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INFLAMMATORY POTENTIAL OF ORGANIC DUST COMPONENTS AND CHEMICALS MEASURED BY IL-8 SECRETION FROM HUMAN EPITHELIAL CELL LINE A549 *IN VITRO**

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Abstract: Due to the complex composition of organic dust it is difficult to point at one or a few components causing pulmonary health problems in different occupational environments. The aim of the present study was to develop a method which aims at measuring the general potential of organic dust to inflict a pulmonary inflammation regardless of the actual composition of the dust. The bioassay is based on measurement of interleukin-8 (IL-8) secretion from a lung epithelial cell line (A549) 24 hours after addition of test compound. The test compounds for method development and characterization were different types of lipopolysaccharide (LPS) and glucans, formaldehyde, methyl methacrylate (MMA), 2,4-dinitro chlorobenzene (DNCB), nickel sulphate, and sodium dodecyl sulphate (SDS). The dust samples were liquid extracts from cotton dust, compost dust, school dust samples, and dust from household waste. The bioassay had a linear dose-response relationship when stimulated with LPS from *Escherichia coli* up to 180 μg/ml. The day-to-day variation was reduced when the estimated potency factor was corrected relatively to the IL-8 secretion obtained with the positive controls. Three types of LPS induced IL-8 secretion in the bioassay. The potency of LPS from *Klebsiella pneumoniae* was 2-3 fold higher than the potency of LPS from *E. coli* and *Pseudomonas aeruginosa*. Two glucans from yeast did not induce IL-8 secretion, whereas Curdlan (glucan from a Gram-negative bacterium) did, but part of the activity was due to a soluble compound perhaps indicating an active non-glucan contamination of the sample. Formaldehyde had a potency similar to that of LPS from *K. pneumoniae*, whereas nickel sulphate had a much weaker potency. In contrast, the contact allergens DNCB and MMA did not induce IL-8 secretion, neither did the irritative ionic detergent SDS. The experiments on liquid extracts of different organic dust samples revealed profound differences. The potency of extracts of the two cotton dust samples was similar to the potency of the extract of compost dust. However, the extract of compost dust was far more cytotoxic. In comparison, the potency of liquid extracts of samples from a school was up to 10 fold higher than the potency of the cotton dust and compost samples. A preliminary experiment on dust from household waste suggested that the insoluble part of dust may have a potency 10^3 to 10^4 fold higher than the potency of liquid extracts of dust.

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INTRODUCTION

Health problems caused by exposure to airborne organic dust range from light irritation of the eyes and mucous membranes in the throat to severe diseases such as ODTS (Organic Dust Toxic Syndrome), allergic and toxic asthma, bronchitis and byssinosis [19, 20, 28]. Pulmonary health problems related to inhalation of

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bioaerosols are seen in many different occupations such as the farming and animal industry, sewer workers, waste recycling workers, etc. [20]. Larsson *et al.* [12] reported that the exposure to swine dust induced an intense inflammatory reaction in the airways, which was assessed especially by an increase in neutrophilic granulocytes in bronchoalveolar lavage.

Airborne organic dust, consisting of many different particles of biological origin, may be very complex in nature. Endotoxin from Gram-negative bacteria and β-1→3-D-Glucan from fungi are important biologically active constituents of organic dust [7, 32]. However, a multitude of other active components may also be present in organic dust, especially from microorganisms such as bacteria and fungi, but also inorganic compounds. Organic dust from different workplaces can have very different composition, and even dust from the same workplace may vary in composition. Therefore it is difficult to point at one or a few components being the main cause of health problems, i.e. the components which are relevant to measure in occupational surveillance programmes.

The establishment of a method which can measure the general potential of the organic dust to inflict a pulmonary inflammation, regardless of the actual components comprising the airborne organic dust, may overcome some of the problems related to risk assessment of complex organic dust.

The epithelial lining of the lung is an important first line of defence against organic dust particle after inhalation. Although it may not be a central part of the immune system, the lung epithelium is involved in the inflammation process. It is now generally accepted that lung epithelial cells, after stimulation with lipopolysaccharide (LPS) or pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor (TNF), produce and release different cytokines such as: interleukin-8 [8, 11, 18, 31], interleukin-6 (IL-6), granulocyte monocyte-colony stimulating factor (GM-CSF) and interferon (IFN) [5]. IL-8 is a pro-inflammatory cytokine, which, together with IL-1, IL-6 and TNF, are the first mediators produced during inflammation.

