Granulocyte Recruitment to Airways Exposed to Endotoxin Aerosols[1,2]

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SUMMARY

Guinea pigs were exposed to aerosols of 0.1 mg of *Salmonella typhosa* endotoxin per ml for 2 or 4 hours, and white cells in blood and in bronchopulmonary lavage were counted at 2, 4, and 6 hours. The lungs of a second group of guinea pigs and hamsters exposed in the same manner were fixed in inflation with osmium tetroxide in fluorocarbon, which retains cells on luminal surfaces of airways. The polymorphonuclear leukocyte counts in cardiac blood were significantly increased, and lymphocyte counts were decreased at 6 hours ($P < 0.05$). The number of cells obtained by bronchopulmonary lavage, which were mostly polymorphonuclear leukocytes, increased at 4 hours to 28 million, and at 6 hours, was 26.5 million ($P < 0.01$), compared to 5 million cells at the same times in air and in water aerosol control preparations. The polymorphonuclear cell counts on airway cross sections, i.e., (polymorphonuclear cells/epithelial cells) $\times$ 100, showed a mean $\pm$ SD peak of 53.9 ± 10.9 after 4 hours in guinea pigs and a peak of 99.7 ± 11.0 after 6 hours in hamsters. Alveoli showed no cell recruitment. Platelets were aggregated on surfaces of arterioles facing bronchioles, although counts in blood were unchanged. Neither leukocytes nor airway cells showed damage to their ultrastructure. The time course for airway recruitment and leukocytosis matches that for symptoms after exposure of workers to dusts from natural fodder or fibers. This suggests that leukocyte recruitment to airways by inhaled endotoxin may be part of the mechanism for the fever and chest tightness in occupational disorders.

Introduction

Inhalation exposure to endotoxin from gram-negative bacilli may be an important factor in several occupational diseases, including asthma (1), bagassosis (2), byssinosis (3), mill fever (4), and detergent enzyme worker's asthma (5). Because each of these exposures is to a complex mixture, Pernis and associates (4) and Cavagna and co-workers (6) studied exposure of human subjects to endotoxin by inhalation. They observed shortness of breath, a nonproductive cough, and a decrease in forced expiratory volume in 1 sec. When Cavagna and co-workers (6) exposed rabbits repeatedly to endotoxin by aerosol, a few rabbits developed pyrexia and hyperventilation initially; many did so after the second or third exposure. Snell (7) also exposed rabbits to aerosols of *Escherichia coli* endotoxin (0127:B8, Difco Corp., Detroit, Mich.); these rabbits showed a decrease in circulating polymorphonuclear (PMN) leukocytes followed by inflammation of airways and alveolar interstitial edema. Cavagna and associates (6) found that daily exposure of rabbits to *E. coli* endotoxin (011:B4, Difco) prolonged for 20 weeks produced bronchitis, peribronchial infiltrates by lymphocytes, and exfoliation of bronchial cells. Brain (8) and Rylander (9) have emphasized the usefulness of counting the free cells lavaged

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from the lung to assay responses to airborne exposures. The purpose of this study was to define the temporal sequence of changes in peripheral blood leukocyte counts, free cells obtained by bronchopulmonary lavage, leukocyte recruitment to airways, and the pathologic features of airways and alveoli produced by inhaled endotoxin.

Materials and Methods

For lavage studies, 9 male guinea pigs (Camm Hartley breed) weighing 225 to 475 g were exposed in a Plexiglas® chamber to an endotoxin aerosol for 2 or 4 hours. To produce the aerosol, 10 mg of *Salmonella typhosa* lipopolysaccharide (Difco, 0901) were suspended in 100 ml of sterile, pyrogen-free water and were placed in a Collison nebulizer. The aerosol was delivered to the domed top of a cuboidal exposure chamber, 1 foot in each dimension, and was removed from a port below the perforated floor. It was operated at a pressure 1 to 2 cm H_2O less than atmospheric. The chamber air and aerosol were exhausted to a water trap. The entire chamber, generator, and trap were operated in a chemical fume hood during exposures. Approximately 25 ml remained in the nebulizer at the end of the 4-hour exposure, indicating that approximately 7.5 mg of endotoxin was aerosolized. A duplicate apparatus was used for exposure of 6 guinea pigs to a sterile, nonpyrogenic water aersol alone. Six control animals were kept in filtered air cages in the laboratory.

