

Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health

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Background: Endotoxin exposure has been shown to be associated with a decreased prevalence of atopic sensitization and symptoms. Yet endotoxin represents only a part of the indoor microbial exposure. Muramic acid, a constituent of peptidoglycan, is present in gram-negative and gram-positive bacteria in the environment and may therefore serve as an additional marker of microbial exposure.

Objective: To study the factors determining the level of indoor exposure to muramic acid/peptidoglycan, as well as its potential association with respiratory health.

Methods: In 553 farm and nonfarm school children from Austria, Switzerland, and Germany, mattress dust muramic acid concentrations were determined, and health was assessed by using IgE measurements and questionnaire information.

Results: The muramic acid concentration was found to be significantly higher in dust from farm children's mattresses than in dust from nonfarm children's mattresses (157 vs 131 ng/mg). Children with higher mattress dust muramic acid concentrations had a significantly lower prevalence of wheezing (odds ratio of highest vs lowest tertile of muramic acid concentration, 0.3; 95% CI, 0.1-0.9), regardless of farming status and endotoxin exposure. The association for asthma was similar, and no association was found with atopic sensitization.

Conclusion: Next to endotoxin, muramic acid provides us with an independent marker of microbial exposure. Unlike endotoxin, muramic acid was inversely associated with wheezing

rather than with atopic sensitization. (*J Allergy Clin Immunol* 2004;113:860-7.)

Key words: Farming, allergy, asthma, peptidoglycan, muramic acid, microbial exposure, hygiene hypothesis

Ever since the hygiene hypothesis proposed a mechanism explaining the increased development of allergies and hay fever in children,¹ efforts have been made to find agents responsible for the proposed mechanism. Household size,^{1,2} early life day care,³ farming,^{4,5} and keeping pets in the first year of life^{6,7} were found to be related to a decreased prevalence of sensitization and possibly asthma. Increased exposure to infections early in life^{8,9} or an increased microbial burden early in life¹⁰ were put forward as other possible causes. One of the substances currently under investigation is endotoxin. Several studies linked increased endotoxin exposure to decreased sensitization rates^{11,12} and possibly lower prevalences of hay fever, asthma, and wheezing.¹³ Determinants of endotoxin exposure have also been studied. The presence of pets,^{14,15} an increasing number of household occupants, older vacuum cleaners, a high relative humidity, and the absence of floor insulation were associated with increased endotoxin concentrations in settled household dust.¹⁵

Endotoxin, however, represents only a part of the total microbial burden, because it occurs only in gram-negative bacteria. Peptidoglycan, in turn, is a major component of the cell wall of all species of the domain Bacteria, but not in species of the domain Archaea. Gram-positive bacteria have a thicker cell wall compared with gram-negative bacteria. The amount of peptidoglycan found in environmental samples reflects the presence of the greater part of environmental bacteria more precisely. However, it may be influenced more strongly by the presence of gram-positive bacteria than by the presence of gram-negative bacteria.

A major component of bacterial peptidoglycan is N-acetyl-muramic acid (2-acetamido-3-O-[(R)-1-carboxy-ethyl]-2-deoxy-D-glucose), which is not found elsewhere in nature.¹⁶ In this article, we refer to this substance as *muramic acid*. Different research groups developed methods to determine the amount of muramic acid in

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Supported by an Austrian grant from the Landesregierung Salzburg, Ressort Gesundheit; the Zurich Lung Association; Union Bank of Switzerland, Switzerland; the Swiss National Science Foundation; the Bavarian Ministry for the Environment; and the Bavarian Ministry for Health, Nutrition and Consumer Safety.

