Toxicity to Endothelial Cells Mediated by Cotton Bract Tannin

Potential Contribution to the Pathogenesis of Byssinosis

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Byssinosis is an occupational disease of textile workers caused by exposure to the bract portion of the cotton plant in the form of mill dust. The authors established an *in vitro* cytotoxicity assay using ^{51}Cr release to assess time- and dose-dependent toxicity of condensed tannin, a component of bracts, on porcine aortic and pulmonary arterial endothelial cells. Tannin produced dose-dependent toxicity to both types of endothelial cells; aortic endothelial cells were more sensitive than were endothelial cells from the pulmonary artery. Skin fibroblasts were relatively insensitive to tannin. Cytotoxicity was not immediate. Release of ^{51}Cr was preceded by a several-hour period during which the endothelial cells underwent profound morphologic changes (as assessed by light and electron microscopy). Even brief exposure of endothelial cells to tannin produced later toxicity and morphologic changes. Condensed tannin causes time- and dose-dependent injury to endothelial cells *in vitro* at doses potentially achievable *in vivo*. (Am J Pathol 1986, 122:399–409)

BYSSINOSIS is a significant occupational lung disease of textile workers. The characteristic acute symptoms of chest tightness, dyspnea, cough, and wheezing typically develop 4–6 hours after exposure to the dusty mill environment. The severity of these symptoms is generally greatest when workers return to the mills after an absence of several days.[1] The acute syndrome is associated with changes in lung function suggestive of reversible airway narrowing; however, prolonged exposure to the mill dust may produce a clinical syndrome similar to chronic obstructive pulmonary disease with irreversible loss of lung function.[2–7] These changes may progress after occupational exposure has ceased.[8]

Several studies have suggested that the byssinotic agent in mill dust is located in the cotton bract – the thin, friable leaves surrounding the cotton boll.[1,9,10] Water-soluble tannins are a major organic component of both cotton bracts and cotton mill dust.[11] Tannins have been proposed as potential contributors to the byssinosis syndrome.[12] Previous studies in our laboratory have identified tannin as a potent platelet agonist, accounting for platelet-stimulating activity present in both cotton bracts and cotton mill dust.[13,14] We now report that, in addition, bract tannin causes dose- and time-dependent toxicity to endothelial cells derived from both porcine thoracic aorta and porcine pulmonary artery. These effects are observed at tannin concentrations consistent with amounts potentially deposited in a worker's airways during the course of a typical work shift.

Materials and Methods

Isolation and Growth of Cells

Porcine endothelial cells from the thoracic aorta were isolated as previously described.[15] Briefly, aortas were obtained from a local slaughterhouse or from an animal purchased for this purpose and euthanized with

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pentobarbital. Tissues were placed in Earle's balanced salt solution (EBSS, GIBCO, Grand Island, NY) with penicillin G, 100 U/ml, and gentamicin (GIBCO), 20 μg/ml, for transport to the laboratory. Aortic endothelial cells were harvested by ligating the vessels of the aortic arch and then clamping the aorta closed at one end. The aorta was then washed free of red blood cells with EBSS and filled with a solution of collagenase (Boehringer-Mannheim Biochemicals, Indianapolis, Ind) in EBSS. After clamping the other end, we incubated the vessel for 15 minutes at 37 C. The collagenase solution was then poured out of the vessel, which was then filled with fresh EBSS. This buffer solution was gently agitated inside the vessel by repeated aspirations in a pipette to dislodge the endothelial cells. A segment of pulmonary artery from the pulmonary valve to the first bifurcation was used to isolate endothelial cells from this site. The vessel was ligated at one end, and the endothelial cells were isolated in a way similar to that by which aortic cells were isolated. The cell suspensions were washed free of enzyme with EBSS and inoculated into 96-well tissue culture plates (Falcon Plastics, Oxnard, Calif) precoated with porcine fibronectin as previously described.[15] The 96-well plates were then examined at intervals for identification of wells containing islands of endothelial cells with typical morphologic features. Such wells were then expanded to 35-mm dishes for verification of their endothelial morphologic features. Cell lines with no evidence of smooth muscle contamination were then frozen in liquid nitrogen for later experiments. Endothelial cells were routinely grown in Medium 199 (M.A. Bioproducts, Walkersville, Md) with 10% fetal bovine serum (FBS, Sterile Systems, Logan, Utah) containing 100 U/ml of penicillin G and 20 μg/ml of gentamicin. When confluent, cells were passaged by treatment of the monolayer with trypsin–EDTA (2.5 mg/ml–60 μM, GIBCO).

Porcine skin fibroblasts were isolated from explants of tissues obtained as follows: after the animal was euthanized as above, the skin was shaved and sterilized with 70% isopropyl alcohol; a strip of skin was then excised, minced, and inoculated into 35-mm dishes in Medium 199 supplemented with 10% FBS and antibiotics as above. Cells of typical fibroblast morphology migrated out of the explants over 5–7 days. When confluent, these cells were passaged and frozen as above.

Preparation of Condensed Tannin

Cotton bract tannin was isolated as previously described[12] and stored lyophilized at −20 C. The cultivar used in these experiments was Acala SJ-2, grown near Raleigh, North Carolina, and harvested prior to senescence in 1982. As noted previously by Taylor,[12] the tannin isolated by this procedure is not a homogeneous compound; it is composed of a heterologous group of complex condensed polyphenols that vary both in molecular weight and in the composition of the subunit monoflavanoids. Tannin concentration was determined by the spectrophotometric method of Bell and Stepanovic.[16] Stock solutions of tannin for use in these assays were prepared immediately before use by dissolving the lyophilized tannin to the appropriate concentration in serum-free Medium 199, which was then filter-sterilized and placed on the cells. At the concentrations used, tannin did not alter the pH of the serum-free medium.

^{51}Cr-Release Assay

Fourth-passage endothelial cells and third-passage skin fibroblasts were grown to confluence in 12-well multiwell plates (Costar, Cambridge, Mass) in Medium 199 with 10% FBS as described above. They were then loaded with ^{51}Cr as follows: ^{51}Cr as sodium chromate (New England Nuclear, Boston, Mass) was diluted in Medium 199 with 10% FBS to yield a final activity of 2.5 μCi/ml, placed on the cells, and incubated for 2 hours at 37 C in a 5% CO_2 tissue culture incubator; at the end of the incubation, the monolayers were washed twice in EBSS for removal of the extracellular ^{51}Cr, and the test solution was placed on the cells in a volume of 1 ml/well. In all assays serum-free Medium 199 was incubated in parallel with the test solutions for determination of the spontaneous release of ^{51}Cr for the appropriate incubation time. Total ^{51}Cr available for release was determined by treatment of parallel wells of ^{51}Cr-loaded cells with 1% Nonidet P-40 (NP-40, Sigma) in EBSS. All test solutions were assayed in quadruplicate wells. At the end of the incubation, 0.5 ml of the 1 ml vol/well was removed and the counts per minute (cpm) in the sample measured in a gamma counter. The ^{51}Cr release was then calculated with the following formula:

$$\%\ ^{51}\text{Cr release} = \frac{\text{mean cpm (sample)} - \text{mean cpm (spontaneous)}}{\text{mean cpm (total)} - \text{mean cpm (spontaneous)}} \times 100$$