Before exposure, 0.3 to 0.5 ml of heparinized cardiac blood was obtained from all animals for total and differential white blood cell counts. Counts were repeated after 2, 4 or 6 hours. White blood cells were counted in a Coulter counter Model B, and differential morphologic features were determined from counting 100 cells on a Wright-stained blood smear.

Another group of 8 guinea pigs was exposed to an aerosol of endotoxin for 4 hours. Cardiac blood was obtained for counting leukocytes and platelets before exposure and at 6 hours, i.e., 2 hours after exposure was completed. Four control guinea pigs were exposed to sterile water, and blood counts were obtained similarly. At 2, 4, or 24 hours after the start of exposure, the animals were anesthetized by intraperitoneal injection of 5 mg of pentobarbital per 100 g of body weight, and a tracheostomy was performed in each. A 3-mm polyethylene tube was inserted into the tracheostomy, and the trachea was tied to the tube. Each animal's lungs were lavaged with 5-ml aliquots of a 0.25 *M* sucrose solution, for a total of 20 ml. The cumulative return volume was measured. Cells were counted from the undiluted fluid using a Spencer AO hemacytometer. The product of cells counted and total volume returned gave the total numbers of cells for each lavage. A 15-ml aliquot was centrifuged at 800 *g* at 2° C for 10 min. The supernate was discarded, and the cell button was resuspended in 5 ml of methanol and recentrifuged. The cell button was resuspended in 0.5 ml of methanol and was smeared onto several slides, air dried, fixed in methanol, and stained with May-Greenwald or Giemsa stain. Differential counts of 1,000 cells per specimen were done under oil immersion lens (1,000 × magnification). Cells were classified as mononuclear cells, airway epithelial cells, or PMN leukocytes.

Immediately after lavage, the lungs were fixed by intratracheal injection of 5 ml of 2 per cent glutaraldehyde buffered with cacodylate. Sagittal sections of these lungs were cut, embedded in paraffin, stained with hematoxylin and eosin, and examined microscopically for cellular infiltrates in alveoli. Animals with such infiltrates were excluded from analysis.

To determine at what airway levels recruitment of leukocytes was occurring, sections of trachea, bronchi, small airways, and alveoli were examined by light and electron microscopy. Eight male guinea pigs weighing 350 g and 12 male and female hamsters weighing 90 to 110 g were exposed to an aerosol of the supernatant of an *E. coli* culture grown on artificial medium. After 2, 4, 6, 12, or 24 hours, animals from both groups were anesthetized with intraperitoneal pentobarbital and underwent tracheostomy. Five milliliters of 2 per cent osmium tetroxide in fluorocarbon FC-80 (Minnesota Mining and Manufacturing Co., St. Paul, Minn.) were instilled into the trachea from a height of 25 cm while the animal continued breathing, as previously described by Thurston and associates (10). After 30 min, the trachea and inflated lungs were removed and placed in 70 per cent ethanol. Ten to 12 1-mm cubes of tissue, including an airway, were cut from sagittal lung sections, dehydrated in increasing concentrations of ethanol and then in propylene oxide, embedded in Epon®, and cut at 1-μm thickness for light microscopy and for trimming and cutting thin sections for electron microscopy. Thin sections were cut with diamond knives, stained with methanolic uranyl acetate and lead citrate, and examined in an AEI EM 6B electron microscope. Counterpart sagittal sections of lung were dehydrated, embedded in paraffin, sectioned, and examined unstained and stained with the periodic acid-Schiff method for histologic study. Sections of Epon-embedded airways 1 μm thick were studied to identify cell types, to count cells, and to distinguish their locations. Alveoli were examined for evidence of cell membrane damage and cell recruitment. Two or 3 animals were sacrificed at each time interval; PMN leukocytes, monocytes, and airway epithelial cells were counted in 3 airways from each animal. Results are expressed as number of PMN leukocytes/number of epithelial cells × 100.

