

EVALUATION OF EXPOSURE TO AIRBORNE BACTERIAL ENDOTOXINS AND PEPTIDOGLYCANS IN SELECTED WORK ENVIRONMENTS

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Laitinen S, Kangas J, Husman K, Susitaival P: Evaluation of exposure to airborne bacterial endotoxins and peptidoglycans in selected work environments. *Ann Agric Environ Med* 2001, 8, 213–219.

Abstract: The aim of this study was to assess workers' exposure to endotoxins and peptidoglycans, as well as associations between workers' reported symptoms and the detected bacterial exposures. From the filter samples, biologically-active endotoxins were analysed with the *Limulus* amoebocyte lysate (LAL) assay. The total amount of endotoxins was analysed as 3-hydroxy (OH) fatty acids with a gas chromatography-mass spectrometry (GC-MS) assay, which was also used to assess peptidoglycans as muramic acid. Biologically-active endotoxins related better to the self-reported symptoms than total endotoxins. Specific 3-OH-14:0 fatty acid in the total endotoxin samples associated better with the symptoms than other 3-OH fatty acids. Half of the surveyed 77 workers reported respiratory symptoms, 27% eye symptoms, and 10% fever or shivering. The proportion of workers with respiratory symptoms was greater when the concentration of endotoxins was over 25 ng/m³. These endotoxin levels were occasionally found in the air of most studied occupational environments. The muramic acid concentrations of peptidoglycans were highest (medians over 100 ng/m³) in the garbage-handling plant and in the grain/vegetable storage houses. The LAL assay for endotoxins, as well as the GC-MS assay analysing muramic acid for peptidoglycans or specific 3-OH fatty acids for endotoxins, seem to be suitable methods for evaluating workers' exposure to airborne bacteria.

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Key words: air, analytical methods, bacteria, endotoxin, peptidoglycan, determination, occupational exposure, health effects.

INTRODUCTION

Endotoxins are substances which trigger the release of mediators from inflammatory cells in the tissues [20]. Endotoxins may play an important role in the development of various symptoms of workers in occupational environments where exposure to bacteria is prevalent [3]. Both pathogenic and saprophytic Gram-negative bacteria have in their cell wall an endotoxin layer on top of the peptidoglycan membrane. In Gram-positive bacteria, peptidoglycans, instead of endotoxins, form the major outer cell membrane. After the death and decomposition

of the bacterial cell, endotoxins remain in the fragments of the cell wall [2]. During the growth of the bacteria, endotoxins can be released into the environment from vesicles budding from the outer membrane [4]. In some studies, an association has been found between exposure to biologically-active endotoxin and workers' symptoms [9, 22, 25, 26, 31, 32]. There is also evidence that peptidoglycan exposure may induce inflammatory responses in humans and animals [7, 13, 18, 32].

The *Limulus* amoebocyte lysate (LAL) assay, in which an enzyme system is activated in the presence of endotoxins, has two major limitations: it detects mainly

Table 1. Study subjects and work environments.

Work environment	N ^R /N ^a	Mean age, range (years)	Smoking ^b (%)	Atopy ^c (%)
Slaughterhouses	11/15	34 (17–44)	36	18
Grain/vegetable storage	7/7	33 (21–49)	71	29
Animal-feed industry	18/19	37 (21–58)	44	16
Garbage-handling plant	2/2	(32–32)	0	0
Cotton mill	10/11	41 (23–53)	30	60
Printing plant	5/7	46 (29–57)	40	0
Wood industry	22/24	44 (23–56)	41	73
Metal-working industry	2/2	46 (36–55)	0	100
Total	77/87	39 (17–58)	38	39

^a N^R/N = number of responded (questionnaire) / number of workers (questionnaire handed). ^b Smoking = current regular smoking. ^c Atopy = ever asthma, allergic rhinitis, or atopic dermatitis

biologically-active endotoxins, and substances other than endotoxins [12] can also activate it. The biological activity of endotoxins is dependent on the bacterial species and may differ for free and cell-bound endotoxins [11, 17, 24]. In the LAL assay, activated enzymes produced a yellow colour with a chromogenic substrate, from which the endotoxin concentration is determined photometrically. The LAL reaction is very sensitive for endotoxins, while other possible activators, such as peptidoglycans, are 10³–10⁵-fold less sensitive than endotoxins in giving a positive reaction [30].