Received for publication November 24, 2003; revised December 22, 2003; accepted for publication January 30, 2004.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2004.01.783

Abbreviations used

ALEX: Allergies and Endotoxin
GLC: Gas-liquid chromatography
TLR: Toll-like receptor

environmental samples.¹⁷⁻¹⁹ Several occupational environments, like swine confinement buildings,¹⁷ a dairy,¹⁸ 2 schools,²⁰ and several other places with exposure to organic dust,¹⁹ have been studied. Few studies are available on determinants of muramic acid in house dust,²¹⁻²³ and no data are available on health effects of muramic acid/peptidoglycan. The determination of muramic acid may be more representative than endotoxin in estimating the total microbial burden; there is also some evidence that peptidoglycan influences the innate immune system. Endotoxin has been shown to act as a ligand for the CD14/Toll-like receptor (TLR)–4 pathway,²⁴ which may result in modulation of the T helper cell response. In contrast, peptidoglycan was reported to activate innate immunity via TLR-2, which induces a functionally different cellular response compared with TLR-4.²⁴ Therefore, muramic acid/peptidoglycan is not only a specific marker for microbial burden, but also a biologically active substance, influencing cellular immune response in a different way compared with endotoxin.

For 812 previously described²⁵ school children from farms and their peers living in the same villages but not on a farm, dust samples were taken.¹³ We analyzed the muramic acid content of a subset of the mattress dust samples and studied the association between muramic acid/peptidoglycan exposure and sensitization, respiratory symptoms, and the diagnoses of asthma and hay fever.

METHODS

Subjects

The Allergies and Endotoxin (ALEX) Study was performed in rural areas of Austria, Germany, and Switzerland, as previously described.^{13,25} Approval to conduct the survey was obtained from the 3 local ethics committees for human studies and from the principals of the schools attended by the children. Written informed consent was obtained from the parents of all children. Participation and consent are shown in Fig 1. Mattress dust samples from the homes of 812 children with German, Swiss, or Austrian nationality were analyzed for endotoxin. The study group was representative of the rural population in the participating countries.²⁵ The muramic acid content of the mattress dust was analyzed for 553 children for whom enough dust was still available after measurement of endotoxin and allergens. Dust samples were collected from November 1999 through February 2000.

Questionnaire

Demographic factors, information about the parents, information about the children's health, and information about their activities on farms were assessed by parental questionnaire. The questionnaire contained questions on sneezing with itchy eyes and wheezing in the past 12 months and on physician-diagnosed hay fever and asthma according to the International Study of Asthma and Allergies in Childhood questionnaire. The children's activities on farms were categorized into rarely (not at all, or rarely) and frequently (now and

then, or frequently) by questions on frequency of visits to the barn and the stable and providing help during hay harvesting. The questionnaire also contained questions about behavior during pregnancy and the first year of life of the child. Furthermore, questions were asked about the age and type of mattress (foam rubber, inner spring, or latex), the cleaning of the mattress, and the presence of pets.

Dust collection

Dust collection was performed by 7 centrally trained field workers (3 in Germany, 2 in Austria, and 2 in Switzerland) following a standardized protocol. New vacuum cleaners (Miele S624; Miele, Gütersloh, Germany) were calibrated to have a flow of 200 L/min using a clean filter, which resulted in an operating power of approximately 800 W. ALK filters (Allergisk Laboratoriet København, Hørsholm, Denmark) were used for dust collection, following the International Study of Asthma and Allergies in Childhood phase II protocol (<http://isaac.auckland.ac.nz/>). Dust samples were collected by vacuuming the mattress of the child for 2 min/m², after removal of all sheets apart from mite-impermeable mattress encasings and plastic sheets. Dust was collected on preweighed ALK filter holders. Particles larger than 2 mm (paper clips, stones, and so forth) were removed with tweezers after dust sampling, and subsequently, the filter holders were reweighed. Samples were stored at 20°C to 22°C until transport to a central location, where the samples were stored at 20°C to 22°C. The endotoxin analysis was described elsewhere.¹³