Results

Exposure to endotoxin increased PMN leukocyte counts in cardiac blood by 2,500 cells per

TABLE 1

RESPONSES TO ENDOTOXIN OF POLYMORPHONUCLEAR (PMN) LEUKOCYTES, LYMPHOCYTES, AND PLATELETS (PER MM^3)

	Time After Exposure, hours		
	0	6	P Value
Endotoxin aerosol (N = 8)			
PMN	5,731	8,225	< 0.05
Lymphocytes	5,535	3,164	< 0.05
Platelets	396,000	519,000	NS
Control water aerosol (N = 4)			
PMN	1,600	2,234	NS
Lymphocytes	2,819	3,180	NS
Platelets	756,750	680,750	NS

mm^3 at 6 hours ($P < 0.05$), whereas the lymphocyte count decreased by 2,370 cells per mm^3 ($P < 0.05$) (table 1). Changes in platelet counts were trivial. Thus the deposition of endotoxin in guinea pig airways that caused recruitment of PMN leukocytes to their surfaces was accompanied by leukocytosis in cardiac blood. Control animals exposed for 6 hours to a sterile water aerosol had no significant changes in PMN leukocytes, lymphocytes, or platelets. The airway response, as shown by the total number of cells lavaged, exceeded 18 million at 2 hours ($P < 0.01$), 28 million at 4 hours ($P < 0.01$), and 26.5 million at 6 hours ($P < 0.01$) (figure 1A). Control animals exposed to air had approximately 3 million cells at each interval, and water aerosol control animals had approximately 5 million at the same intervals. Most of the cells counted in exposed animals were PMN leukocytes (figure 1b). The increases in lavaged total cell count and PMN leukocytes were significantly different from air and water control counts at both 4 and 6 hours ($P < 0.01$). At 2 hours, the PMN counts were significantly greater in the endotoxin group ($P < 0.05$) than the group exposed to water. There were less than 4 million mononuclear cells at 2 and 4 hours, but there were 5 million at 6 hours and 14 million at 24 hours. When compared to control animals challenged with water aerosol, this difference was significant ($P < 0.02$). By 24 hours, mononuclear cells comprised 27 per cent of lavaged cells, compared to 10 per cent at 7 hours ($P < 0.05$). No significant changes were seen in epithelial cell counts.

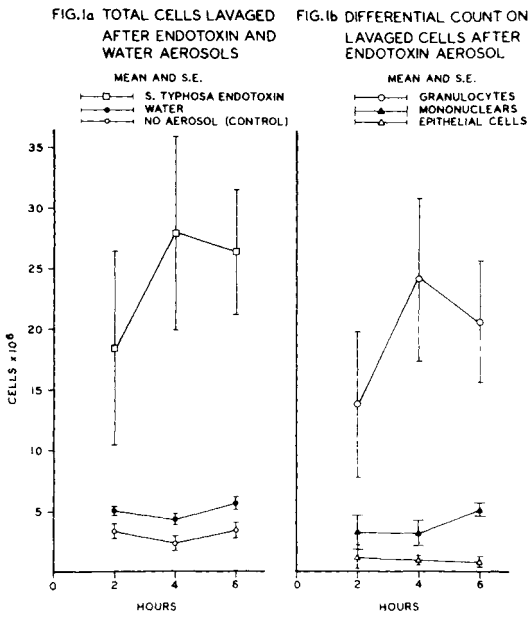

Fig. 1.A. Total number of cells obtained by lavage and the standard errors of counts from guinea pigs exposed to *Salmonella typhosa* endotoxin, to sterile water, and to air (no aerosol) compared at 2, 4, and 6 hours. Exposure was for 2 or 4 hours. The point at zero time is the average for the water and air control cells obtained at 2 hours. B. Counts of granulocytes, polymorphonuclear leukocytes, mononuclear cells, and airway epithelial cells, compared at 2, 4, and 6 hours for guinea pigs exposed to endotoxin.

The histologic and morphometric studies showed that both air and water aerosol control animals of both species had 2 to 3 PMN leukocytes per 1,000 normal airway epithelial cells (0.2 to 0.3 per cent). The ultrastructure of airway cells and lamina propria was normal. In guinea pigs and hamsters exposed to endotoxin for 2 hours, PMN leukocytes not only lined the basal lamina of the airways, but were also between epithelial cells and were beginning to line the airway surface (table 2). The counts were 21.0 ± 9.9 PMN leukocytes per 100 epithelial cells in guinea pigs and 50.5 $\pm$ 12.2 PMN leukocytes per 100 epithelial cells

