

Effects of different extraction protocols on endotoxin analyses of airborne grain dusts

by Stephen A Olenchock, PhD, Daniel M Lewis, PhD, Judith C Mull, MS¹

OLENCHOCK SA, LEWIS DM, MULL JC. Effects of different extraction protocols on endotoxin analyses of airborne grain dusts. *Scand J Work Environ Health* 1989;15:430-435. The detection of gram-negative bacterial endotoxins in occupational dusts, specifically those from agricultural environments, is of increasing importance in research on occupational lung disease. In this study, the quantitative chromogenic *Limulus* amoebocyte lysate test for the detection of endotoxins in airborne dusts from spring wheat and oats was examined. Different extraction fluids were tested, as were the effects of time on extraction and of repeated freeze-thaw cycles on the extracts. The data suggest that the chromogenic method can be used effectively in the analysis of environmental dusts or their frozen extracts for endotoxin quantitation. Water appears to be the preferable extraction medium, and the length of extraction time may affect the results.

Key terms: agriculture, oat dust, occupations, spring wheat dust.

Gram-negative bacteria and their endotoxins appear to be ubiquitous contaminants of materials and dusts related to agricultural occupations. Endotoxins are known to stimulate a wide range of profound biological effects (1, 2), and the lung is a target organ for endotoxin-induced damage (3, 4). Reports of endotoxin levels in animal confinement buildings (5-8), cotton textile mills (9, 10), port grain terminals (11), poultry-processing plants (12), and tower silos (13) attest to the potential for respiratory impairment in members of the diverse work force that inhales endotoxin-laden dusts. Controlled exposures of humans to endotoxin-laden cotton dusts have led to the definition of an association between decreases in acute pulmonary function and the level of airborne endotoxins (14-16). Cotton mill studies in The People's Republic of China have also shown a dose-response relationship between endotoxin levels in cotton dust and chronic lung impairment such as chronic bronchitis among cotton workers (17). In the acute studies, the thresholds for no pulmonary function change were defined as 80 ng/m³ for cotton workers who smoke (16), but the results from a larger study of a mixed population indicated a calculated zero change threshold of as little as 9 ng/m³ (15).

The two controlled-exposure studies that obtained zero pulmonary function change thresholds for endotoxin levels used two markedly different techniques to quantify the endotoxins. Haglund & Rylander (16) used the *Limulus* tube clot technique, while Castellan et al (15) used a spectrophotometric modification of the *Limulus* gel technique. Other investigators have uti-

lized different techniques when analyzing environmental dusts for endotoxin contamination. The microtiter modification of the *Limulus* test was used to study endotoxins in raw cotton components (18), while in another study the endotoxic respirable dust potential of baled cotton was determined by a kinetic *Limulus* test (19). Endotoxins in compost samples have been detected by gas chromatography (20), and other investigations have assessed endotoxins in swine confinement buildings (5) and different agricultural environments (21) with a quantitative chromogenic modification of the *Limulus* test.

In an attempt to develop scientific standardization of the tests used to determine endotoxins in environmental dusts, the Work Group on Agents in Organic Dusts in the Farm Environment recommended the use of the quantitative chromogenic modification of the *Limulus* amoebocyte lysate test (22). This same technique was recommended again at an international symposium on work-related respiratory disorders among farmers because of its accuracy and reproducibility (23).

The purpose of this paper is to evaluate the efficacy of the quantitative chromogenic *Limulus* amoebocyte lysate test in determining endotoxin contamination in environmental dusts. Specifically, the detection of endotoxins in airborne spring wheat and oat dusts was followed when various extraction protocols were used.

Materials and methods

Endotoxin analyses

Throughout this study, sterile, pyrogen-free plasticware was used. Sterile, nonpyrogenic water and saline were obtained commercially (Travenol Laboratories, Inc, Deerfield, Illinois, United States) and used throughout this study unless otherwise noted. The en-

¹ Immunology Section, Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, West Virginia, United States.