The aim of the present study was to develop a method for screening the hazards of chemicals and organic dust components in the working environment. The method is based on quantitative measurements of IL-8 secretion from a human pulmonary cell line (A549) after stimulation *in vitro.* A549 is a human lung epithelial cell line originating from a 58 year old male with alveolar lung carcinoma. This cell line shares many characteristics with alveolar type II cells and is regarded as such [14].

MATERIALS AND METHODS

Chemicals. Pure chemical test substances: Formaldehyde (Sigma product No. F 1268); methyl methacrylate (Sigma product No. M 1283), 2,4-dinitro chlorobenzene (DNCB) (Sigma product No. C 6396), nickel sulphate ($NiSO_4 \bullet 6H_2O$) (Sigma product No. N 4882), sodium dodecyl sulphate (SDS) (Sigma product No. L 4509).

Purified compounds from bacteria and fungi: LPS from *E. coli* serotype O55:B5 (Sigma product No. L 2637), LPS from *Pseudomonas aeruginosa* serotype 10 (Sigma product No. L 8643), LPS from *Klebsiella pneumoniae* (Sigma product No. L 1770), Curdlan consisting mainly of β-1→3-D-Glucan from the Gram-negative bacterium *Alcaligenes faecalis* (Sigma product No. C 7821), Zymosan cell wall extract from *Saccharomyces cerevisiae* (Sigma product No. Z 4250), purified glucan (β-1→3-D-Glucan) from *Saccharomyces cerevisiae* (Sigma product No. G 5011). The LPS compounds were dissolved in growth medium, and the glucans were held in a homogenous suspension in medium.

Organic dust samples: Liquid extracts of cotton dust and 5 week old compost of biodegradable household waste were kindly donated by Drs. T. Sigsgaard and V. Roepstorff, University of Aarhus, Denmark. The extracts had been prepared by washing dust (1 : 10 w/v) in either 0.125 M ammonium hydrogen carbonate ("Bract IV" and K5U II" dust samples) or pyrogen free water ("Bract II" sample), followed by filtering, freeze drying of the extract, and finally dissolving in a small volume of pyrogen free water (1 ml added to the freeze dried extract from 1.25 g dust) [23, 24, 29]. Two dust samples from a Danish school with frequently cleaning were kindly donated by Dr. C.K. Wilkins, National Institute of Occupational Health, Denmark. These samples were extracted in water and sterile filtrated (sample AB: 0.1635 g in 5.0 ml; sample 31L: 0.1786 g in 5.0 ml). Airborne dust from household waste was generated in a rotating drum, collected on a filter and resuspended in pyrogen-free water, sterilized with 0.5% formalin [9], washed and resuspended in media. The insoluble fraction was held in a homogenous suspension.

Cells. The A549 (ATCC) lung epithelial cell line was grown in Hams F'12 medium supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine and 10% foetal bovine serum (FBS) (all reagents from GIBCO BRL, Life Technologies, Denmark). The A549 cells were grown at 36.5°C and 5% CO_2 in Nucleon tissue culture flasks (GIBCO BRL, Life Technologies, Denmark).

The bioassay. A549 cells (10^6) were first cultured for three days. Hereafter the culture medium was replaced. After additional 24 hours the monolayer was treated with trypsin, and 1×10^5 cells were transferred to each well in a Nucleon 24-well plate (GIBCO BRL, Life Technologies, Denmark), where they were cultured to confluency (48 hr with change of medium after 24 hr). Hereafter the culture medium was replaced with 1.0 ml fresh media containing the test compound in question (or positive control compound), and the plate was incubated for additional 24 hours. Each test compound was tested in triplicate at a series of different concentrations. Finally, the culture medium from each well was collected separately and stored at -80°C until analysis for IL-8 content.