Muramic acid analysis

Ten milligram dust was methanolized (85°C, 18 hours) in 3 mL 4-mol/L methanolic HCl in a glass tube. The tube was cooled at 20°C to 22°C and then transferred to a 10-mL vial in liquid nitrogen. Subsequently, the sample was dried in a vacuum centrifuge (speed vac UV S400; Life Sciences International, Frankfurt/Main, Germany). Two hundred microliters pyridine and 100 μ L acetic anhydride were added, and after ultrasonication, the sample was incubated at 85°C for 30 minutes and dried in a vacuum centrifuge. The sample was suspended in 1 mL 50% ethyl acetate in hexane and allowed to precipitate. The supernatant was transferred to a small column (approximately 1-1.5 mL in a small Pasteur pipette) of silica gel 60 (230-400 mesh) in 50% ethyl acetate in hexane, which before this step had been washed twice with this solvent mixture. Then the column was washed 5 times with 50% ethyl acetate in hexane (1 mL each time). The sample was then extracted twice with 0.5 mL ethyl acetate. The combined supernatants of this step were transferred to the column of silica gel, which was finally eluted with 8 mL ethyl acetate. The eluted sample was dried in a stream of nitrogen, dissolved in chloroform, and analyzed by combined gas-liquid chromatography (GLC)/mass spectrometry (HP5890/MSD 5970; Hewlett-Packard, Böblingen, Germany). GLC separation was achieved on a column (12 m $\times$ 0.33 μ m in diameter) of Ultra-1 (Hewlett-Packard), applying a temperature program of 120°C over a period of 3 minutes, then increasing by 5°C/min to 320°C, which was maintained for 10 minutes. For quantification of *N*-acetyl-muramic acid in dust samples, authentic *N*-acetyl-muramic acid (10 μ g; Sigma, Deisenhofen, Germany) was methanolized (0.5 mL, 4-mol/L methanolic HCl), acetylated (50 μ L pyridine and 25 μ L acetic anhydride), and purified on a column of silica gel 60 as described for the dust samples above. The standard was dissolved in 100 μ L chloroform, and for determination of a calibration curve, volumes equal to 5, 10, 25, 50, and 100 ng were analyzed by GLC/mass spectrometry and quantified by determination of the specific ion at *m/z* 187. Areas increased linearly with the amounts injected; thus, the calibration was verified and could subsequently be used for muramic acid analyses. The assay coefficient of variation was 10.6%, which was determined by analyzing 3 samples 6 times on different days. The detection limit was 50 ng/mg dust, and all samples with

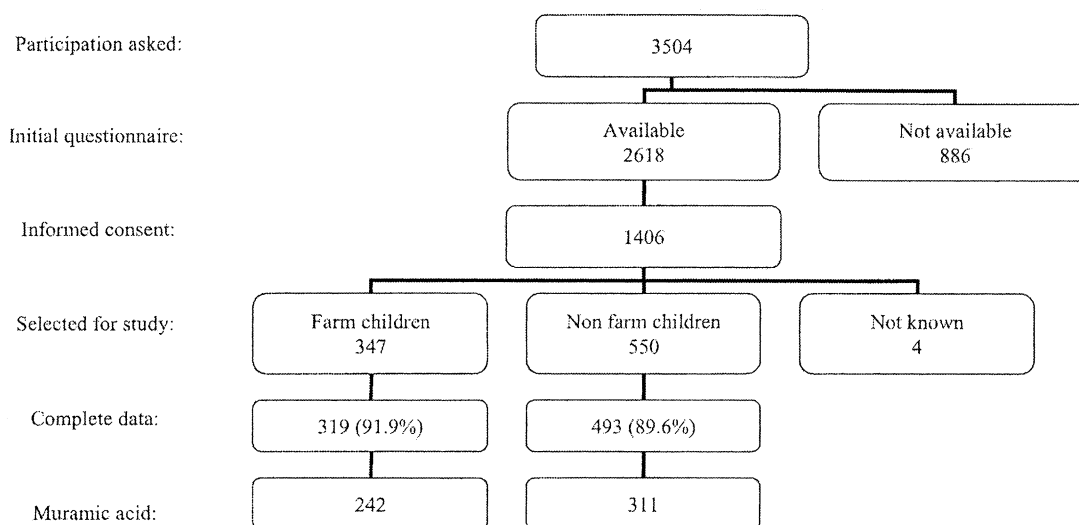


FIG 1. Participation and consent in the ALEX Study.

nondetectable muramic acid concentrations ($n = 25$) were assigned a value of 25 ng/mg. Mattress dust muramic acid load ($\mu\text{g}/\text{m}^2$) was calculated by multiplying with the total amount of dust collected and dividing by the surface area of the mattress.