Reprint requests to: Dr SA Olenchock, Chief, Immunology Section, NIOSH, 944 Chestnut Ridge Rd, Morgantown, WV 26505, USA.

dotxin analyses were performed in duplicate according to a quantitative chromogenic modification of the *Limulus* amoebocyte lysate test (QCL-1000; Whittaker Bioproducts, Walkersville, Maryland, United States).

Commercial lipopolysaccharides

Lipopolysaccharides (LPS) from different bacteria were obtained commercially, reconstituted with water or saline, and diluted (with the same water or saline) to a final concentration of 5000 EU/ml, as defined by the manufacturer, and assayed.

In the assessment of the effect of possible differences in the extraction media on the resulting endotoxin determinations, two separate vials (100 mg contents) from the same manufacturer's lot number of LPS from *Escherichia coli* 0127:B8 (DIFCO Laboratories, Detroit, Michigan, United States) were reconstituted with 20 ml of water or saline, and the endotoxin activities were determined.

In evaluating the differences in endotoxin activities, commercial LPS samples from various bacterial sources and different preparations [Wesphal (W), Boivin (B)] were reconstituted to a concentration of 1 mg/ml or 5 mg/ml (depending upon the initial content of the vial) with water and assayed. LPS from *E. coli* (W) 0127:B8, *Salmonella typhosa* (B) 0901, *S. typhimurium* (B), *S. typhimurium* (W), and *S. enteritidis* (B) were obtained from DIFCO; samples of LPS from *E. coli* K12 D3lm4 (Re), *E. coli* K12, mm294 (complete core), and *S. minnesota* Re 594 were obtained from List Biological Laboratories (Campbell, California, United States).

Grain dusts

Airborne dusts from spring wheat and oats were collected as described previously (24) from active port grain terminals in the northern United States. The standard extraction protocol for bulk dusts in our laboratory is rocking in water (in this case 500 mg of dust in 10 ml of water) for 60 min at room temperature. The mixture is then centrifuged for 10 min at 1000 g, and the supernatant fluid is decanted and assayed for endotoxin content.

Extraction solutions

The effect of different extraction solutions on the efficacy of the quantitation of endotoxin levels in grain dust samples was studied. One percent (volume/volume) solutions were made in water from Tween 20 (polyoxyethylene sorbitan monolaurate) (Technicon Corporation, Tarrytown, New York, United States), Tween 80 (polyoxyethylene sorbitan monooleate) (Fisher Scientific Co, Fairlawn, New Jersey, United States), Triton X-100 (polyethylene glycol p-isooctylphenyl ether) (Fisher Scientific Co), and Saponin (saponin glycosides) (1 % weight/volume; Sigma Chemical Co, St Louis, Missouri, United States). In

addition, 1 % and 0.01 % (weight/volume) solutions were made from human, bovine, and chicken egg albumin (Sigma Chemical Co).

Each solution was used in place of water to extract dusts from both spring wheat and oats by our standard protocol. Dilutions were made with the extraction solutions.

In the standard protocols for our laboratory, the commercial chromogenic *Limulus* amoebocyte lysate assay requires a standard that is reconstituted with water before the standard curve is measured. In order to compare the effects of different extraction solutions on the efficacy of endotoxin removal from the grain dust samples, we made separate standard curves by reconstituting the endotoxin standard with two different extraction solutions, Tween 20 or Saponin. All the standards were from the same manufacturer's lot number, and the dilutions for the standard values were made with the same solutions that were used for the reconstitution.

Timed extractions

We made standard extractions of bulk dusts by rocking the dusts for 60 min. The effect of time on the extraction efficacy was measured. Aliquots (10 g) of spring wheat dust and oat dust were placed in separate sterile, nonpyrogenic, 75-cm² tissue culture flasks (Corning Glass Works, Corning, New York, United States) and mixed on a tube rotator (Scientific Equipment Products, Baltimore, Maryland, United States) for 2 h at room temperature to insure that the dusts were homogeneous. Nine samples (500 mg each) were taken from both dusts, and each was extracted by rocking in sterile, nonpyrogenic water for different lengths of time at room temperature. The endotoxin measurements were made after extractions of 15 and 30 min and after extractions of 1, 2, 4, 8, 12, 24, and 36 h.