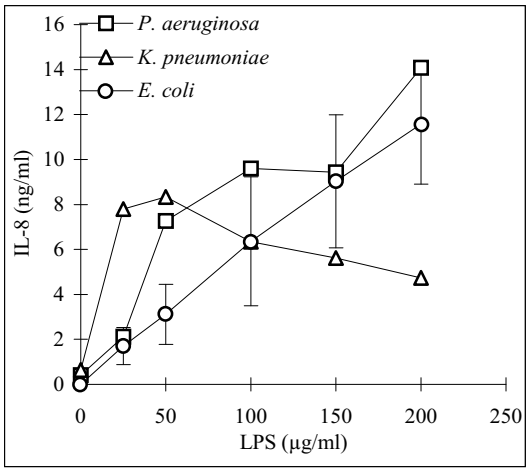

Figure 1. Dose-response curves produced after stimulation of A549 with LPS from *E. coli*, *P. aeruginosa* and *K. pneumoniae* in 24 hours. The curves are drawn between the mean of several samples (n = 9). The standard deviation (vertical lines) is shown for LPS from *E. coli*.

The concentration of IL-8 in each of the collected medium samples was determined in duplicate using a standard IL-8 ELISA kit (BIOTRAK^{TM}, Amersham LIFE SCIENCE, Denmark).

In the data evaluation the concentration of IL-8 was plotted versus the concentration of test compound used in the *in vitro* stimulation. If a linear dose-response curve was obtained the potency factor of the test compound (ng IL-8 secreted from 10^5 A549 cells in 24 h per µg of test compound) was estimated from the slope of the curve. When tested in a broad range of concentrations, some of the test compounds tended to have a bell-shaped dose-response curve indicating cytotoxicity (and reduced IL-8 secretion) at the highest concentration. In this case the initial part of curve, being approximately linear, was used for the estimation of the potency factor of the test compound.

Biological assays tend to show some degree of day-to-day variation. To reduce the day-to-day variation the estimated potency factor for a test compound was normalized relatively to the IL-8 secretion obtained with the positive control samples being either 100 µg/ml of LPS from *E. coli* or 10 ng/ml of TNF-α. Each control was divided by the target value (the mean of all the controls) and used to correct the potency factors.

RESULTS

The A549 response to LPS. Preliminary experiments revealed that the A549 cell line did not produce TNF-α or IL-1α when stimulated with LPS from *E. coli*. Therefore only the IL-8 secretion was measured in the medium samples after stimulation of the cells.

The basal parameters of the assay were optimized using LPS from *E. coli* as a model stimulant. Method evaluation [21] revealed a linear dose-response relationship when the cells were stimulated with LPS from *E. coli* in concentrations up to 180 µg/ml. Limit of detection of LPS was 17 µg/ml. When the assay was performed on 4 different days during a period of 1 month some degree of day-to-day variation in the estimated potency factor was observed (Tab. 1). This day-to-day variation was reduced when the estimated potency factor was corrected relatively to the IL-8 secretion obtained with the positive TNF control (Tab. 1).

Table 1. Potency factors (calculated from the linear part of the dose-response curves) of different types of LPS and glucans. For each test substance results from different days are shown together with the potency factor corrected with the control values relatively to the mean of the control.

Test substance	IL-8 induction ng IL-8/µg	LPS corrected IL-8 induction ng IL-8/µg	TNF corrected IL-8 induction ng IL-8/µg
LPS (*E. coli*)	0.04	0.09	0.03
	0.05	0.07	0.03
	0.06	0.07	0.04
	0.08	0.06	0.03
LPS (*P. aeruginosa*)	0.04	0.08	n.d.
	0.09	0.06	
	0.16	0.10	
LPS (*K. pneumonia*)	0.12	0.44	n.d.
	0.38	0.23	
	0.38	0.25	
Curdlan	0.02	0.01	
	0.0043	0.015	0.011
	0.0105	0.012	0.014
Curdlan liquid insoluble	0.0054	0.005	0.006
Glucan	0	0	n.d.
Zymosan	0	0	n.d.

n.d. - Not determined.

Figure 1 shows three examples of dose-response curves for the stimulation of A549 cells with LPS of different origin. Stimulation of A549 with LPS from *K. pneumoniae* produced a quite different response compared to stimulation with LPS from the two other strains. In this case a bell-shaped curve was obtained, having a peak in IL-8 secretion at an LPS concentration of about 50 µg/ml. At higher concentrations of LPS the IL-8 secretion drops and levels off probably due to some cytotoxic effects. When A549 was stimulated with LPS from *P. aeruginosa*, the linearity of the dose-response relationship was not as convincing as the one obtained with LPS from *E. coli*, and a large day-to-day variation in the estimated potency factor was observed (Tab. 1). In these experiments a positive control containing 100 µg LPS from *E. coli* was included. When the estimated potency factors were corrected relatively to this control, the day-to-day variation was reduced for all three types of LPS. After correction, LPS from *K. pneumoniae* seemed to be more potent than LPS from the two other species of Gram-negative bacteria.