Because of the above limitations, a gas chromatography-mass spectrometry (GC-MS) assay has been developed as an alternative approach to measure the total amount of endotoxins [16]. The GC-MS assay detects 3-hydroxy (OH) fatty acids as chemical markers of endotoxins. Lipopolysaccharides (LPS) are structural components of endotoxin molecules. They consist of a lipid part - lipid A - and a polysaccharide part. Lipid A has a similar structure in different Gram-negative bacteria, and is responsible for the toxic effects of endotoxins. The 3-OH fatty acids in lipid A can be quantified from both biologically-active and inactive endotoxins [27]. It has not been shown to date if measuring total endotoxins by the GC-MS assay has any use in assessing health effects [5].

In the present study, biologically-active endotoxins (measured with the LAL assay) and the total amount of endotoxins (measured with the GC-MS assay) in filter-collected air samples from various occupational environments were assessed. The endotoxin concentrations were compared with the amounts of airborne cultivable bacteria and peptidoglycans, analyzed as muramic acid. In addition, associations between workers' recent symptoms and the bacterial exposures were analysed quantitatively.

MATERIALS AND METHODS

Study subjects. The study was conducted in 14 occupational environments (Tab. 1) where samples for airborne endotoxins, peptidoglycans, and cultivable bacteria were collected. Studied workplaces included two storage houses (grain, onions), two slaughterhouses (poultry, reindeer), three animal-feed processing plants (animal material, grain), three wood processing plants (pulp mill, paper mill, recycled paper mill), two workplaces using humidifiers (printing plant, cotton mill), one garbage-handling plant, and one workplace using metal-working fluids.

The workers in the study had been employed in their jobs on average for 10 years (range 1–39 years). Symptoms that occurred during 24 hours after the air sample collection were recorded with a self-administered questionnaire, which was given to 87 workers and returned by 77 (89%) (Tab. 1). Questions on the following symptoms were included: respiratory symptoms (cough and other symptoms of chronic bronchitis, chest tightness, wheezing, sore/hoarse throat and nasal symptoms), fever or chills, eye symptoms, skin symptoms, nausea, and diarrhoea. Questions on atopy (history of asthma, allergic rhinitis or atopic dermatitis) and smoking were also included. The questions were derived from Tuohilampi [28] or British Medical Research Council [19] questionnaires. The worker's self-reported symptoms were compared with results of his personal or the stationary air sample, which was taken in the immediate vicinity of the worker.

Statistical analysis. Fisher's exact test was used to detect differences in reporting symptoms and various concentrations of endotoxins. Values of $p < 0.05$ were considered statistically significant.

Determination of endotoxins and peptidoglycans.

Air samples for endotoxin and peptidoglycan assays were collected at a velocity of 2.2 l/min on glass fibre filters (85/220, Macherey-Nagel Corp., Düren, Germany) on the first working day after at least a 2-day absence from work. Sampling time varied from 1–8 hours, depending on workers' activities in a bioaerosol-contaminated area during the workshift. The collected filters were extracted with nonpyrogenic water during the first hour after the sampling. The extracts were divided into two parts. From one part, biologically active endotoxins were analysed using the endpoint chromogenic LAL assay (Coatest® Endotoxin Test Kit, Chromogenix, Mölndal, Sweden). From the other part, the total amount of endotoxins and peptidoglycans were analysed with the GC-MS assay.

Modification of the method according to Mielniczuk *et al.* [21] was used to evaluate the total amount of endotoxins by analysing 3-hydroxy fatty acids of LPS. The extract was lyophilised before an internal standard (3-OH-13:0, Larodan AB, Malmö, Sweden) and 3.6 M methanolic HCl were added. The sample was hydrolysed at 85°C for 18 hours. After adding water, the methyl esters of hydroxy fatty acids were extracted with hexane from a methanolic phase and separated from the methyl esters of nonhydroxy fatty acids in a silica column (Sep-Pak®, Millipore Corp., Bedford, MASS, USA). The methyl esters of hydroxy fatty acids were derivatized in pyridine with bis(trimethylsilyl)trifluoroacetamide (TMS) at 80°C for 15 minutes.