Freeze-thaw studies

The effect of repeated freeze-thaw cycles on the level of detectable endotoxins in water extracts of the airborne grain dusts was studied. The standard extraction protocol was followed. Endotoxin measurements of the same extracts were made on day 0, never frozen, and at 2- to 3-d intervals for the next 30 d after freezing (-85°C). The same extracts were thawed and frozen again on each test day.

Results

Commercial lipopolysaccharides

Commercial LPS from *E. coli* were reconstituted in sterile, nonpyrogenic water or saline and diluted to a concentration of 5000 EU/ml, on the basis of the manufacturer's stated concentration. Analyses of the samples for endotoxin activity resulted in endotoxin levels of 3934.72 EU/ml for LPS dissolved in water

and 2743.66 EU/ml for saline-dissolved LPS. The data showed a 30.3 % reduction in endotoxin detection when the sample was dissolved in saline.

The next studies on endotoxin detection with commercial LPS samples were conducted with water. Each vial was reconstituted to a concentration of 5000 EU/ml, and the endotoxin concentrations, as determined by the chromogenic modification of the *Limulus* amoebocyte lysate test, are shown in figure 1. The endotoxin activities of the different samples ranged from 1405.83 EU/ml for *S typhimurium* (W) to 3934.72 EU/ml for *E coli* (W) 0127:B8. LPS samples for *S typhimurium*, isolated by two different methods, Boivin and Wesphal, resulted in little difference in detectable endotoxin. LPS from three rough strains of bacteria were analyzed also. These samples contained low, but detectable levels of endotoxin activity [ie, *E coli* K12, D3lm4 (Re), 23.27 EU/ml; *E coli* K12 mm294 (complete core), 109.69 EU/ml; and *S minnesota* R594 (Re), 0.31 EU/ml].

Table 1. Endotoxin concentrations in extraction solutions.

Agent	Concentration (%)	Endotoxin (EU/ml)
Water	-	<0.125 ^a
Tween 20	1	<0.125
Tween 80	1	<0.125
Triton X-100	1	<0.125
Saponin	1	0.171
Albumin		
Human	1	72.23
Bovine	1	1.73
Chicken	1	72.71
Human	0.01	14.37
Bovine	0.01	<0.125
Chicken	0.01	20.27

^a <0.125 EU/ml was the lowest detectable level for the analyses. Average of duplicate tests.

Table 2. Endotoxin levels in spring wheat dust and oat dust after extraction with different solutions.

Solution	Spring wheat dust (EU/mg)	ΔWater ^a (%)	Oat dust (EU/mg)	ΔWater ^a (%)
Water	1569.75 ^b	-	2562.50 ^b	-
Tween 20	2687.38	71.2	2833.08	10.6
Tween 80	1950.94	24.3	2257.17	-11.9
Triton-X-100	1785.56	13.7	28.47	-98.9
Saponin	2094.99	33.5	2781.18	8.5
Albumin				
Human 1 %	1438.80	- 8.3	1371.54	-46.5
Bovine 1 %	782.84	-50.1	1146.49	-55.3
Chicken 1 %	1742.88	11.0	1795.04	-29.9
Human 0.01 %	1460.13	- 7.0	1811.54	-29.3
Bovine 0.01 %	1742.88	11.0	2409.74	- 6.0
Chicken 0.01 %	1214.73	-22.6	2413.96	- 5.8

^a Percentage of change from water extraction.

^b Average of duplicate tests.

Effect of extraction solutions

Before the effect of different extraction solutions on the detectable endotoxin levels could be determined, the endotoxin contamination of the solutions was quantified. Table 1 lists the concentrations of endotoxin in the extraction solutions. The data show that human and chicken albumin contained modest endotoxin contamination. The other solutions were basically free of endotoxins.

