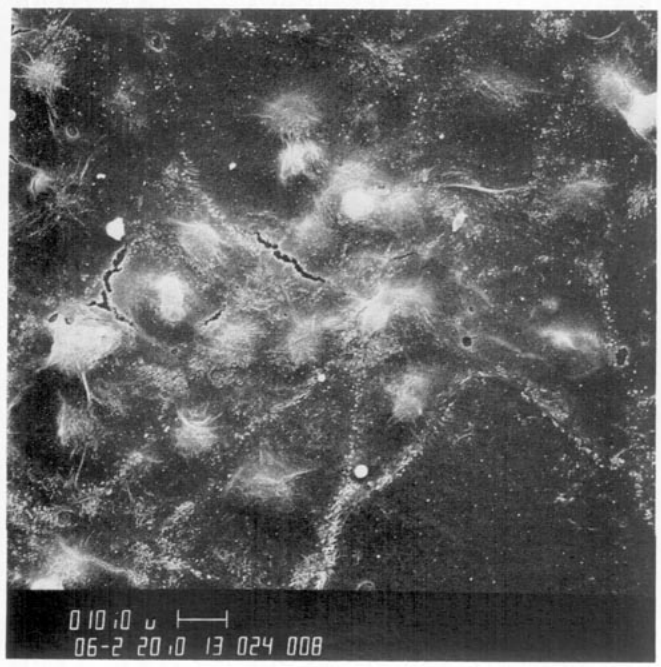

FIGURE 6. Scanning electron micrograph of the same cells depicted in Figure 5. Again the pycnotic nuclei are readily apparent as are numerous folds and microvilli in the membrane. Magnification = 600×; bar = 10μm.

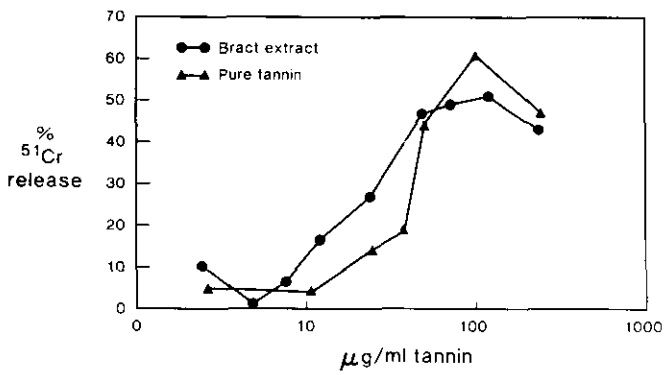

FIGURE 7. Dose–response curves for the release of ^{51}Cr from monolayers of pulmonary artery endothelial cells mediated both by purified tannin (▲-▲) and aqueous cotton bracts extract (●-●). For ease of comparison, the concentrations of bracts employed are expressed in terms of their tannin content. The values shown are the mean of three independent experiments.

workers with byssinosis have a decreased number of circulating platelets after exposure to the dusty mill environment (*3*). This suggests that platelets may be activated and sequestered, perhaps in the lungs, in these workers. Tannins could contribute to this platelet activation since they are potent agonists of human platelets. They promote platelet aggregation, the nonlytic secretion of 5-hydroxytryptamine from platelet dense granules and the formation of thromboxane A_2 in a time and dose-dependent manner (*9,13*). Tannins may also be involved in the recruitment of neutrophils into the airways, an event that has been demonstrated in animals exposed to cotton mill dust (*14,15*). Recent studies from our laboratory demonstrated that purified tannins interact with rat alveolar macrophages to evoke the nonlytic, time- and dose-dependent secretion of a high molecular weight neutrophil chemotactic factor (*16*). Finally, tannins also dramatically alter the electrophysiology of intact sheets of canine tracheal epithelial cells. Exposure of the mucosal surface of these cells to tannin impaired active sodium and chloride transport as measured by a decrease in short-circuit current (*7*). This, in turn, could alter water homeostasis in the airways with a resulting decrease in the mucociliary clearance of compounds from the airways.

The cytotoxicity of purified tannins toward porcine aortic and pulmonary arterial endothelial cells also can now be added to the list of biological activities of tannin that may contribute to the pathogenesis of byssinosis. These studies have demonstrated that tannin causes nearly immediate sublethal structural changes in the morphology of these cells and ultimately produces lethal injury upon continued exposure. The observation that tannin causes a rapid nonlethal change in morphology of these cells raises the possibility that functional changes accompany these structural changes. The potential functional changes most relevant to the pathogenesis of byssinosis are alterations in the adherence of platelets to the endothelial cells and changes in the ability of the endothelial cells to uptake and inactivate 5-hydroxytryptamine.

Endothelial cells normally provide a nonthrombogenic surface, i.e., platelets do not adhere to the plasma membrane of intact endothelial monolayers (*17*). In one preliminary experiment performed in our laboratory (data not shown), we found that a 15-min exposure of endothelial cells to tannin resulted in a 10- to 15-fold increase in the number of platelets bound to these cells. If this observation is substantiated in subsequent studies, it would suggest that the interaction between tannin and endothelial cells could enhance localized platelet binding, and therefore activation, in the pulmonary microvasculature.

Endothelial cells from the pulmonary vasculature also actively take up and inactivate 5-hydroxytryptamine (*18*). It is believed that this represents a mechanism for ensuring that the circulation does not contain high levels of this potent smooth muscle contracting bioamine. If tannin inhibits the ability of endothelial cells to take up and degrade 5-hydroxytryptamine, a significantly higher amount of this amine release upon local platelet activation could reach the airway smooth muscle. This, in turn, would result in increased bronchoconstriction.

In summary, these studies have shown that tannin is a potent cytotoxin for pulmonary endothelial cells and that the cytotoxicity of tannin is not diminished by other water-soluble compounds present in bracts. If these effects observed *in vitro* also occur *in vivo* as a result of the tannin present in inhaled cotton dust, the action of tannin on endothelial cells may contribute to the pathogenesis of byssinosis.

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