TABLE 2

CELL COUNTS* AFTER AEROSOL EXPOSURE OF RODENTS TO *SALMONELLA TYPHOSA* ENDOTOXIN (0.1 mg/ml) FOR 4 HOURS

Hours After Exposure	Guinea Pig		Hamster		Hamster (*E. coli*)†	
	(mean)	*(SD)*	*(mean)*	*(SD)*	*(mean)*	*(SD)*
0	0.2	0.1	0.3	0.1	0.3	0.1
2	21.0	9.9	50.5	12.2		
4	53.9	10.9	40.9	18.9		
6	28.0	9.7	99.7	11.0	103.6	37.4
12					61.6	9.7
24	9.1	9.1**	20.6	5.6††	12.4	4.8

*Number of polymorphonuclear leukocytes/number of epithelial cells X 100.
†Cultural supernatant.
**Mononuclear cells, 2 per cent; all other times, < 1 per cent.
††Mononuclear cells, 7 per cent; all other times, < 1 per cent.

in hamsters. At 4 hours, the counts of PMN leukocytes had doubled to 53.9 ± 10.9 per 100 epithelial cells in guinea pigs, but had not increased from the 2-hour value in hamsters, 40.9 ± 8.9 per 100 epithelial cells. By 6 hours, counts in guinea pigs were decreasing (28.0 ± 9.7), but counts in hamsters increased to 99.7 ± 11.0 per 100 epithelial cells. Counts dropped to 20 per cent of the maximum at 24 hours. The time course appeared to be the same in hamsters given *E. coli* cultural supernatant. Mononuclear cells represented less than 1 per cent of all leukocytes at all times through 6 hours in both species, but at 24 hours, they had increased to 2 per cent (mononuclear/epithelial cells) in guinea pigs and to 7 per cent in hamsters.

The aerosol exposure of hamsters to bacteria-free supernatant cultural medium in which *E. coli* had been growing showed practically the same counts (103.6 ± 37.4 per 100 epithelial cells) at 6 hours, with a stepwise decrease at 12 and 24 hours. The migration of PMN leukocytes through capillaries to airway lumens was preceded by margination of granulocytes within capillaries. The PMN leukocytes appeared to stick to capillary walls. This suggested that they moved from capillaries through lamina propria to the epithelium, and hence, to airway lumens. Alveolar spaces were normal, but there was patchy interstitial thickening with apparent edema at 6 hours. In addition, platelets were adherent to the chord of the arteriole adjacent to bronchus or bronchiole (figures 2 and 3). This was observed in each of 54 sections from both species. Ultrastructural examination of these adherent platelets and migrating granulocytes showed they were normal (figure 3).

Discussion

A partially purified endotoxin (11) derived from *S. typhosa,* delivered by aerosol to guinea pigs and hamsters, recruited PMN leukocytes to the lumens of airways. With this recruitment, PMN leukocytes increased, and lymphocytes decreased in blood. During 4 hours of exposure, PMN leukocytes were recruited exclusively, as measured by bronchopulmonary lavage and by fixing recruited cells on lumens of airways with osmium tetroxide in fluorocarbon. Two hours after exposure, however, the number of granulocytes was decreased and the number of mononuclear cells was increased both in lavage cell counts and in cells counted on airway lumens. This trend continued, so that at 24 hours, 37 per cent of lavaged cells were mononuclear.

Platelets were aggregated on the walls of arteries and arterioles that faced airways, and all stages of the crossing of PMN leukocytes from capillaries and vessels through walls of airways were observed. These findings suggested that endotoxin either interacts with airway cells to generate a leukocyte chemotactic factor or is a chemotaxin directly, as it is *in vitro* (12). The response was similar to that observed after hamsters inhaled polyphenolic monomers and vegetable tannins (13). Counting cells in cross sections of airways and counting cells after recovery of free cells by pulmonary lavage provide 2 methods for estimating cells present on airways and alveoli; both methods quantify and localize responses to inhaled materials (9). In response to endotoxin, leukocytes migrated across airway epithelium from the trachea to terminal bronchioles, but not into alveoli. Because inhaled endotoxin recruited platelets to