The endotoxin levels detected in the airborne dusts from spring wheat and oats after extraction with different solutions are listed in table 2. In addition, the percentage of change from the "standard" water extraction for each solution is presented. While the majority of solutions resulted in the extraction of lower levels of detectable endotoxin than when water was used, extraction with Tween 20 and Saponin resulted in increases of detectable endotoxins for both dusts. In both cases, however, the increase over water extraction alone was less for oat dust than for spring wheat dust.

Although the data suggest that Tween 20 or Saponin resulted in potential improvements when used as extraction solutions instead of water, it must be remembered that table 2 reflects data that were obtained with the use of a standard curve that was made with water, as required by the chromogenic *Limulus* amoebocyte lysate test protocol. To determine if these solutions affected the chromogenic assay, we made standard curves from endotoxin standards of the same lot number with the Tween 20 and Saponin solutions (figure 2). The coefficient of correlation for both curves was >0.99. However, the standard curve made with Saponin was not usable because valid absorbance readings could not be achieved after repeated attempts. Therefore, only the curves for Tween 20 and water are shown in figure 2.

The endotoxin levels in spring wheat dust and oat dust when a Tween 20 extract was used with a water standard curve were 2687.38 EU/mg and 2833.08 EU/mg, respectively (table 2). When the Tween-20 extract was used with the Tween-20 standard curve, however, the detectable endotoxin levels were only 37.16 EU/mg and 87.57 EU/mg for spring wheat dust and oat dust, respectively, because of the marked change in the slope of the standard curves.

Effect of extraction time

Figure 3 illustrates the effect of extraction time on the level of detectable endotoxins in homogeneous samples of both spring wheat dust and oat dust. For both dusts, the peak extractable endotoxins were determined after extraction for 2 h. The levels dropped after that time and remained low through 36 h of extraction. The standard 1-h extraction yielded 68.8 and 77.4 % of the peak levels for spring wheat dust and oat dust, respectively.

Effect of repeated freezing and thawing

Single water extracts of spring wheat dust and oat dust were used in the study of the effect of freezing and thawing on the detectable endotoxin levels. The results of the 30-d experiment are shown in figure 4. Both extracts were analyzed at the same times, and a new endotoxin kit was used each day. The kits were from a single lot number until day 23, when a new lot number was used. Day-to-day variation from the original, unfrozen, extract could be observed.

Discussion

Quantitation of gram-negative bacterial endotoxins in dusts from various workplaces such as animal confine-

ment and processing facilities, cotton textile mills, port grain terminals, and tower silos provides new insight into the potential respiratory hazards of workers who breathe the dusts. Recent reports indicate that accurate measurement of airborne endotoxin levels can be used to estimate the threshold of acute pulmonary function response to inhaled cotton dusts (14–16). In addition, the acute pulmonary response to cotton dust correlated with the endotoxin levels and not the gravimetric dust levels. Thus there is need for an accurate and reproducible method for detecting airborne endotoxin levels. While most laboratories that study airborne endotoxins rely on the *Limulus* amoebocyte lysate gelation test for their studies (25), the chromogenic modification of the *Limulus* test has been endorsed by two groups of scientists who study agriculturally related

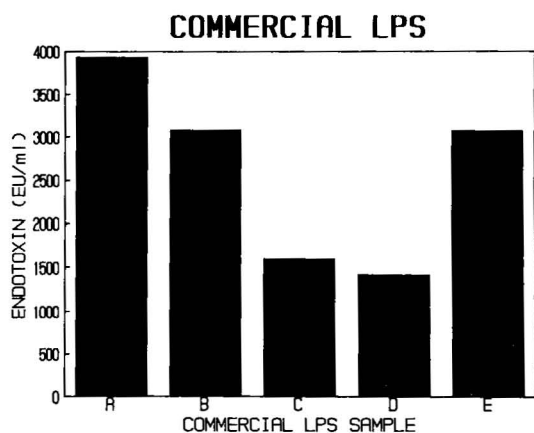


Figure 1. Detectable endotoxin units (per ml) in commercial lipopolysaccharide samples that were reconstituted with sterile, nonpyrogenic water to a final concentration of 5000 EU/ml (manufacturer's label concentration). [A: *Escherichia coli* (W) 0127:B8, B: *Salmonella typhosa* (B) 0901, C: *S typhimurium* (B), D: *S typhimurium* (W), and E: *S enteritidis* (B)]