The A549 response to glucans. Figure 2 shows stimulation curves for three different glucans. Zymosan and Glucan, both from baker's yeast *S. cerevisiae*, produced a very small or lacking response. In contrast,

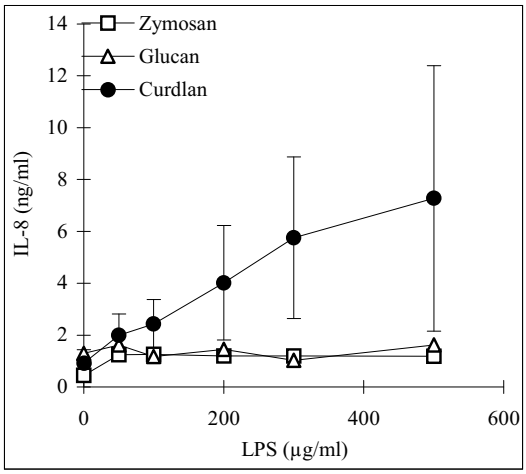

Figure 2. Dose-response curves produced after stimulation of A549 with glucans in 24 hours. Curdlan originates from *A. faecalis*. Zymosan and Glucan originate from *S. cerevisiae*. Each curve is drawn between the mean of several samples (n = 6). The standard deviation (vertical lines) is shown for Curdlan.

Curdlan, being a β-1→3-D-Glucan from a Gram-negative bacterium *A. faecalis*, resulted in an induction of IL-8 secretion from the A549 cells. This potency of the soluble part of Curdlan was not caused by a contamination with LPS, since Limulus Amoebocyte Lysate test (LAL-test) of this sample turned out negative (results not shown). However, centrifugation of the sample and resuspension of the insoluble fraction of Curdlan in an equal volume of media, revealed that the soluble fraction itself could induce IL-8 secretion (Tab. 1).

The A549 response to pure chemicals. Formaldehyde, nickel sulphate, methyl methacrylate (MMA), dinitro chlorobenzene (DNCB) and sodium dodecyl sulphate (SDS) are all known contact allergens or irritants. In general these agents were tested at very low concentrations to prevent cytotoxic effects. These effects can be detected in microscopy analysis as profound changes in cell morphology. The estimated potency factors are presented in Table 2.

Formaldehyde resulted in a bell-shaped dose-response curve with maximum value at 3 µg/ml. At higher concentrations cytotoxic effects were seen. Nickel sulphate resulted in a linear dose-response curve up to 700 µg/ml $NiSO_4$. At concentrations of $NiSO_4$ above 100 µg/ml cytotoxic effects were observed.

Stimulation with MMA did not result in a significant induction of IL-8 secretion, and the values could not be distinguished from the background level. The same was the case for SDS (concentrations above 100 µg/ml were cytotoxic) and DNCB (cytotoxic above 10 µg/ml). It should be noticed that DNCB was dissolved in dimethyl sulfoxid (DMSO) and then added to the culture media. Hence in all sample wells the concentration of DMSO was adjusted to 0.01% which neither did induce IL-8 secretion from the cells in the blank samples.

Table 2. Potency factors (calculated form the linear part of the dose-response curves) of different pure chemical test substances. For each test substance results from different days are shown together with the potency factor corrected with the control values relatively to the mean of the control.

Test substance	IL-8 induction ng IL-8/µg	LPS corrected IL-8 induction ng IL-8/µg	TNF corrected IL-8 induction ng IL-8/µg
Formaldehyde	0.08 0.12	0.17 0.28	0.11 0.14
Nickel sulphate	4.77×10^{-4} 8.39×10^{-4}	1.24×10^{-3} 1.06×10^{-3}	0.71×10^{-3} 1.09×10^{-3}
Methyl methacrylate	0 0	0 0	0 0
Dinitro chlorobenzene	0 0	0 0	0 0
Sodium dodecyl sulphate	0 0	0 0	0 0

The A549 response to dust samples. The aim of these preliminary experiments was to see whether the A549 cell assay could be used to distinguish between different aqueous extracts of organic dust samples. Since the method of sample extraction did not allow for a determination of the extraction efficacy the concentration of test substance was expressed as the weight of dust per ml. Consequently, the estimated potency factors for dust should not be compared with the potency factors of the tested chemicals or purified microbial toxins. However, the estimated potency factors of extracts from the different dust samples can be compared (Tab. 3).