The quadruplicate wells of this assay consistently gave results with a standard error of less than 10% of the mean value. The spontaneous release of chromium increased linearly with incubation time, reaching between 12% and 18% of the total available for release after a 6 hours' incubation. Although these experiments were performed with the test material dissolved in serum-

free Medium 199, we have found that the spontaneous release of ^{51}Cr in this system is independent of the protein concentration of the medium. During a 6-hour incubation, ^{51}Cr-loaded endothelium cells released the same amount of radioactivity whether the medium was serum-free or contained either FBS (1% or 10%) or 0.5% bovine serum albumin. The endothelial cells showed no change in morphology after the ^{51}Cr-loading or following a 6-hour incubation in serum-free medium alone. Although ^{51}Cr can show some nonuniformity in binding to intracellular proteins, over the incubation times used in these experiments, the appearance of the isotope in the supernatant in excess of the spontaneous release represents cell injury in the form of loss of membrane integrity.[17]

Endotoxin Experiments

Endotoxin derived from *Enterobacter agglomerans* (the major species of gram negative bacillus found on cotton bracts[18]) was a gift of Dr. Robert Jacobs of Cotton, Incorporated. Cytotoxicity assays using this endotoxin were performed by loading endothelial cells with ^{51}Cr as described above. Subsequently, varying concentrations of endotoxin dissolved in serum-free Medium 199 were incubated on the cellular monolayers as described above for 6 hours. Serum-free Medium 199 without endotoxin provided the control for spontaneous release of radionuclide. At the end of the incubation, ^{51}Cr in the supernatant was measured and ^{51}Cr release calculated as described above.

Microscopy

During the incubations with test solutions, the cells were observed and photographed with a Zeiss IM 35 microscope equipped with phase-contrast optics. Selected cells were processed for scanning electron microscopy (SEM) by fixation in 2% glutaraldehyde buffered to pH 7.25 with 2 M sodium cacodylate. The samples were then dehydrated in graded ethanol, critical-point-dried, coated with palladium-gold, and viewed in an ETAC scanning electron microscope operated at 20 keV. To assure accurate comparsions of cellular morphology, we directed the electron beam at right angles to the cell sheet for all SEM samples. Transmission electron microscopy (TEM) was performed on selected cells by growing the monolayers on an Aclar membrane (Allied Chemical, Morristown, NJ). After fixation of the cells in the above buffered glutaraldehyde, the membranes were postfixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.2. They were then stained with 2% aqueous uranyl acetate, dehydrated in graded ethanol, and embedded in Spurr's low-viscosity resin (Electron Microscopy Sciences, Fort Washington, Pa). The blocks were then sectioned in two directions – both parallel and perpendicular to the plane of the cellular monolayer. The sections were then poststained with 0.3% aqueous lead citrate and examined with a Philips 201 transmission electron microscope operated at 60 keV.

Statistical Methods

The mean values of the quadruplicate wells were calculated as described above for determination of percent release values for a given time or concentration point. The standard errors of these quadruplicate wells were in all cases less than 10% of the corresponding mean values. Results were then described as the mean ± standard error for each time or concentration point of three separate identical experiments done on different days with different cohorts of cells. The tannin dose producing 50% of the maximal effects (EC_{50}) was calculated for each of triplicate experiments for aortic and pulmonary arterial endothelial cells, and the results were compared with a two-tailed *t* test.

Results

Cotton bract tannin produced dose- and time-dependent toxicity to porcine endothelial cells. Figure 1 shows the results of increasing tannin dose on ^{51}Cr release for the fixed incubation time of 6 hours. It is apparent that, for endothelial cells, a rapid increase of

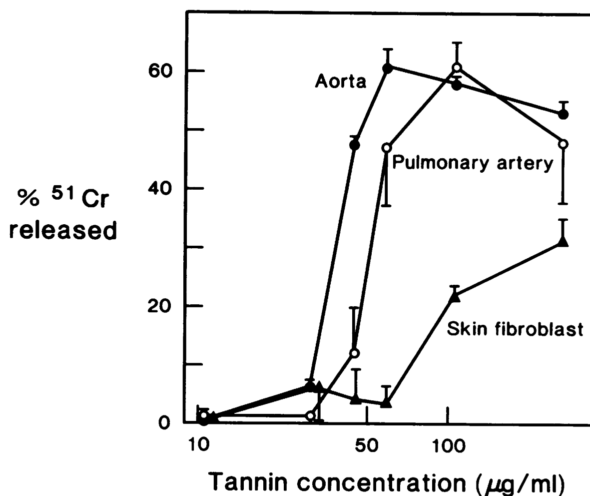

Figure 1 – A dose-response curve of ^{51}Cr release to increasing tannin concentrations for both aortic and pulmonary arterial endothelial cells as well as for skin fibroblasts. The incubation time was 6 hours; the assay was performed as described in the text. Each point represents the mean value of triplicate experiments (± standard error) performed on different days on different cohorts of cells (●——●, aorta; ○——○, pulmonary artery; ▲——▲, skin fibroblast).

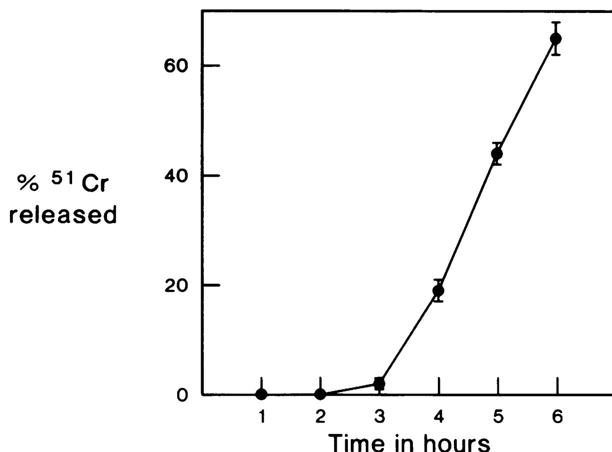

Figure 2—A time course of tannin exposure to aortic endothelial cells. The dose used was 50 μg/ml, a tannin concentration shown in Figure 1 to be maximally toxic to aortic cells. For each time point there was a parallel incubation of serum-free medium over ^{51}Cr-loaded cells for determination of the appropriate spontaneous release value. Each point represents a mean value (± standard error) of triplicate experiments performed on different days on different cohorts of cells. Pulmonary artery cells exhibited a similar pattern of a lag phase followed by a linear increase in ^{51}Cr release (data not shown).