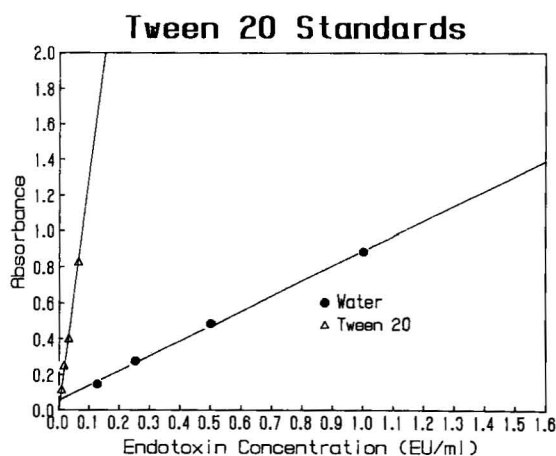


Figure 2. Standard curves of absorbance and endotoxin concentration for endotoxin standards (from the same manufacturer's lot number) reconstituted and diluted with sterile, nonpyrogenic water or with a 1% (volume/volume) Tween 20 solution.

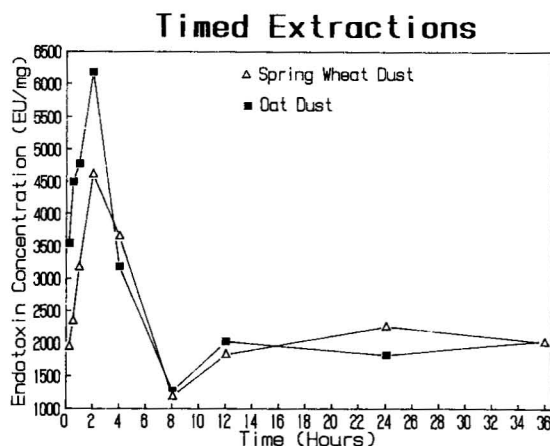


Figure 3. Detectable endotoxin levels in homogeneous samples of spring wheat dust or oat dust at various times (hours) of extraction in sterile, nonpyrogenic water.

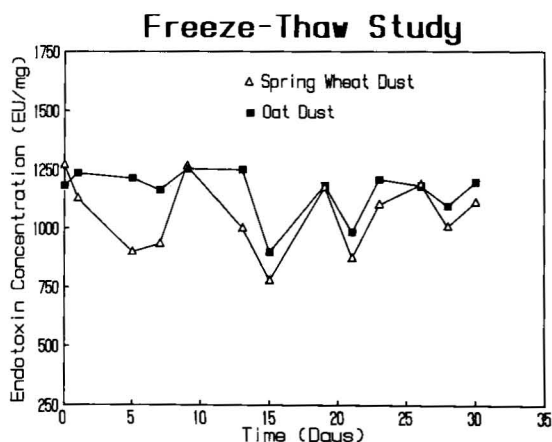


Figure 4. Detectable endotoxin levels in a single water extract of either spring wheat dust or oat dust that was frozen and thawed repeatedly during a period of 30 d.

lung diseases (22, 23). Our study is a first attempt to examine the chromogenic modification of the *Limulus* amoebocyte lysate test to determine its usefulness for environmentally derived dust studies. Specifically, different extraction protocols were examined with two different airborne grain dusts.

Data from reconstitution studies with commercial LPS suggest that water is the medium of choice, although commercial LPS samples from different organisms may vary in their endotoxin activities when reconstituted with water. The problem of whether these differences are a result of the relative toxicities of the different LPS samples or of inconsistency in the product preparation was not addressed fully in these experiments. However, it is interesting to note that no difference in endotoxin activity was noted when two preparations of *S. typhimurium*, Boivin and Wesphal, were examined. This result may imply that the differences in the endotoxin activities of the LPS samples could be due to the relative toxicities of the LPS from different source microorganisms.