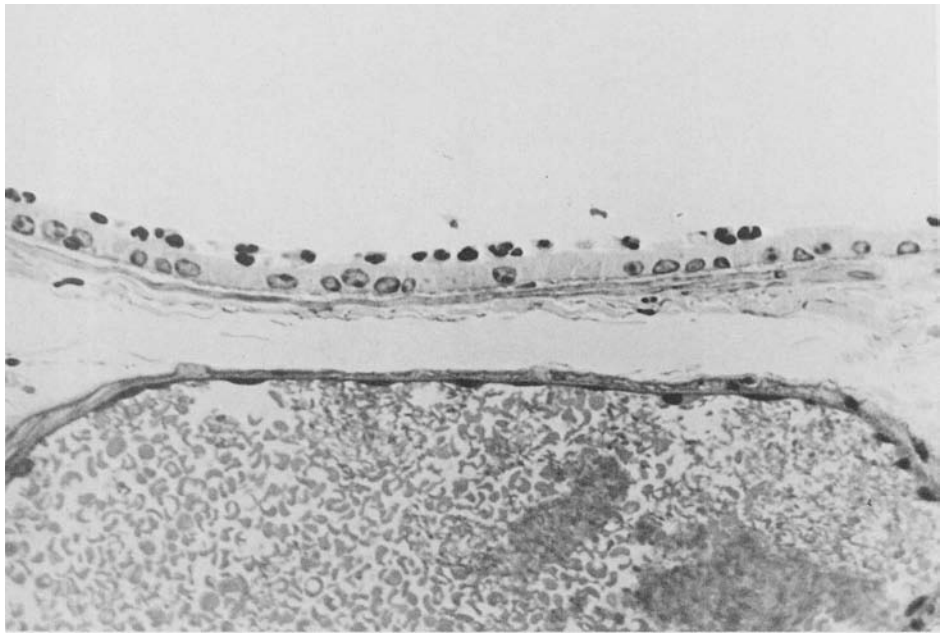

Fig. 2. A chord of a 1-μm section of bronchiole and arteriole shows many polymorphonuclear leukocytes on the lumen of the airway and platelets aggregated in the adjacent arteriole (original magnification: × 100).

the chord of arterioles facing bronchioles, its further effects on platelets and coagulation within the lung need study. Whether it aggregates platelets and recruits leukocytes alone or acts by releasing an agent from airway cells is unanswered. The role of complement in this *in vivo* system also begs to be evaluated. Recently, Kane and associates (14) showed that in guinea pigs, endotoxin activates both the classic and the alternate complement pathways. Pennington and co-workers (15) demonstrated that intravenous injection of endotoxin aggregated platelets and leukocytes in pulmonary arteries and veins and increased pressures. Circulating vasopressors derived from blood cells contributed to this vascular response.

Pernis and associates (4) demonstrated in rabbits that inhalation of endotoxin aerosols produced dyspnea and fever 30 to 50 min after exposure. When given intravenously, an *E. coli* extract produced fever and leukopenia followed by leukocytosis in rabbits (16). In the present experiments, the blood counts of PMN leukocytes increased; the lymphocyte counts decreased, but the platelet counts were not changed 6 hours after a 4-hour exposure to endotoxin was initiated. The differences may be due to species, may be a function of the type of endotoxin (*E. coli* 0127:Bs, Difco versus *S. typhosa* 0901 Difco), or may be due to the concentrations deposited per g of body weight or to the time course of the experiments. Further studies should resolve these differences. Snell and Ramsey (16) confirmed that bacterial endotoxin is absorbed from the being, is antigenic, and has pharmacologic activity. In addition, it produced alveolar edema followed by interstitial pneumonitis and mild bronchial and bronchiolar inflammation. Cavagna and associates (6) showed that 20 weeks of endotoxin inhalation in rabbits produced bronchitis, bronchiolitis, and alveolar septal thickening as pulmonary resistance and antibodies against endotoxin increased.

This time course and magnitude resemble those shown for cotton dust (17) and its purified products (12). This resemblance (3) and the clinical similarity between responses of textile workers to vegetable dusts and to inhalation of endotoxin (6, 9) have suggested that the "Monday morning" chest tightness and shortness of breath among processors of cotton and soft hemp, may, in part, be due to endotoxin. It is even more likely that "mill fever," with fever, malaise, and peripheral blood leukocytosis, is due to endotoxin. Gram-negative bacilli are