Sensitization

Serum was collected from the children and tested for specific IgE to a mixture of aeroallergens containing grass pollen, birch pollen, mugwort pollen, *Dermatophagoides pteronyssinus*, cat dander, dog dander, and *Cladosporium herbarum* (sx1 Immunocap, Pharmacia, Uppsala, Sweden). Results were obtained in kilounits per liter. Cut-off values of 0.35 kU/L (comparing RAST class 1 or higher with RAST class 0) and 3.5 kU/L (comparing RAST class 3 or higher with RAST classes 0, 1, and 2) were both used to study sensitization, and the cut-off value of 0.35 kU/L was used to stratify the children by sensitization.

Statistical analyses

The muramic acid and endotoxin concentrations as well as the muramic acid, endotoxin, and dust loads were log-normally distributed, and therefore ln-transformed. Pearson correlation coefficients were calculated. Differences for subject or home characteristics between farming and nonfarming children were tested with χ^2 tests. Adjusted geometric means and 95% CIs of the muramic acid concentration for different subject or home characteristics were calculated as least-square means by using linear regression analysis in SAS version 8.2 (Cary, NC). The Tukey-Kramer method was used to adjust for multiple comparisons. Locally weighted regression smoothers were used to study the unadjusted association of respiratory symptoms and diagnoses with increasing muramic acid exposure. Adjusted odds ratios were calculated by using multiple logistic regression analysis. Odds ratios are shown for an increase in muramic acid concentration or load equal to the interquartile range of the distribution (108 ng/mg and 145 $\mu\text{g}/\text{m}^2$, respectively), and in the case of nonlinearity, for the 2 highest tertiles of the muramic acid concentration (98.5–167.5 ng/mg and >167.5 ng/mg) versus the lowest (<98.5 ng/mg). Significance was assumed at $P < .05$.

RESULTS

Only mattress dust samples for which a sufficient amount of dust was still available (553 of 812) were

analyzed for muramic acid. The analyzed samples contained initially on average about twice as much dust compared with the samples that were not analyzed (respective geometric means, 0.86 g/m^2 vs 0.39 g/m^2 ; t test: $P < .05$). On the other hand, the endotoxin concentration in analyzed samples was not significantly different from the concentration in samples that were not analyzed. Farm children were represented more often in the group for whom the dust samples were analyzed than in the group for whom the dust samples were not analyzed (44% vs 30%; χ^2 test, $P < .05$), whereas the prevalence of sensitization, respiratory symptoms, or diagnoses of asthma or hay fever was not significantly different for children with analyzed versus nonanalyzed samples.

The geometric mean muramic acid concentration in dust from mattresses of rural children (median age, 9 years; range, 6–14 years) was 128 ng/mg (95% CI, 121–135; range, 25–2011). Prevalences of different symptoms and diagnoses are described elsewhere for the whole group ($n = 812$) and were not significantly different for the subgroup described here. There was some correlation between the muramic acid and endotoxin concentration of the dust ($r = 0.44$; Fig 2, A), whereas the correlation between the muramic acid and the endotoxin loads ($r = 0.74$; Fig 2, B) was high. The latter is most probably caused by the strong influence of the amount of dust collected from the mattress. The muramic acid concentration of the dust was not correlated with the amount of dust ($r = 0.14$; Fig 2, C), and the muramic acid concentration and the muramic acid load were correlated highly ($r = 0.77$).

In Table I, adjusted geometric means and 95% CIs of the mattress dust muramic acid concentration are shown for separate categories of each potential predictor of the muramic acid concentration. For 439 children, complete data were available. Furthermore, Table I shows the distribution of each potential predictor for farm children and nonfarm children. Apart from the variables listed in

Table I, the analysis was adjusted for field worker (and thereby also for country, because field workers operated only in 1 country), month of sampling, endotoxin concentration, and dust load. The results show that independently from all other variables, farm children had a higher mattress dust muramic acid concentration compared with nonfarm children. Furthermore, the mattress dust muramic acid concentration was increased in homes heated with wood or coal (compared with gas, oil, or electric heating) and in dust from mattresses that were cleaned less often. When taking multiple comparisons into account, results did not change. When looking at farm children only, children who often helped with the work in the stables had increased mattress dust muramic acid concentrations (data not shown). Samples taken in Switzerland, and samples taken in January and February compared with November and December, had higher muramic acid concentrations (data not shown).