Water was used, therefore, to extract two samples of airborne grain dusts. These samples were chosen to be representative of a variety of agricultural dusts, and we could obtain a sufficient quantity of well-mixed dust so that consistent repeated sampling could be made. It can be seen from the data that the dusts from spring wheat and oats provided different values throughout these tests. Agricultural dusts would be expected to be unique and different from each other in endotoxin contamination because of the diversity of the growth area, the varieties of plants, and the storage and transport of the bulk grains.

Because extraction with water alone may harbor its own disadvantages, especially when the extraction of whole bacterial cells is the research objective (26), several agents were added to the water to test the efficacy of extraction. Of the solutions that we examined, only 1 % Tween 20 and 1 % Saponin increased the detectable endotoxin concentration for both dusts. However, examination of the characteristics of the endotoxin standard curve when the wetting agents were used revealed that the data were artificially elevated. As shown for the calculations of endotoxins when the Tween 20 standard curve was used with Tween 20 extraction, the true values were markedly lower. These results imply that these agents affected the chromogenic assay.

The length of extraction time can affect the detectable endotoxin concentration in extracts, as demonstrated by the timed extractions of spring wheat and oat dusts (figure 3). Extraction of both dusts for 2 h provided maximal levels of detectable endotoxin. The levels declined rapidly, and, by 12 h of extraction, they stabilized at approximately 30–40 % of the maximal level. One can only speculate at this point in the research as to the cause of the lowered detectable endotoxins with longer extraction times. It is possible that the endotoxins adsorbed onto the tubes, or onto par-

ticles, and were no longer available in the supernatant fluid. It is possible also that the molecules joined into larger structures and hid the active moieties. Another possibility lies in the environmental dusts themselves. Perhaps interactions with an agent(s) in the dust resulted in an inactivation or alteration of the *Limulus*-reactive moiety, either through oxidation or another chemical interaction. Whatever the cause, the data do suggest that the length of extraction should be held constant within a given study. An examination of other environmental dusts should determine if the 2-h extraction maximum is consistent or if it varies with different dusts.

Finally, the freeze-thaw experiments demonstrated that endotoxin analyses of spring wheat and oat dusts can be performed on extracts that have been frozen. Our experiments illustrated the worst case situation wherein the extracts underwent repeated freeze-thaw cycles for 30 d.

In conclusion, the recommended chromogenic modification of the *Limulus* amoebocyte lysate test can be used effectively in the analysis of environmental dusts for endotoxin quantitation. Of the extraction media that we tested, water is a preferable extraction medium, and the length of extraction should be examined for each dust if sufficient quantities of a thoroughly homogenized bulk dust are available.

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Mention of company names or products does not constitute endorsement by the National Institute for Occupational Safety and Health.

References

1. Bradley SG. Cellular and molecular mechanisms of action of bacterial endotoxins. *Ann Rev Microbiol* 1979; 33:67–94.
2. Morrison DC, Ulevitch RJ. The effects of bacterial endotoxins on host mediation systems. *Am J Pathol* 1978; 93:527–617.
3. Brigham KL, Meyrick B. Endotoxin and lung injury. *Am Rev Respir Dis* 1986;133:913–27.
4. Rylander R, Snella M-C. Endotoxins and the lung: cellular reactions and risk for disease. *Prog Allergy* 1983; 33:332–44.
5. Attwood P, Versloot P, Heederik D, De Wit R, Boleij JSM. Assessment of dust and endotoxin levels in the working environment of Dutch pig farmers: a preliminary study. *Ann Occup Hyg* 1986;30:201–8.
6. Clark S, Rylander R, Larsson L. Airborne bacteria, en-