^{51}Cr release, and hence cytotoxicity, occurred over a narrow concentration range of tannin. Following this rapid rise, a maximal level was achieved that was less than the total ^{51}Cr available for release by detergent (1% NP-40 in EBSS). Additionally, the aortic endothelial cells appeared to be more sensitive to tannin effects, compared with pulmonary artery endothelial cells. Calculation of the tannin dose producing 50% of the maximal ^{51}Cr release (EC_{50}) for both cell types yields an EC_{50} for aortic cells of 39 ± 1 μg/ml (mean ± SEM) and for pulmonary artery cells of 50 ± 2.4 μg/ml. These values are significantly ($P \leqslant 0.05$) different from each other and were obtained in experiments performed on cells of the same passage derived from the same animal. Both cell types were of similar density at confluence—5×10^4 cells per square centimeter of dish surface.

Tannin also caused some toxicity to porcine skin fibroblasts. However, as can be seen in Figure 1, the ^{51}Cr release curve for fibroblasts is shifted at least 1 log unit to the right, compared with both endothelial cell types.

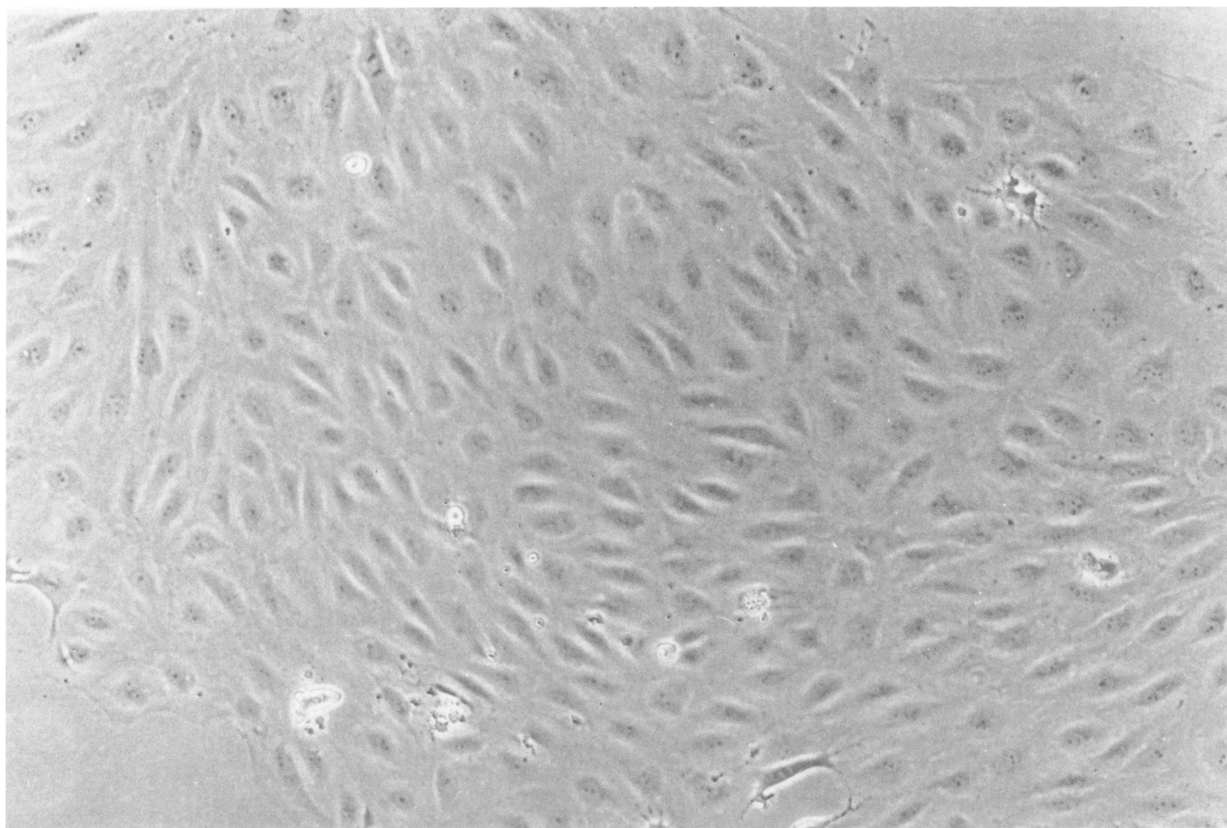

Figure 3—A phase-contrast photomicrograph of confluent, fourth-passage aortic endothelial cells. The cells grow in a compact monolayer of polygonal-shaped cells and show no change after a 6-hour incubation in serum-free medium. (×125)

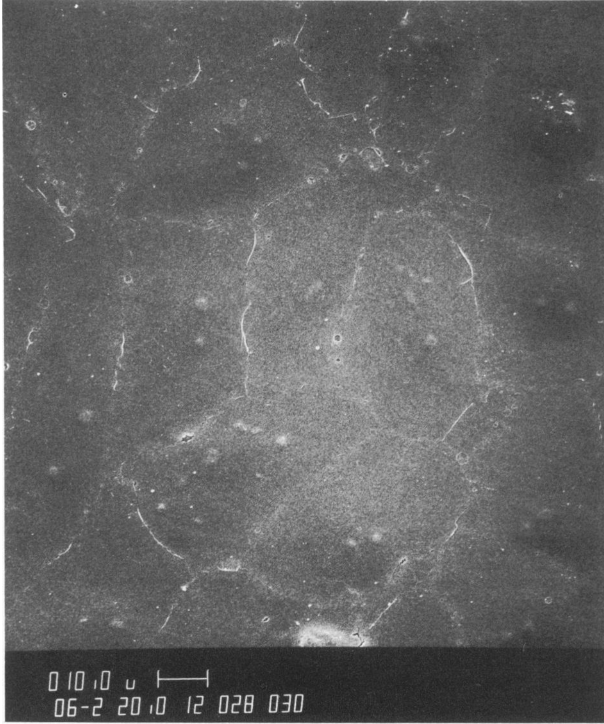

Figure 4—A scanning electron microscopic view of the same aortic cells shown in Figure 3. The specimen was prepared and viewed as described in the text. (×600; bar = 10 μ)

Because maximal response was not reached in the fibroblast assays, an EC_{50} could not be calculated. We found that tannin concentrations in excess of those shown in the figure caused difficulties in maintaining a neutral pH in the medium.

Tannin-mediated cytotoxicity was not immediate. Figure 2 shows a time course of ^{51}Cr release from aortic endothelial cells to a fixed dose of tannin. With a dose that produced the maximal ^{51}Cr release after a 6-hour incubation (50 μg/ml) in Figure 1, it can be seen that cytotoxicity is delayed. After the onset of ^{51}Cr release, the amount of the isotope in the culture supernatant increased with time in a linear fashion. Pulmonary artery cells showed a similar delay in toxic effects (data not shown).