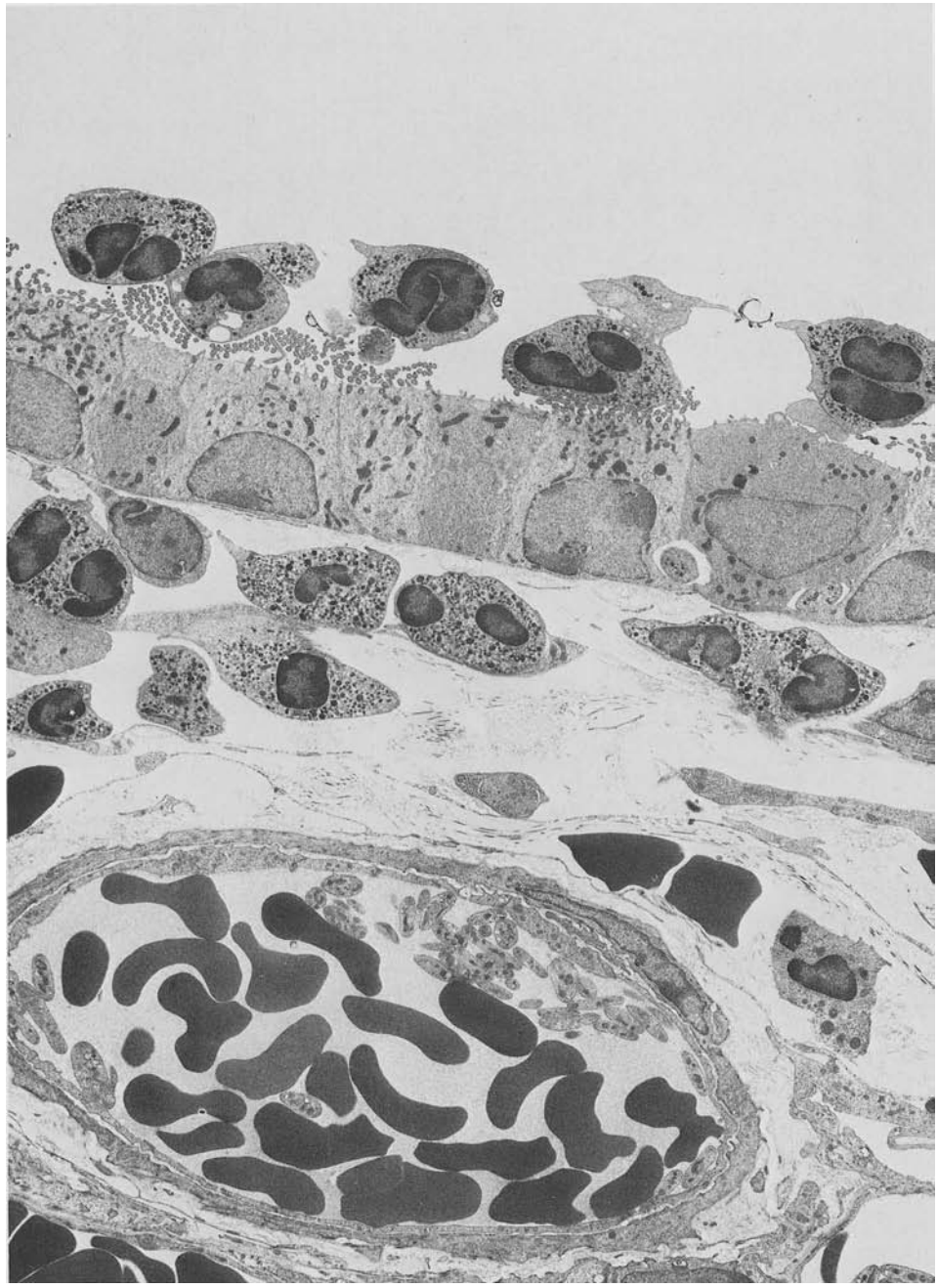

Fig. 3. A low power electron photomicrograph shows a chord of a bronchiole and an arteriole with platelets aggregated on its endothelial surface facing the bronchiole. There are many polymorphonuclear leukocytes beneath the airway and on its lumen and occasional erythrocytes free within the lamina propria (original magnification: × 4,500).

easily demonstrated on vegetable products (4, 18), and 0.1 μg of inhaled endotoxin affects hamsters and guinea pigs. This dose was calculated from an efficiency of aerosol delivery to lung of 0.1 per cent (19) for 0.1 mg of endotoxin per ml. Although it has been calculated that the concentrations of endotoxin in textile mills are too low to produce symptoms or functional impairment in man (20), the precision of these measurements and their application to the entire industry should be questioned. To answer this question, it will be necessary to synthesize exclusive cotton chemicals and test them in the absence of endotoxins (21, 22).

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