Extracts of two dust samples (31L and AB) from a school resulted in an increase in IL-8 secretion up to “9 mg dust per ml”. It should be emphasized than only minute

Table 3. Potency factors (calculated from the linear part of the dose-response curves) of different organic dust samples. Results from correction of the potency factor with the control values relatively to the mean of the control is also shown.

Test substance Liquid extracts	IL-8 induction ng IL-8/µg	LPS corrected IL-8 induction ng IL-8/µg	TNF corrected IL-8 induction ng IL-8/µg
Cotton dust (Bract II)	4.2×10^{-6}	9.18×10^{-6}	n.d.
Cotton dust (Bract IV)	8×10^{-6}	1.72×10^{-5}	n.d.
Compost dust (K5U II)	4×10^{-6}	7.69×10^{-6}	n.d.
School dust (31L)	1.4×10^{-5}	1.11×10^{-4}	2.06×10^{-5}
School dust (AB)	9.6×10^{-6}	5.31×10^{-5}	8.19×10^{-6}
Insoluble fraction of household waste dust	0.048	0.01	n.d.

n.d. - Not determined.

amounts of dust were available, and many of the values were below the background level.

Cotton dust extracted in water (Bract II) and in NH_4HCO_3 (Bract IV) resulted in approximately similar linear dose-response curves in the stimulation range up to "100 mg dust per ml". At the highest concentrations of Bract IV minor cytotoxic reactions were observed. In contrast, the compost dust extract (K5U II) produced a bell-shaped curve, and cells stimulated with extract of 100 mg/ml K5U II showed a strong cytotoxic response when examined by microscopy.

The insoluble fraction of formalin treated dust from ordinary household waste, resulted in a high IL-8 secretion, 75 ng when the A549 cells were stimulated with 1.6 mg. This gives a high potency factor (Tab. 3). The dose-response curve was bell-shaped, indicating a cytotoxic response at concentrations higher than 1.6 mg dust.

DISCUSSION

Preliminary experiments revealed that the A549 cell line did not produce TNF-α or IL-1α when stimulated with LPS from *E. coli*. Similar results were obtained by Spriggs *et al.* [30] working with A549 and other tumour epithelial cell lines, and Agace *et al.* [2] in their studies on the human kidney epithelial cell line A498 and the human bladder epithelial cell line J82. In agreement with the reports of Cromwell *et al.* [6] the bronchial epithelium was shown to be a potent source of IL-8 production. Therefore, only IL-8 secretion from the A549 cells was measured in the present study.

Depending on the employed concentration range of test compound the dose-response curve was either linear or bell-shaped if the test compound was cytotoxic at the higher concentrations. The potency factor of the test compound (i.e. its capability to induce IL-8 secretion from A549) was estimated from the slope of the dose-response curves. For bell-shaped curves the potency factor was estimated from the initial slope of the curves. The initial velocity of the dose-response curves corresponds to the maximal velocity of Michaleis-Menton first order kinetics. For complex samples, however, it is not possible to give a rational suggestion of the dominating kinetics, but *a priori* we expect that a first order kinetic model would be a fairly good approximation. Therefore, the initial slope will be the best possible measurement of the IL-8 induction of the sample.

Several reports emphasize that TNF-α strongly stimulates IL-8 production from lung epithelial cells [6, 11], and TNF-α may therefore be considered an appropriate positive control for the general ability of the cells to produce and secrete IL-8. Pugin *et al.* [22] suggested that epithelial and endothelial cells are stimulated with LPS through binding to a soluble receptor (sCD14). Hence, LPS may serve as a positive control for the presence of specific receptors on the surface of the epithelial cells. The present study demonstrated that correction of the estimated potency factors relatively to the IL-8 response obtained with the positive controls resulted in a reduced day-to-day variation (Tab. 1).