In addition to cytotoxicity as measured by ^{51}Cr release, cotton bract tannin produced dose- and time-dependent alterations in porcine endothelial cellular morphology. Figure 3 shows a phase-contrast photomicrograph of aortic endothelial cells incubated in serum-free medium. These cells exhibit the typical morphologic features of vascular endothelial cells in culture.[19] Figure 4 shows a scanning electron microscopic view of the same cells. Both photomicrographs dem-

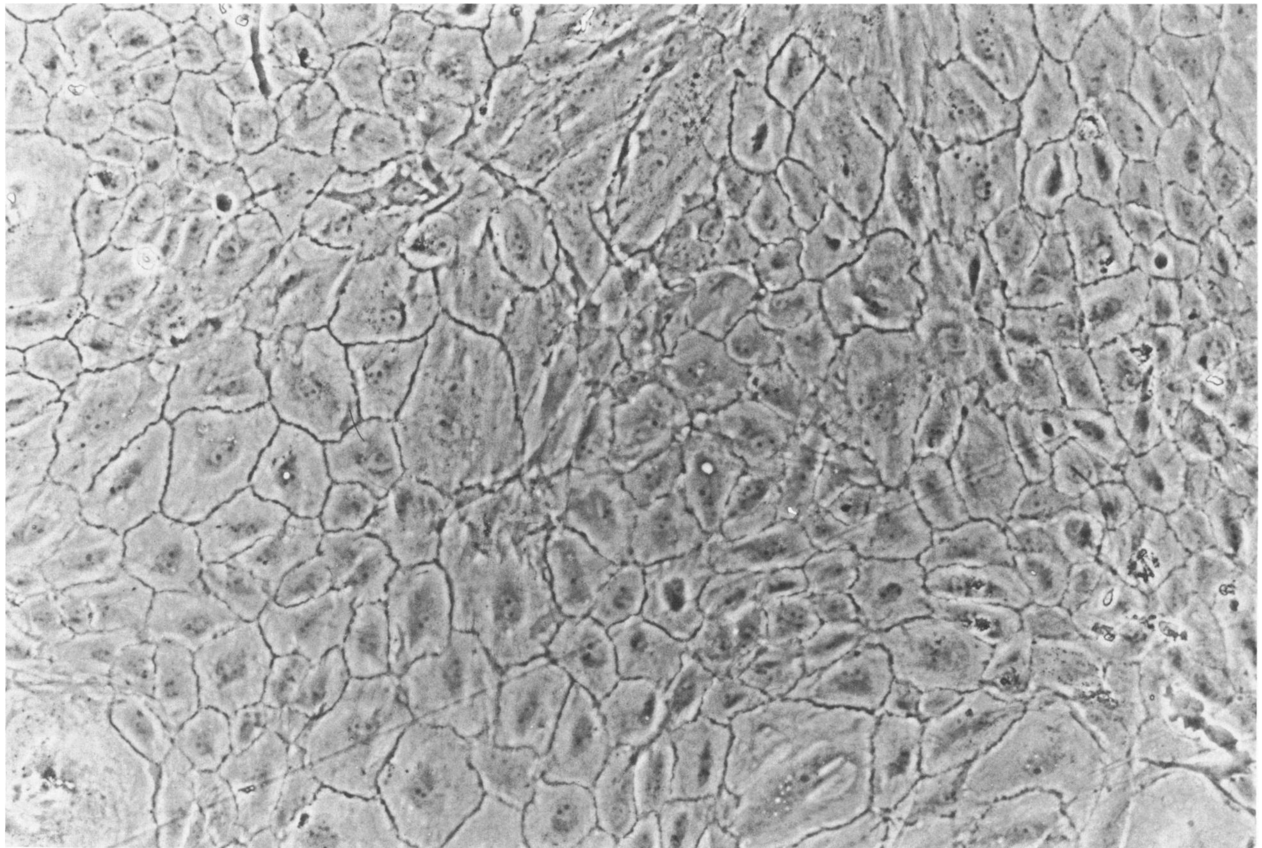

Figure 5—A phase-contrast view of an aortic endothelial cell monolayer 30 minutes after the addition of 50 μg/ml of condensed tannin dissolved in serum-free medium as described in the text. The monolayer is intact; the prominence of the intercellular junctions is apparent. (×125)

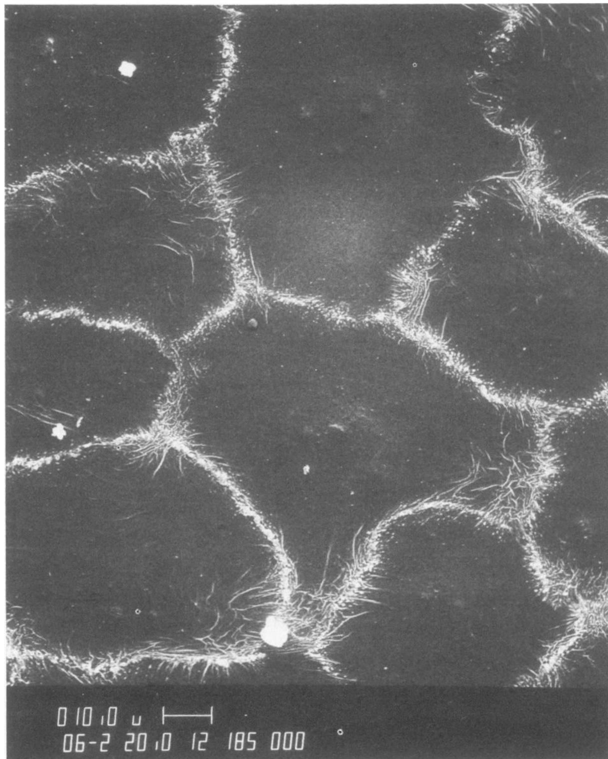

Figure 6—A scanning electron microscopic view of the same aortic cells depicted in Figure 5, 30 minutes after the addition of 50 μg/ml of tannin in serum-free medium. The specimen was prepared and viewed as described in the text. The intercellular junctions are strikingly apparent. In addition, the cellular surfaces show multiple microvilli and some folding of the membranes. (×600; bar = 10 μ)

onstrate a uniform monolayer of polygonal-shaped cells with tight intercellular junctions and easily discernible nuclear bulges. This morphology showed no change after a 6-hour incubation in serum-free medium.

Within minutes after addition to the wells of a tannin dose of 50 μg/ml, both aortic and pulmonary artery cells underwent a consistent and marked change in their appearance. Figure 5 shows a phase-contrast photomicrograph of aortic endothelial cells after a 30-minute exposure to a tannin dose of 50 μg/ml, a maximally cytotoxic dose in Figure 1. The intercellular junctions, normally barely seen (Figures 3 and 4), have become quite prominent. No gaps were observed between the cells. On scanning electron microscopy the cells show the same prominence of intercellular junctions (Figure 6). In addition, the cellular membranes show multiple folds and microvilli. As shown in Figure 2, these morphologic changes occurred well before any cytotoxicity as measured by ^{51}Cr release was observed.