Table II shows odds ratios (adjusted for potential confounding, including mattress dust endotoxin concentration or mattress endotoxin load) for sensitization, wheezing, sneezing with itchy eyes, hay fever, and asthma. In bivariate analyses, an increasing muramic acid concentration was associated with a decreasing sensitization rate, but this association disappeared after adjusting for the endotoxin concentration. No relation was found between muramic acid load or concentration and sneezing with itchy eyes, hay fever, and asthma. For wheezing in the past 12 months, however, a decreased risk was seen with increasing mattress dust muramic acid concentration (ng/mg), and a similar trend was seen with increasing mattress muramic acid load ($\mu\text{g}/\text{m}^2$). All odds ratios in Table II were calculated for an increase of the mattress dust muramic acid concentration, or the mattress muramic acid load of a magnitude equal to the interquartile range of the distribution. Locally weighted regression smoothers were used to study the assumption of a linear risk increase, whereas subjects were stratified according to sensitization status (data not shown). Among nonsensitized children, the inverse association appeared to be roughly linear, but among sensitized children, the association followed an inverse J-shaped trend. Therefore, the association between tertiles of the mattress dust muramic acid concentration and the prevalence of wheezing or an asthma diagnosis, stratified by sensitization, was investigated.

Fig 3 shows the prevalence (in blue) of wheezing and asthma for the 3 tertiles of the mattress dust muramic acid concentration. The corresponding adjusted odds ratios (and 95% CIs) are shown in black, comparing the 2 highest tertiles with the lowest, while adjusting for the mattress dust endotoxin concentration, the child living in a farming family, and other potential confounders. A decreasing risk of wheezing was seen for an increasing mattress dust muramic acid concentration. This trend was mostly seen for wheezing and asthma among non-sensitized children. Among sensitized children, the highest tertile seemed to follow the same trend, whereas children in the middle tertile tended to have a higher risk of wheezing and asthma compared with children in the

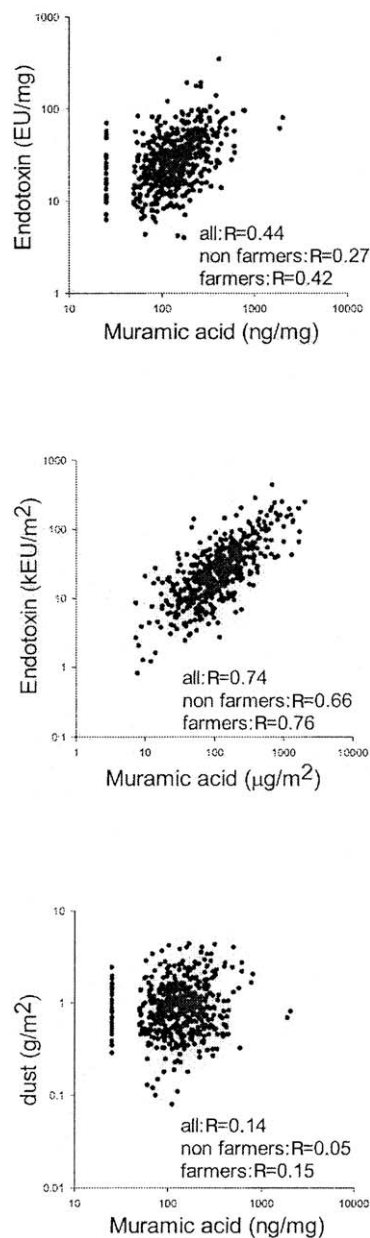


FIG 2. Correlation among muramic acid, dust, and endotoxin in mattress dust.

lowest tertile. This association was also seen in the subgroup of nonfarming children (data not shown).

DISCUSSION

Our study shows that muramic acid, in addition to endotoxin, can be considered as a marker for microbial exposure. Independent of the endotoxin concentration, increasing mattress dust muramic acid concentrations

TABLE I. Characteristics and percentage of children in each category; the geometric mean muramic acid concentration (and 95% CI) is shown for each category, n = 439.