It is well known, that LPS of different origin may show large differences in pyrogenic properties when tested in animal models [10, 25]. Perhaps in agreement the present study revealed that LPS from *E. coli* and *P. aeruginosa* had about the same potency whereas LPS from *K. pneumoniae* was more potent.

Fungal spores are often found in large numbers in bioaerosols from occupational environments in agriculture and in the waste collection and recycling industry [19, 20]. In controlled exposure experiments it has been demonstrated that inhalation of glucans can inflict pulmonary inflammation [26]. However, the dose needed was high, and the results from controlled exposure of human volunteers suggest that glucans are less potent than LPS. In the present study glucans of fungal origin did not possess the ability to induce IL-8 secretion from epithelial cells, whereas β-1→3-D-Glucan from the Gram-negative bacterium *Alcaligenes faecalis* had a potency factor about one third of the potency of LPS from *E. coli* (Tab. 1). A fraction of this activity was water soluble indicating a potent non-glucan contamination. However, the difference between the potency of β-1→3-D-Glucan from the Gram-negative bacterium (Curdlan) versus β-1→3-D-Glucan from yeast is still unexplained.

In the present study different chemical compounds with known irritative or contact allergenic properties were tested to elucidate the specificity of the A549 bioassay. Both formaldehyde and nickel sulphate are well known contact allergens giving rise to type IV hypersensitivity [1, 4, 13, 16, 17]. In the A549 bioassay formaldehyde and nickel sulphate induced IL-8 secretion (Tab. 2). Formaldehyde had a potency similar to that of LPS from *K. pneumoniae*, whereas nickel sulphate had a much weaker potency. In contrast, the strong contact allergen dinitro chlorobenzene (DNCB) [3, 27] did not induce IL-8 secretion from A549 epithelial cells. Neither did methyl methacrylate (MMA) which has been reported to induce occupational contact allergy among workers handling this compound [15]. Finally, the strong, irritative ionic detergent sodium dodecyl sulphate (SDS) did not induce IL-8 production in A549 epithelial cells.

Based on results from tests with pure chemicals and LPS and glucan of different microbial origin it is tempting to speculate that the induction of IL-8 secretion from A549 epithelial cells may be dependent on the presence of specific receptors. This may explain why some, but not all low molecular weight allergens have a potential for induction of IL-8 secretion, and also that an unspecific irritative stimulus will not give rise to a specific IL-8 response. Research is needed to elucidate the basic mechanisms of induction of secretion of IL-8 from epithelial cells. However, our results demonstrate that the specificity of the assay is considerably broader than that of the Limulus Amoebocyte Lysate assay.

The experiments on liquid extracts of different organic dust samples revealed profound differences between these

samples with respect to their potency (Tab. 3). Detailed chemical and microbial analysis of the dust extracts was not performed, but the potency was not related to the content of endotoxin in the samples, as measured with the Limulus Amoebocyte Lysate assay (results not shown).

The potency factor for the extracts of the dust samples was estimated on the basis of the weight of dust per ml, and not on the actual concentration in the extracts. Since the extraction efficacy is unknown, it makes no sense to compare these values with the potency factors obtained for LPS, glucans and pure chemical compounds. However, it may be valid to compare the estimated potency factors of liquid extracts of the different dust samples (Tab. 3). Several reports have documented that exposure to cotton dust and compost dust may inflict severe pulmonary health problems [20, 24, 29]. In the present study the potency of extracts of the two cotton dust samples (Bract II and Bract IV) was similar to the potency of the extract of compost dust (K5U II). However, the extract of compost dust was far more cytotoxic indicating a health damaging potential which is not related to induction of IL-8 secretion. Only small amounts of dust were sampled from the schools (31L and AB), and the uncertainty of the estimated potency factor for these two samples was high. Nevertheless, the results indicate a very high potency of these samples, up to 10 fold higher than the potency of the cotton dust and compost samples (Tab. 3). This high potency of dust from indoor air suggests that organic dust, even at low concentration, may be an important parameter governing the indoor air quality.

The potency factor for the insoluble fraction of organic dust from ordinary household waste was only a factor of 10 below the potency factors of the LPS samples. This indicates that the insoluble fraction of organic dust rather than soluble components has a high inflammatory potential.

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