Morphologic changes are also apparent when the cells are examined by transmission electron microscopy. Figure 7 shows an intercellular junction of a normal aortic endothelial cell monolayer; a tight, overlapping junction is apparent. Figure 8 shows an intercellular region of similar cells after a 30-minute exposure to 50 μg/ml of tannin. Multiple membrane folds and microvilli can be seen. Figures 9 and 10 show these same tannin-treated cells, but with the sample sectioned parallel to the plane of the monolayer. The cellular border regions show no gaps between adjacent cells, but again the membrane folds and microvilli are obvious.

When tannin exposure was continued, an additional morphologic change in the cells was noted. Figures 11 and 12 show phase-contrast and scanning electron microscopic views of aortic endothelial cells after 6 hours of constant exposure to a tannin dose of 50 μg/ml. In these photomicrographs the monolayer is intact. However, the nuclei appear pyknotic; and in the scanning electron microscopic view, the cellular surfaces continue to show substantial folding and microvilli. These findings suggest that the cellular membranes may have collapsed over cytoskeletal elements. This second morphologic change correlated with the appearance of ^{51}Cr in the supernatant, and thus presumably reflects cell lysis. Pulmonary artery cells underwent the same morphologic changes over a similar time course (data not shown).

When aortic and pulmonary arterial endothelial cells

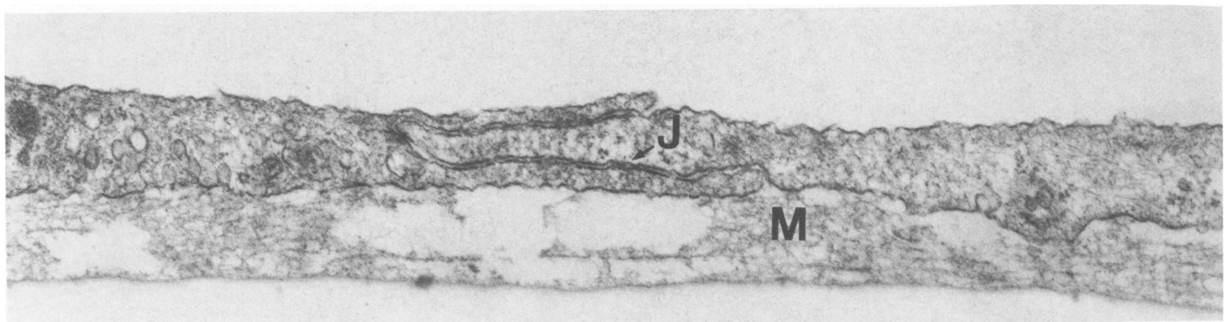

Figure 7—A transmission electron micrograph of aortic endothelial cells after a 30-minute incubation in serum-free medium, processed as described in the text. The sample was sectioned perpendicular to the plane of the monolayer. The intercellular junction is seen (*J*), as is the subcellular matrix material (*M*). (×22,500)

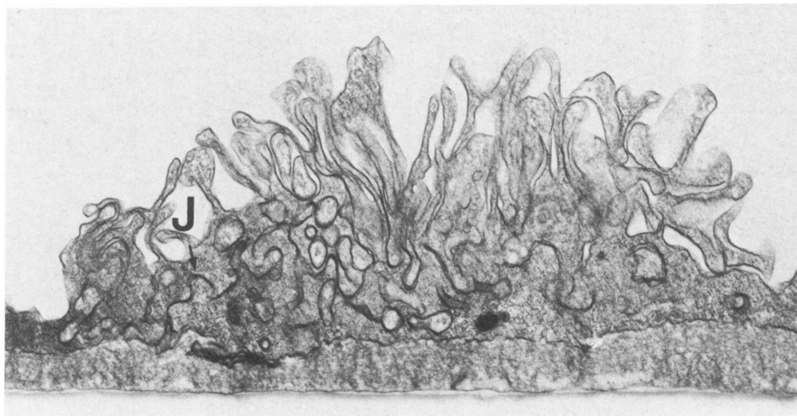

Figure 8—Transmission electron micrograph view of aortic endothelial cells following a 30-minute exposure to 50 μg/ml of tannin, processed as described in the text and sectioned like the cells in Figure 7. The intercellular junction (*J*) is difficult to identify among the many folds and microvilli. (×21,000)

were only briefly exposed to cytotoxic tannin doses, a measurable, albeit more modest, release of ^{51}Cr occurred. Figure 13 shows ^{51}Cr released into the supernatant as measured 6 hours after a brief tannin exposure. These aortic monolayers were incubated with 50 μg/ml of tannin for times ranging from 1 to 30 minutes. At the end of the tannin exposure, the ^{51}Cr in the supernatant was counted, the monolayers washed three times with EBSS, and the incubation continued in serum-free medium for a total of 6 hours. At the end of the incubation ^{51}Cr release was calculated as described. As previously shown in Figure 2, no ^{51}Cr release in excess of the spontaneous rate was found in the supernatant during the short tannin incubation. As can be seen in Fig-