	Category	Nonfarming (n = 260) %	Farming (n = 179) %	χ^2 *	Mass spectrometry muramic acid
					(all children) (ng/mg)§
Farming family	No	100.0	0.0	—	131 (113-152)
	Yes	0.0	100.0		157 (135-182)†
Frequency of child's barn visits (present)	Rarely	83.9	29.1	‡	146 (127-169)
	Frequently	16.2	71.0		141 (120-165)
Child helps with hay harvesting (present)	Rarely	83.1	20.1	‡	140 (122-161)
	Frequently	16.9	80.0		147 (126-171)
Frequency of child's stable visits (present)	Rarely	72.7	16.8	‡	137 (117-160)
	Frequently	27.3	83.2		151 (131-173)
Raw milk during pregnancy	No	72.3	34.6	‡	147 (129-168)
	Yes	27.7	65.4		140 (121-161)
Heating	Gas, oil or electric	85.8	38.0	‡	133 (116-152)
	Wood or coal	14.2	62.0		155 (134-179)†
Age of mattress	≤6 y (median)	53.1	41.3	†	140 (123-160)
	>6 y	46.9	58.7		147 (128-169)
Mattress material	Foam rubber	21.2	33.0	†	155 (134-178)
	Inner spring	66.2	57.0		139 (122-158)
	Latex	12.7	10.1		138 (113-168)
Extra mattress cover	No	49.6	45.3		146 (128-168)
	Yes	50.4	54.8		141 (123-161)
Weeks ago mattress last cleaned	≤20 (median)	50.0	54.2		135 (119-154)
	>20	50.0	45.8		152 (133-174)†
Indoor pets	None	57.7	71.0	†	132 (119-146)
	Cat only	31.9	19.6		145 (127-165)
	Dog only	3.5	3.9		160 (119-215)
	Cat and dog	6.9	5.6		138 (111-172)

* χ^2 Test for difference between farming and nonfarming († $P < .05$; ‡ $P < .01$).§Adjusted for fieldworker, month of sampling, endotoxin concentration, dust load, and all other variables in the table († $P < .05$).**TABLE II.** Adjusted* odds ratios for symptoms with increasing† muramic acid concentration and muramic acid load.

	Muramic acid concentration (ng/mg)	Muramic acid load ($\mu\text{g}/\text{m}^2$)
Sensitization (SX1 screening)		
>0.35 kU/L	0.90 (0.68-1.18)	0.92 (0.63-1.35)
>3.50 kU/L	0.94 (0.69-1.30)	1.01 (0.64-1.60)
Sneezing and itchy eyes (past 12 mo)	0.80 (0.52-1.24)	0.73 (0.38-1.40)
Hay fever	0.87 (0.54-1.39)	0.74 (0.37-1.50)
Wheezing (past 12 mo)	0.62 (0.41-0.94)§	0.55 (0.29-1.04)‡
Asthma	1.17 (0.69-2.00)	1.08 (0.51-2.26)

*Adjusted for age, sex, study area, family history of asthma or hay fever, educational level of the parents, number of older siblings, and endotoxin concentration or load.

†Odds ratios were calculated for an increase of the exposure by a magnitude of the interquartile range (108 ng/mg and 145 $\mu\text{g}/\text{m}^2$).‡ $P < .10$.§ $P < .05$.

were associated with a lower frequency of wheezing and possibly asthma among rural school children.

The analysis of muramic acid in mattress dust samples of the original ALEX Study subjects¹³ could be performed in only 553 of 812 subjects because no dust was available for the remaining 259 subjects. This raises the question of whether selection bias occurred.

The following arguments sustain the fact that the occurrence of selection bias was unlikely. First, the endotoxin concentration in analyzed samples was similar compared with the endotoxin concentration in samples that were not analyzed for muramic acid. Second, the low correlation between the amount of dust and the muramic acid concentration ($r = 0.14$) indicated that this