Table 3. The medians (MD) of muramic acid concentrations in the air of the studied workplaces. Figures over the limit of detection are presented.

Work environment	Samples (n)	Muramic acid concentrations (ng/m ³) / MD (range)
Slaughterhouses	13	39 (7.4–300)
Grain/vegetable storage	4	940 (170–1700)
Animal-feed industry	3	22 (8.7–180)
Garbage-handling plant	3	120 (120–940)
Wood industry	1	12
Cotton mill	5	28 (27–97)

A VG Trio-2 GC-MS system (Manchester, UK) was used for analysis of samples. The gas chromatograph was a Hewlett-Packard (HP, Avondale, PA, USA) model 5890, equipped with HP-5 capillary column (50 m, 0.31 mm i.d.). Injections were made using a Hewlett-Packard model 7673 autosampler in splitless mode. The temperature of the column was programmed from 120°C (1 min) to 260°C at 10°C/min. Both the interface and the injector were kept at 260°C. The TMS derivatives were analysed in the EI mode. For quantitative analysis, a selective ion monitoring (SIM) mode was used by measuring the ions *m/z* 175.31 (cleavage of C₃-C₄ linkage), 259.49 (M-CH₃; 3-OH-10:0), 287.55 (M-CH₃; 3-OH-12:0), 301.58 (M-CH₃; 3-OH-13:0), 315.61 (M-CH₃; 3-OH-14:0) and 343.67 (M-CH₃; 3-OH-16:0).

Table 2. The medians (MD) of the endotoxin concentrations in the air of the studied workplaces analysed by LAL^a and GC-MS^b assays.

Workplace	Samples (n)	Endotoxin concentrations (ng/m ³)			
		LAL assay		GC-MS assay	
		MD	(range)	MD	(range)
Slaughterhouses	stationary (10)	190	(0.02–940)	1,700	(1,100–2,500)
	personal (6)	87	(1.4–520)	9,300	(760–64,000)
Grain/vegetable storage	stationary (5)	17,000	(1,700–38,000)	9,500	(5,210–18,000)
	personal (4)	5,600	(4,000–8,000)	3,100	(2,700–11,000)
Animal-feed industry	stationary (13)	6.5	(0.3–20)	1,800	(390–51,000)
	personal (17)	19	(0.2–50)	8,600	(1,000–66,000)
Garbage-handling plant	stationary (8)	120	(0.9–1,400)	3,000	(1,500–11,000)
	personal (1)	260		650	
Wood industry	stationary (22)	3.3	(0.1–51)	1,000	(96–7,700)
	personal (11)	6	(1.0–36)	840	(500–4,200)
Cotton mill	stationary (5)	11	(1.9–223)	160	(29–310)
	personal (4)	120	(14–960)	1,000	(240–1,800)
Printing plant	stationary (5)	0.05	(0.03–0.1)	260	(74–280)
Metal-working industry	stationary (4)	6.7	(1.6–27)	2,900	(2,100–3,100)

^aLAL = *Limulus* amoebocyte lysate. ^bGC-MS = gas chromatography-mass spectrometry.

Table 4. The medians (MD) of cultivable bacteria concentrations in the air of the studied workplaces.