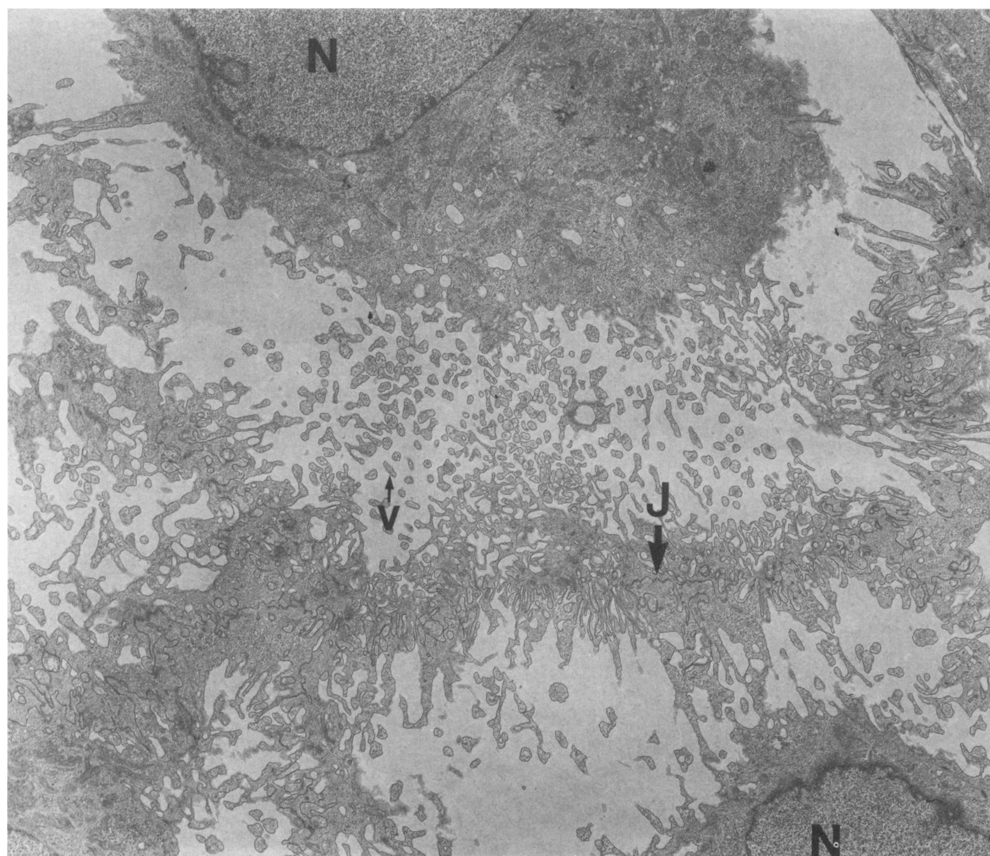

Figure 9—A transmission electron microscopic view of cells treated identically to those in Figure 8 but sectioned parallel to the plane of the monolayer. The region near the intercellular junction (*J*) is seen to be relatively prominent. The plane of the section passes above the main cell body and shows numerous microvilli (*V*) cut transversely. Two cellular nuclei (*N*) are also seen. (×4500)

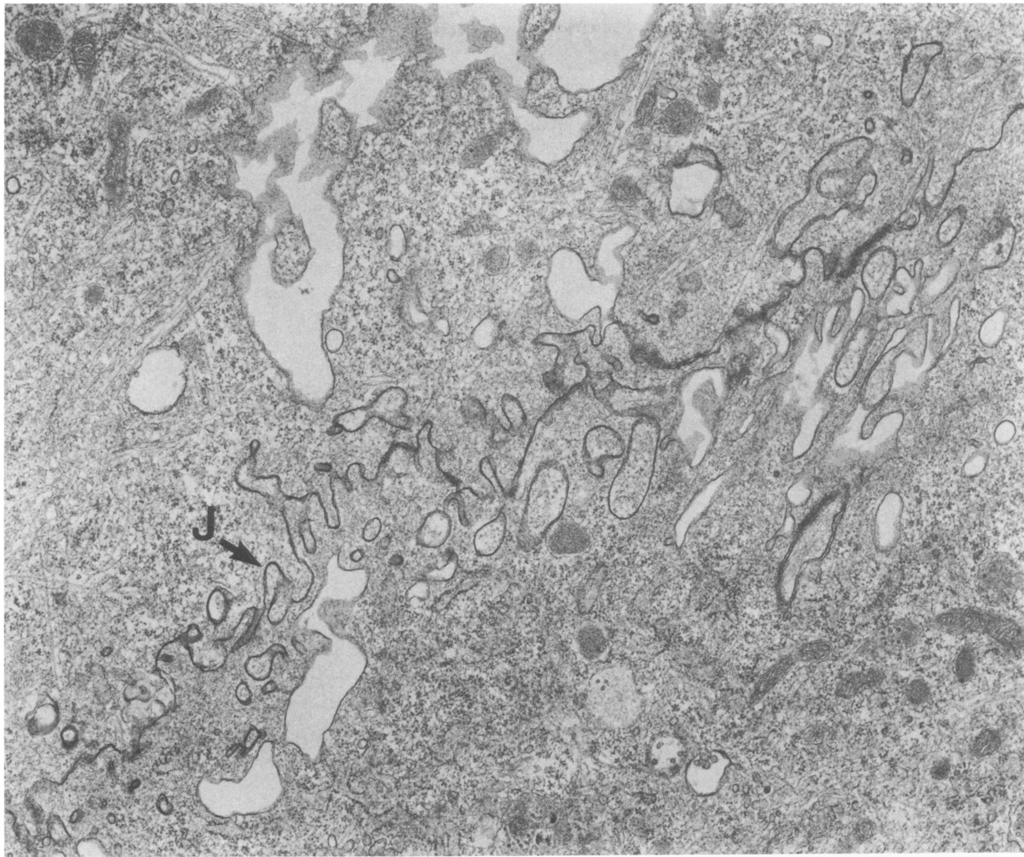

Figure 10—A higher power transmission electron micrograph of the same cells illustrated in Figure 9. The intercellular junction (*J*) is intact, with the membrane showing numerous folds. (×15,000)