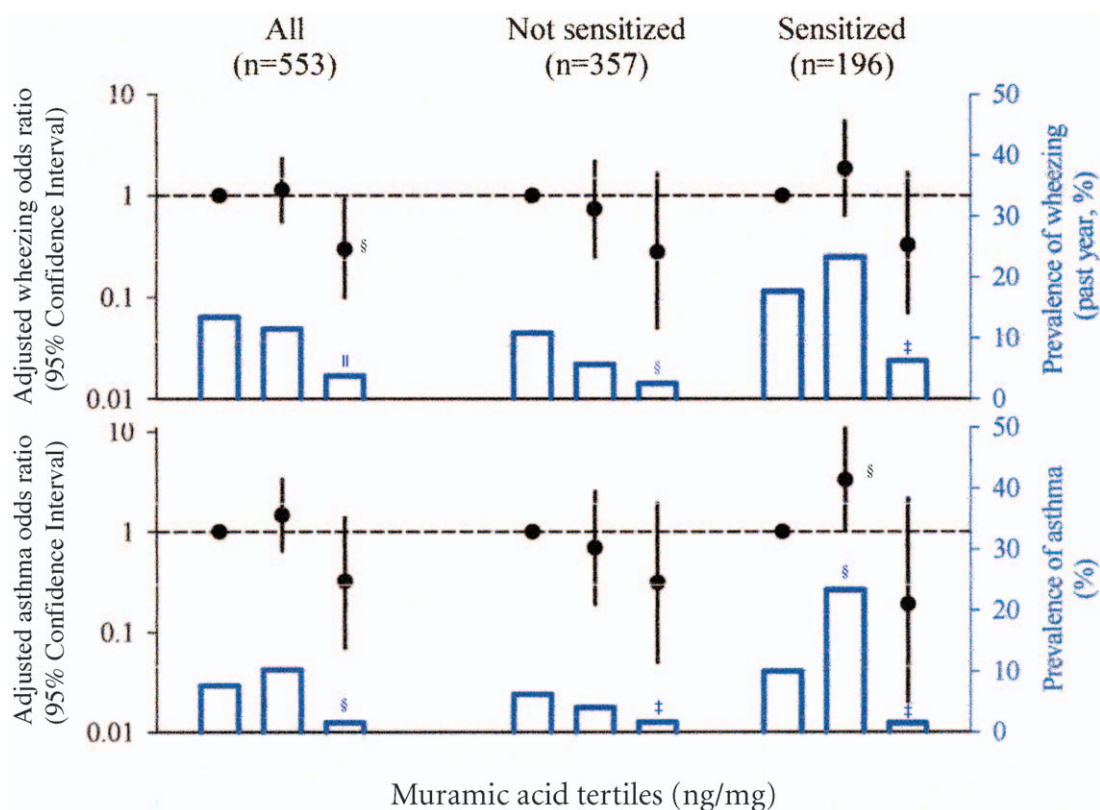


FIG 3. Prevalence (in blue) and adjusted* odds ratios (in black) for wheezing (past 12 months) and asthma diagnosis with increasing† muramic acid concentration (ng/mg; † $P < .10$; § $P < .05$; || $P < .01$). *Adjusted for age, sex, study area, family history of asthma or hay fever, educational level of the parents, number of older siblings, living on a farm, and mattress dust endotoxin concentration. †Odds ratios compare children in the second (98.5–167.5 ng/mg) and third tertile (>167.5 ng/mg) with children in the first tertile (<98.5 ng/mg) of the mattress dust muramic acid concentration.

forced selection of subjects with relatively high dust exposure was most likely not biased. Third, the prevalence of sensitization, respiratory symptoms, and diagnoses of asthma and hay fever was also not significantly different when comparing the two groups, indicating that bias because of this selection was not to be expected. Therefore, and keeping earlier considerations²⁵ in mind, we concluded that the observations made here were applicable to the rural population in the countries in which the study took place, and may not be extrapolated to urban populations. Muramic acid concentrations found in our study (geometric mean, 128 ng/mg) were somewhat higher when compared with previously reported concentrations of 23.1 ng/mg and 50.5 ng/mg dust from 2 beds,²² and were much higher than concentrations found by Liu et al²⁰ in floor dust from schools. These differences are probably in part attributable to methodological differences in sampling or analysis and to differences between school floors and mattresses. In addition, some studies reported airborne muramic acid concentrations in several different occupational environments^{17,19}; however, the outcomes are difficult to compare with our results.