Work environment	Samples (n)	Concentrations of bacteria (cfu/m ³ × 10 ³)	
		EMB medium	R2A medium
		MD (range)	MD (range)
Slaughterhouses	3	11 (4.7-42)	46 (21-98)
Grain/vegetable storage	4	25 (16-28)	120 (58-160)
Animal-feed industry	12	0.35 (0.03-25)	4.7 (0.8-33)
Garbage-handling plant	3	1.9 (0.3-13)	13 (1.2-76)
Wood industry	13	0.50 (0.002-52)	2.5 (0.03-69)
Cotton mill	6	7.0 (0.5-14)	4.9 (1.2-11)
Printing plant	2	0.01 (0.01-0.02)	0.14 (0.1-0.2)
Metal-working industry	5	0.85 (0.09-41)	8.6 (1.7-60)

The amounts of 3-OH fatty acids were determined against an internal standard. The number of 3-OH fatty acid moles was divided by four and multiplied by 8,000 (the average molecular weight of LPS) estimating the total amount of endotoxin in the sample as a result. Final endotoxin results are expressed as ng/m³, assuming that 1 ng is equal to 12 EU (Endotoxin Units).

Peptidoglycans were determined using muramic acid as a chemical marker. From the same sample as endotoxins, the muramic acid was analysed as trifluoroacetylated methyl glycoside using GC-MS assay with chemical

ionisation according to Elmroth *et al.* [6]. The sample was first treated like that for 3-hydroxy fatty acids. After hydrolysis, an internal standard (N-methyl-D-glycamine, Buchs, Switzerland) and water were added, and the muramic acid in the methanolic phase was separated by hexane extraction. A derivatization was performed in acetonitrile with trifluoroacetic anhydride at 60°C for 15 minutes. The GC-MS analysis for the muramic acid derivative was performed with the same equipment as that for 3-OH fatty acids and isobutane as the reagent gas. The initial column temperature (70°C for 1 min) was increased at 8°C/min to 200°C and the temperature of the GC-MS interface was 160°C. The ion source temperature was 150°C. The mass spectrometer was used in the negative ion-chemical ionization mode and quantitation was conducted with SIM-monitoring of TIC analysis. The ion m/z 480 [M-CH₃CHCOOCH₃]⁻ and m/z 567 [M]⁻ for muramic acid and m/z 657 and m/z 674 for the internal standard were followed in the analysis. Peptidoglycan results are reported as nanograms of muramic acid in a cubic meter of air (ng/m³).

Determination of airborne cultivable bacteria.

Airborne cultivable bacteria were sampled with a six-stage impactor (Graseby Andersen Ltd., Kent, UK) on EMB and on R2A agar plates (Difco Laboratories, Detroit, USA) at a flow rate of 28.3 l/min and 1.5 meter above the ground. The EMB culture medium is quite selective for Gram-negative bacteria [15]. The total cultivable bacteria, both Gram-positive and Gram-negative grew on the nonselective R2A culture medium.

Table 5. Workers' self-reported symptoms by biologically active endotoxins, %.

Endotoxin level (ng/m ³)	Number of exposed workers (n)	Any reported symptom	Fever or shivering	Respiratory symptoms	Chest tightness	Eye symptoms	Skin symptoms	Nausea or diarrhoea
< 25	43	63	2	35	9	21	35	14
≥ 25	26	92	27	81	27	38	31	15
P ^a		> 0.1	0.0036	0.0002	0.0568	> 0.1	> 0.1	> 0.1

^a Differences between groups were tested with Fisher's exact test.

Table 6. Workers' (N = 61) self-reported symptoms compared to the predominant 3-hydroxy fatty acids detected in the air samples analysed for total endotoxins.

3-OH fatty acid	Any reported symptom (n/N ^a)	Fever or shivering (n/N)	Respiratory symptoms (n/N)	Chest tightness (n/N)	Eye symptoms (n/N)	Skin symptoms (n/N)	Nausea or diarrhoea (n/N)
3-OH-10:0	4/5	0/5	2/5	1/5	2/5	0/5	0/5
3-OH-12:0	14/24	3/24	9/24	3/24	4/24	8/24	2/24
3-OH-14:0	18/20	4/20	16/20	6/20	10/20	7/20	6/20
3-OH-16:0	10/12	1/12	5/12	0/12	2/12	4/12	1/12
61 workers	75%	13%	52%	16%	29%	31%	15%

^a n = number with the symptom(s), N = total in the group.

The EMB agar plates were incubated at 37°C for 48 hours. The R2A agar plates were incubated at 20°C for seven days. After incubation, the colonies were counted and the number of colony-forming units (cfu) was determined according to the positive-hole correction method. The results are expressed as cfu per cubic meter of air.