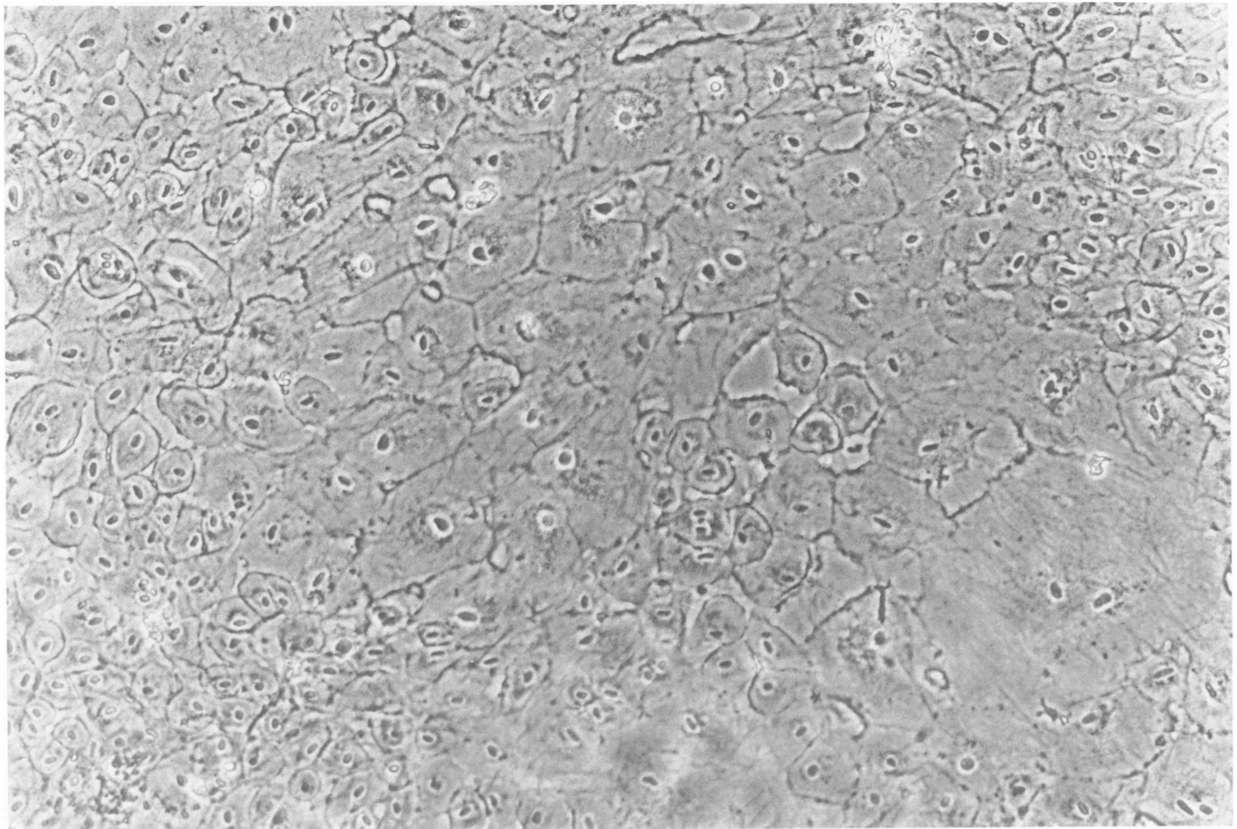

Figure 11—A phase-contrast photomicrograph of aortic endothelial cells after 6 hours of constant exposure to a tannin dose of 50 μg/ml as decribed in the text. The intercellular junctions continue to be quite prominent when compared with control cells (Figure 3). An additional finding in this figure is the condensed, pyknotic nuclei. The latter change correlates with the appearance of ^{51}Cr in the supernatant as described in Figure 2. (×125)

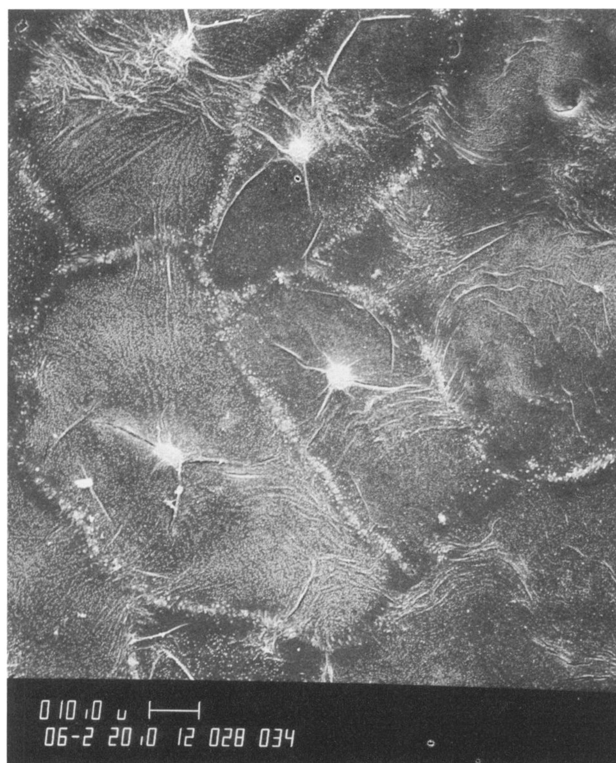

Figure 12—A scanning electron microscopic view of the same aortic endothelial cells illustrated in Figure 11, prepared as described in the text. The folds and microvilli are apparent, as well as the shrunken nuclear bulges. (×600; bar = 10 μ)

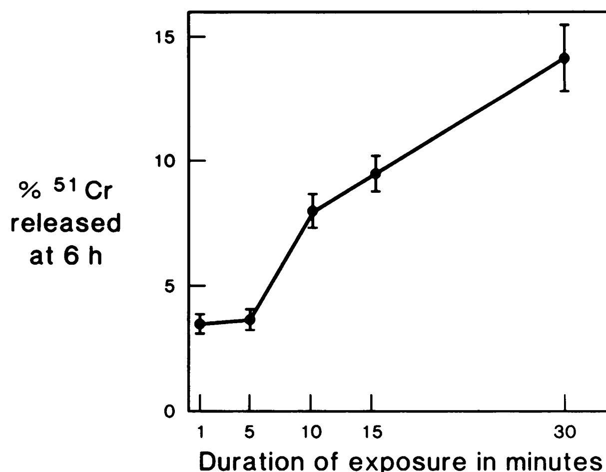

Figure 13—The effects of brief tannin exposure on subsequent ^{51}Cr release by aortic endothelial cells. Confluent monolayers of cells were loaded with ^{51}Cr as described in the text. The cells were then exposed to 50 μg/ml of tannin in serum-free medium for time periods ranging from 1 to 30 minutes. At the end of the various incubation times, quadruplicate wells were washed three times with EBSS (the initial supernatant was saved for measurement of ^{51}Cr) and fresh serum-free medium without tannin placed on the cells. The incubation was then continued for a total of 6 hours, at which time the ^{51}Cr release was determined as described in the text. Parallel wells of cells not exposed to tannin were washed with EBSS at intervals to determine the spontaneous ^{51}Cr release appropriate to the individual time point and to correct for any ^{51}Cr release caused by the washing. As suggested by Figure 2, no radioactivity in excess of the spontaneous control value was noted in any of the wells during the initial tannin incubation. Each point shown represents the mean ± SEM of quadruplicate wells of a single experiment.

ure 13, even brief exposure of aortic monolayers to tannin caused cytotoxicity 6 hours later, and the extent of this increased with lengthening exposure time. Both endothelial cell types in the limited-exposure experiments also displayed altered morphologic features at 6 hours, but in a pattern different from that in the previous experiments. Figure 14 shows a phase-contrast photomicrograph 5½ hours after the end of a 30-minute tannin exposure of pulmonary artery cells as described above. The cells appear to show retraction from each other and detachment from the substratum of the culture dish, as well as extensive vacuolization. As with all the morphologic changes described previously, this phenomenon was uniform over the plate and did not appear to be confined to a subpopulation of cells.

The results of endotoxin exposure on endothelial cells are shown in Table 1. High concentrations caused some ^{51}Cr release; no morphologic changes were observed in the cells for any endotoxin dose. As with the tannin experiments, aortic endothelial cells were somewhat more sensitive to endotoxin than were pulmonary artery cells. The endotoxin concentration in our tannin preparation was measured by Dr. Janet Fisher of the University of North Carolina, Chapel Hill, using the limulus lysate assay: it was found to be present at a maximum concentration of 66 pg/μg of tannin.