There was some correlation between endotoxin and muramic acid ($r = 0.44$), indicating that both substances are markers for microbial exposure, but because the correlation coefficient was not so high, indicating a partial correlation, they seem to occur largely independent of each other. Endotoxin is only present in gram-negative bacteria, whereas muramic acid is a constituent of peptidoglycan, which forms the rigid backbone of the bacterial cell wall of gram-negative and gram-positive bacteria. The peptidoglycan of the gram-positive cell wall is much thicker than that of the gram-negative cell wall, and this may result in concentration differences between the 2 dust contaminants. The high correlation between the muramic acid load and endotoxin load of the mattress could be a cause of concern, and therefore, the muramic acid concentration was used instead. To avoid reporting spurious associations between health outcomes and muramic acid concentrations, all analyses were adjusted for the endotoxin concentration.

Several factors that may influence microbial exposure were studied for their relation to the muramic acid concentration. Farm children had higher muramic acid exposure than non-farm children, as was shown for endotoxin.²⁶ This finding is probably explained by higher

exposure to microbial contaminants in general, because most of the farms in the study area were traditional dairy farms, where the children are in close contact with the farm animals. Independent of being a farm child or not, mattress dust from homes heated with wood or coal had increased muramic acid concentrations. Using wood or coal for heating may be indicative of a more traditional way of life, possibly associated with increased microbial exposure. Using wood or coal for heating may also spread airborne dust through the house more easily. Furthermore, dust from less frequently cleaned mattresses also contained more muramic acid, pointing to the fact that muramic acid exposure from the mattress may also be influenced by cleaning.

Some studies have shown that peptidoglycan, of which muramic acid is a major component, activates the innate immune response via TLR-2 and stimulates the production of TNF- α , IL-1, and IL-6 by human monocytes, although the dose necessary for induction was very high compared with lipopolysaccharide.^{24,27,28} Dust containing muramic acid was furthermore shown to stimulate the production of IL-6 and IL-8 in lung epithelial cells, although the effect could not be separated from the effects of other constituents of the dust.²²

Because of limited numbers of samples and subjects, no previous epidemiological studies have investigated the potential health effects of muramic acid exposure. In this study, the prevalence of wheezing in the past year decreased with increasing mattress dust muramic acid concentrations. The effect persisted after adjusting for potential confounders, after adjusting for the endotoxin concentration or stratifying by the median of the endotoxin concentration, and also after adjusting for being part of a farming family. Moreover, exclusion of families who reported taking measures to reduce allergen exposure ($n = 105$) did not considerably change the results (data not shown). Sensitivity analyses separate for farm and nonfarm children showed that the inverse association between muramic acid exposure and wheezing was apparent for both groups of children (data not shown), suggesting that the effect was not limited to a highly exposed group (farm children) but was also seen among rural children not living on a farm.

Muramic acid exposure was most strongly associated with the prevalence of wheeze in the past 12 months. In Germany, Austria, and Switzerland, atopic wheezing children are more likely to be labeled as asthmatic patients than nonatopic wheezing children. We therefore stratified the analyses for wheeze and asthma into children with atopic and nonatopic phenotypes as measured by sensitization. It is intriguing that microbial exposure as assessed by muramic acid concentrations of mattress dust was inversely related to wheeze and asthma, whereas no association with atopy was seen. This observation contrasts with the assumption of 1 interpretation of the hygiene hypothesis, proposing that the potential beneficial effect of infectious or "unhygienic" exposure is mediated via T_H1-like immune responses, resulting in reduced atopic responses. Yet

asthma is only in part attributable to atopy,²⁹ and the regulation of immune responses other than those eventually leading to the production of IgE antibodies is likely to be involved. Peptidoglycan has been shown to modulate the innate immune response on a pathway other than endotoxin, acting as a ligand for TLR-2 functioning in innate immunity.²⁴ TLR-2 genes were also more strongly expressed in farm children compared with nonfarm children.³⁰ It is thus interesting to speculate that exposure to endotoxin and muramic acid/peptidoglycan modulates different pathways of the innate immune system and thus different phenotypes of asthma, although the molecular processes behind this observation are as yet unknown.

The results reported herein therefore suggest that ubiquitous environmental exposure to bacteria, in particular gram-positive bacteria, is inversely associated with the prevalence of asthmatic symptoms, supporting the hygiene hypothesis. Thereby, the inception of asthma may relate to the lack of activation of innate immune responses as triggered by certain microbial products.

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