RESULTS

In the studied workplaces, the biologically-active endotoxin concentrations varied from under 0.02 to 38,000 ng/m³ (Tab. 2). The total endotoxin concentrations varied from 29–66,000 ng/m³, and the concentration was often much higher than that of biologically-active endotoxins. However, the variation in the total endotoxin concentrations was wide even within the same workplace. The amount of peptidoglycans, analysed as muramic acid, varied from under the limit of detection to 1700 ng/m³ (Tab. 3). Only in 29 (28%) of the analysed peptidoglycan samples (total 104), were the concentrations above the limit of detection (over 5 ng/m³). The concentrations of cultivable bacteria on EMB medium (mainly Gram-negative bacteria) ranged from 2–52,000 cfu/m³, and the concentrations of bacteria on R2A medium (total cultivable bacteria) ranged from 30–160,000 cfu/m³ (Tab. 4). In the samples from most workplaces, except the cotton mill, more bacterial colonies grew on R2A medium than on EMB medium. The highest concentrations of biologically-active endotoxins, muramic acid and cultivable bacteria were detected in the grain/vegetable storage houses.

About 50% of the 77 questionaired workers complained of having respiratory symptoms during the 24 hours after the bacterial air samples were collected. The most common respiratory symptoms, reported by 36%, were nasal obstruction, discharge, or sneezing. Phlegm, muscle aches, fatigue, eye irritation, sore throat, and dermatitis on the face were also common symptoms (>20%). Symptoms reported by 14–18% were chest tightness, dermatitis on hands, joint pains, dry or bleeding nose, nausea or diarrhoea, and hoarseness. Ten percent reported fever, shivering, or cough. The proportion of workers with respiratory complaints and fever or shivering was significantly higher when the air concentration of biologically-active endotoxins was higher than 25 ng/m³ (Tab. 5). All 13 workers exposed to the concentration of endotoxins above 150 ng/m³ reported at least one symptom. There was no correlation between skin symptoms or nausea/diarrhoea and the concentration of endotoxins. Of those with hand dermatitis symptoms, 81% were atopics by history (allergic rhinitis, asthma or atopic dermatitis).

No correlation was detected between any reported symptoms and the total amount of endotoxins analysed by GC-MS assay. The proportion of symptomatic persons was highest when the total concentration of endotoxins

was 2,000–5,000 ng/m³. The method used for determination of total endotoxins detects all 3-OH fatty acids. The presence of specific 3-OH fatty acids seemed to relate to workers' symptoms (Tab. 6). At least one symptom, mostly respiratory or nausea/ diarrhoea, was reported by 18/20 workers when 3-OH-14:0 was the predominant airborne 3-OH fatty acid.

DISCUSSION

Exposure to biologically-active endotoxins seemed to be associated with reported symptoms of workers, as in some other studies [9, 22, 25, 26, 31, 32]. Precise information about dose-response relationships between endotoxin exposure and experienced symptoms is not available in the literature. Heederik *et al.* [9] has reported acute shortness of breath as being related to endotoxin exposures over 100 ng/m³. In the present study, the number of workers with respiratory complaints or fever/shivering was significantly higher when the concentration of biologically-active endotoxins in the air was over 25 ng/m³. Reporting of eye symptoms and chest tightness was higher when the airborne concentration of biologically-active endotoxins was over 150 ng/m³. Excluding workers with atopy or symptoms of chronic bronchitis from the analysis did not change the above results.

The Dutch Expert Committee on Occupational Standards has recommended a health-based occupational exposure limit of 4.5 ng/m³ (50 EU/m³) over an eight-hour period of endotoxin exposure [10]. This guideline value is based on lung function studies and it is applicable for the assessment of exposure to biologically-active endotoxins analysed by the LAL assay. Furthermore, Rylander [23] has suggested that 10 ng/m³ of endotoxin will not lead to an increased risk for airway inflammation in normal or atopic subjects. He also implies that there is no risk of organic dust toxic syndrome (ODTS) if the level of airborne endotoxins does not reach 200 ng/m³.