- dotoxin and fungi in dust in poultry and swine confinement buildings. *Am Ind Hyg Assoc J* 1983;44:537—41.
7. Jones E, Morring K, Olenchock SA, Williams T, Hickey J. Environmental study of poultry confinement buildings. *Am Ind Hyg Assoc J* 1984;45:760—6.
 8. Thedell TD, Mull JC, Olenchock SA. A brief report of gram-negative bacterial endotoxin levels in airborne and settled dusts in animal confinement buildings. *Am J Ind Med* 1980;1:3—7.
 9. Gokani VN, Doctor PB, Ghosh SK. Isolation and identification of gram-negative bacteria from raw baled cotton and synthetic textile fibers with special reference to environmental GNB and endotoxin concentrations of textile mill. *Am Ind Hyg Assoc J* 1987;48:511—4.
 10. Olenchock SA, Christiani DC, Mull JC, Ye T-T, Lu P-L. Endotoxins in baled cottons and airborne dusts in textile mills in the People's Republic of China. *Appl Environ Microbiol* 1983;46:817—20.
 11. DeLucca AJ II, Godshall MA, Palmgren MS. Gram-negative bacterial endotoxins in grain elevator dusts. *Am Ind Hyg Assoc J* 1984;45:336—9.
 12. Olenchock SA, Lenhart SW, Mull JC. Occupational exposure to airborne endotoxins during poultry processing. *J Toxicol Environ Health* 1982;9:339—49.
 13. Olenchock SA, May JJ, Pratt DS, Morey PR. Occupational exposure to airborne endotoxins in agriculture. In: Watson SW, Levin J, Novitsky TJ, ed. Detection of bacterial endotoxins with the limulus amoebocyte lysate test. New York, NY: Alan R Liss Inc, 1987:475—87.
 14. Castellan RM, Olenchock SA, Hankinson JL, et al. Acute bronchoconstriction to endotoxin and other dust factors. *Ann Intern Med* 1984;101:157—63.
 15. Castellan RM, Olenchock SA, Kinsley KB, Hankinson JL. Inhaled endotoxin and decreased spirometric values: an exposure response relation for cotton dust. *N Engl J Med* 1987;317:605—10.
 16. Haglund P, Rylander R. Exposure to cotton dust in an experimental cardroom. *Br J Ind Med* 1984;41:340—5.
 17. Kennedy SM, Christiani DC, Eisen EA, et al. Cotton dust and endotoxin exposure-response relationships in cotton textile workers. *Am Rev Respir Dis* 1987;135:194—200.
 18. Fischer JJ, Morey PR, Foarde KK. The distribution of gram-negative bacteria and endotoxin on raw cotton components. *Am Ind Hyg Assoc J* 1986;47:421—6.
 19. Millner PD, Perkins HH Jr, Harrison RE. Methods for assessment of the endotoxic respirable dust potential of baled cotton. In: Jacobs RR, Wakelyn PJ, ed. Proceedings of the twelfth cotton dust research conference. Memphis, TN: National Cotton Council, 1988:3—5.
 20. Kirschner D, Que Hee SS, Clark CS. Method for detecting the 3-hydroxymyristic acid component of the endotoxins of gram-negative bacteria in compost samples. *Am Ind Hyg Assoc J* 1985;46:741—6.
 21. Olenchock SA. Quantitation of airborne endotoxin levels in various occupational environments. *Scand J Work Environ Health* 1988;14(suppl 1):72—3.
 22. Popendorf W. Report on agents. *Am J Ind Med* 1986;10:251—9.
 23. Rylander R. Role of endotoxins in the pathogenesis of respiratory disorders. *Eur J Respir Dis* 1987;71:136—44.
 24. Peach MJ III, Olenchock SA, Sorenson WG, Major PC. Relevance of grain dust collection techniques to respiratory disease studies. In: Dosman JA, Cotton DJ, ed. Occupational pulmonary disease: focus on grain dust and health. New York, NY: Academic Press, 1980:507—12.
 25. Rylander R, Burrell R. Conference report. *Ann Occup Hyg* 1988;32:553—6.
 26. Gould M, Olenchock S. Endotoxin extraction method for organic dusts. In: Rylander R, Burrell R, Peterson Y, ed. Proceedings of endotoxin inhalation workshop. Memphis, TN: National Cotton Council, 1988:204—5.

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