Discussion

In these experiments we have shown that cotton bract tannin causes dose- and time-dependent toxicity to porcine endothelial cells. Even before lethal cellular injury occurs, tannin caused profound morphologic changes in the cells. Our observation of a several-hour delay in the onset of lethal injury suggests that tannin does not act simply by immediate disruption of the cellular membrane. It is intriguing that the dose-response curve for tannin-mediated toxicity is very similar to our previous description of tannin-induced activation of human and bovine platelets.[13] Accumulation of activated platelets in the airways is suggested in a rabbit model of byssinosis,[20] and workers with an acute byssinotic attack show a drop in their circulating platelet counts.[21]

These studies have also shown a differing sensitivity to tannin between ascending aortic and pulmonary arterial endothelial cells. In contrast, skin fibroblasts were much less susceptible than either endothelial cell type. Such a hierarchy of response suggests that tannin-mediated cytotoxicity may not be a generalized cellular phenomenon. Neither of the endothelial cell types used in these experiments, however, necessarily parallels the behavior of the pulmonary capillary endothelial cells.

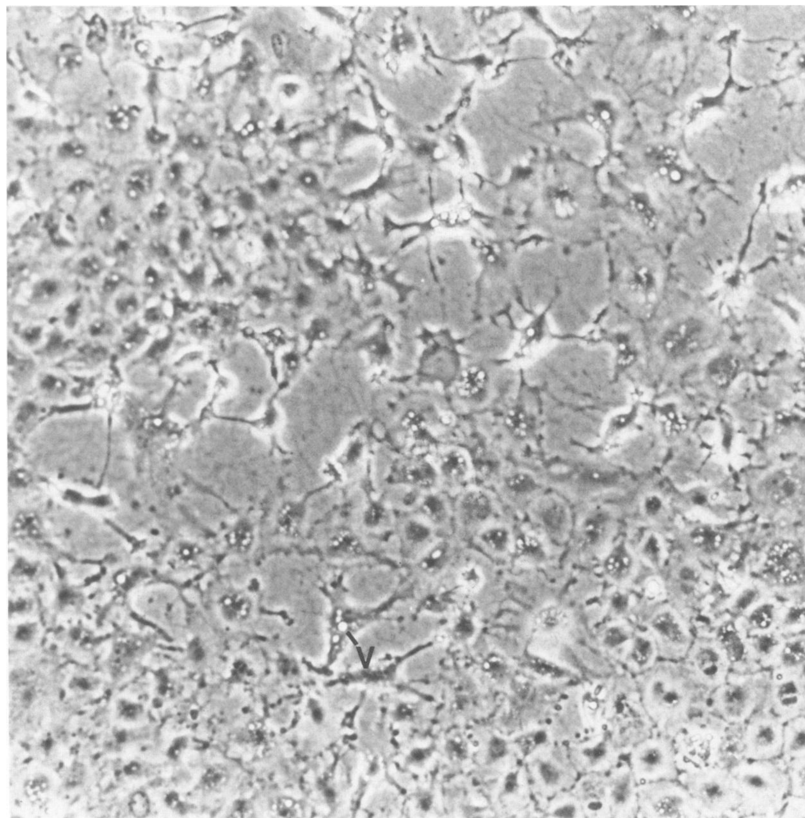

Figure 14—A phase-contrast photomicrograph of pulmonary artery endothelial cells from an experiment in design identical to that described for aortic cells in Figure 13. The cells are viewed 5½ hours after the end of a 30-minute incubation in 50 μg/ml of tannin. The monolayer is disrupted, with the cells retracting from each other and detaching from the dish surface. Many of the cells show multiple vacuoles (*v*); these were noted to begin appearing at 3–4 hours after the end of the tannin exposure. (×125)

Cotton mill dust is a complex mixture of substances, including ground cotton and other plant material, fungi, bacteria, pesticides, and soil.[22,23] The major botanical component of the dust is the bract, the thin, friable leaves surrounding the cotton boll, which may comprise up to 70% of the dry weight of the dust.[11] Studies from several laboratories have suggested that the etiologic agent (or agents) of byssinosis is located in the cotton bracts,[1,9,10] and tannins comprise up to 4.2% of the dry weight of the bract.[24] Currently, aqueous bract extracts have been shown to mediate multiple activities of potential importance in byssinosis: these include smooth-muscle contraction,[25] neutrophil chemotaxis,[26,27] and platelet activation.[13,14] Recent evidence has shown a strong association between the amount of endotoxin in a dust sample and acute byssinotic symptoms in human subjects.[28] However, such an association does not establish a cause; in fact, there is no reason to assume that byssinosis is caused by a single agent in the bracts, because several of the biologic activities described above could act synergistically to produce the syndrome. The endotoxin concentration in our tannin preparation (66 pg endotoxin/μg tannin) is far too low to account for our observed cellular toxicity, as shown in Table 1. Lipopolysaccharide has been shown to produce cellular detachment and cause cytotoxicity in bovine aortic endothelial cells.[29] The degree of ^{51}Cr release over 6 hours reported by these authors is in close agreement with our data obtained with *E agglomerans* endotoxin (Table 1).

Table 1—Endothelial Cytotoxicity to Endotoxin

	Endotoxin concentration		
Cell type	0.1 μg/ml	1.0 μg/ml	10 μg/ml
Aorta	3.1 ± 0.5%	4.0 ± 0.4%	7.8 ± 0.3%
Pulmonary artery	1.0 ± 1.2%	1.0 ± 0.6%	3.0 ± 0.1%

Porcine endothelial cells were assayed for cytotoxicity to endotoxin as described in the text. Values given are mean ± standard error of percent ^{51}Cr released for triplicate experiments. Significant, dose-dependent cytotoxicity was seen with aortic cells for all endotoxin doses. For pulmonary artery cells only a concentration of 10 μg/ml of endotoxin yielded ^{51}Cr release significantly different from the spontaneous release value.

It is implicit in the studies in this report that significant doses of tannin could reach the pulmonary endothelial cells of a cotton worker. Using an adult of average weight (70 kg), breathing an average tidal volume, we estimate that a typical worker can deposit 19–20 μg/hour of tannin in his airways. This estimate is only a rough guide, because the amount of tannin in different bract samples can vary widely.[24] One would also anticipate wide variations in the local concentrations of tannin: for example, the microenvironment immediately surrounding a dust particle would be expected to have quite high amounts of tannin. Once solubilized

on the airway epithelium, the tannin would need to move across the epithelium and interstitium to reach the endothelial cells. Recent work has shown that aqueous bract extracts profoundly alter the functional integrity of canine tracheal epithelium, causing increased paracellular diffusion of manitol suggestive of a loosening of cell-to-cell contact.[30,31] These data suggest that bract tannin in the doses used in these experiments could conceivably reach target endothelial cells. Additionally, as we have noted, byssinosis may be associated with platelet activation, and any agent responsible for this effect must presumably cross the endothelial barrier to the microcirculation.

In conclusion, the cellular pathophysiology of byssinosis is unknown. These experiments suggest that condensed tannin, a substance present in substantial quantities in the cotton bract, has profound toxic effects on porcine endothelial cells *in vitro* at doses potentially achievable *in vivo*. Moreover, even brief, sublethal exposure of endothelial cells to tannin may affect their morphologic features and integrity.

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