In the present study, the mean concentrations of biologically-active endotoxins, both in personal and in stationary samples, exceeded 4.5 ng/m³ at all workplaces except the printing plant. There may be an elevated risk for ODTS in grain and vegetable storage houses, where the concentrations of biologically active endotoxins were mostly higher than 200 ng/m³. Individual endotoxin values were over 200 ng/m³ also in the cotton mill, the garbage-handling plant and the slaughterhouses. This means that workers in occupational environments where airborne Gram-negative bacteria are present in the air, may have an increased risk of respiratory diseases.

The GC-MS analysis, which is based on the total load of bacterial material in the air, gives much higher concentrations of airborne endotoxins than the LAL assay, which detects the biologically-active endotoxins. This finding is in agreement with two earlier studies [27, 32]. On the other hand, no association between the total

amount of endotoxins as measured by the GC-MS assay and the reported symptoms could be observed. However, some 3-OH fatty acids were associated with reported symptoms. At least one of the symptoms inquired about was reported by 90% of the workers who were exposed to airborne endotoxins containing predominantly 3-OH-14:0 fatty acid. These were mainly respiratory and eye symptoms, nausea and diarrhoea. Helander *et al.* [11] have suggested that differences in the acute pulmonary toxicity of endotoxins could be related to the chemical composition of the endotoxin. In their study, endotoxins of *Enterobacteriaceae*, which include predominantly 3-OH-14:0 fatty acid, had a higher biological activity than endotoxins of those bacteria, such as *Agrobacterium* sp., which include also other 3-OH fatty acids.

The highest concentrations of muramic acid were detected in the air of grain/vegetable storage houses, garbage-handling plant and slaughterhouses, where the concentrations of total cultivable bacteria (both Gram-positive and Gram-negative) on the R2A medium were also higher than in the other workplaces. Association of muramic acid concentrations with bacterial levels measured on the EMB medium, which favours Gram-negative bacteria, as well as with endotoxins, was weak. Fox *et al.* [8] have found higher content of muramic acid in Gram-positive bacteria than in Gram-negative bacteria.

In most of the air samples collected in the present study for muramic acid analysis, the concentrations were below the limit of detection. Therefore, the concentrations of muramic acid were not compared with workers' symptoms. One study [29] using similar analysis to the present study reported concentrations of muramic acid in swine dust varying from 300–1,900 ng/m³, which correspond with the highest figures in our study. Another study group [14] detected lower muramic acid levels in a stable and a dairy (means 11 and 20 ng/m³) by using a different method of analysis. Analysis methods to detect environmental peptidoglycans are still developing [1]. It is probable that in the future a specific and sensitive method will be available for occupational air measurements. Further studies by using new methods of analysis are needed to study the effect of inhaled peptidoglycans on humans in occupational environments.

CONCLUSIONS

In bacterial exposure, biologically-active endotoxins analysed with the LAL assay seem to associate better with workers' reported symptoms than the total endotoxin levels from the GC-MS analysis. Also, the levels of some specific 3-OH fatty acids from endotoxins seem to be useful in evaluating workers' risk for adverse health effects caused by airborne bacteria. In the present study, workers reported more respiratory and ODS-symptoms when they were exposed to levels higher than 25 ng/m³ of biologically-active endotoxins in the air of their workplaces. In the processing of cotton, grain, vegetables,

wood, garbage or metal-working fluids contaminated with Gram-negative bacteria, and slaughtering animals, the concentration of biologically-active endotoxins often seemed to be over 25 ng/m³ in the air of the workplace. Very high air levels of muramic acid occurred in grain, vegetable and garbage handling.

Acknowledgements

This study was financed by the Academy of Finland and is gratefully acknowledged. Dr. Ilkka Helander (VTT Biotechnology and Food Research, Finland) and Dr. Lennart Larsson (University of Lund, Sweden) are gratefully acknowledged for their help in starting analysis of chemical markers with the GC-MS assay. The authors also wish to thank Nina Helppi, Juha Laitinen and Jussi Susitaival for their technical assistance.

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