THE EFFECT OF SMOKING ON CYTOKINE RELEASE AFTER EXPOSURE TO ORGANIC DUSTCOMPONENTS IN A WHOLE BLOOD ASSAY

T. Sigsgaard[1], E. van der Maarl[1-2], N. Bak[1-2], E.C. Bonefeld-Jørgensen[1], Ø. Omland[1] and G. Doekes[2]

Institute of Environmental and Occupational Medicine
Aarhus University, Denmark
Institute of Environmental Epidemiology
Wageningen Agricultural University, the Netherlands

Abstract

Studies of human volunteers who were exposed to swine dust have revealed a range of effects on respiratory mucosa after exposure. This experimental study measured the in vitro cytokine release in blood of healthy farmers after incubation with aqueous extracts of LPS, §-1,3-D-glucan and *Aspergillus fumigatus* in a whole blood assay (WBA).

From an ongoing study of young farmers we recruited 46 healthy farmers. From each farmer 10 ml of EDTA blood was collected and exposed to aqueous extracts of LPS from *E. coli*, §-1,3-D-Glucan (Curdlan) and *Aspergillus fumigatus.* No release of cytokines was seen in the WBA after exposure to *Aspergillus fumigatus* suspensions. Smoking strongly attenuated in vitro cytokine release from blood cells in a WBA. The cytokine release in the present study was from 1.5 times higher for IL-6 after LPS II exposure to 4.6 times higher for TNF after exposure to §-1,3-D-glucan. The release of cytokines was almost the same for 0.5 µg/ml LPS as for 250 µg/ml §-1,3-D-glucan. In conclusion this study shows that smoking is a confounder in studies of the release of proinflammatory cytokines in a WBA. Furthermore LPS is a 500 times more potent inducer of the proinflammatory cytokine release compared to §-1,3-D-glucan in WBA on a weight basis.

Introduction

Occupational exposure to organic dust induces a wide range of symptoms from the respiratory tract. These symptoms have been reported in farmers [1-5] as well as in workers from other occupations from garbage handling to cotton milling [6-10].

Studies of human volunteers who were exposed to swine dust for 3 hours weighing swine have revealed a range of effects of this exposure on the upper and lower respiratory mucosa. Cell counts and concentrations of the proinflammatory cytokines TNFα, IL-1α, IL-1§ and IL-6 increase dramatically in BAL as well as NAL 7 to 24 hours after this exposure [11]. The increase in cytokine concentration is followed by an increased bronchial reactivity to metacholine and in some persons also fever. Systemic effects can also been shown by measuring increased cytokine concentrations in the circulation. Studies of grain workers have revealed that the cross shift increase in peripheral blood cytokine concentration can occur without measurable effects on lung function parameters [12]. Furthermore the physiologic changes in lung function or release of proinflammatory cytokines were shown to be almost identical after exposure to either corn dust extract or endotoxin from Gram negative bacteria (LPS) in healthy non-atopic non-smoking volunteers. The only difference found was a somewhat higher concentration of IL-1§ following LPS exposure [13].

Laboratory studies with exposure of human lung epithelial cell cultures to extracts of organic dusts including dust from farming have shown a marked effect of barn dust in a cytotoxic assay [14]. However, no effect was demonstrated after exposure to pure endotoxin or §-1-3-D-glucan in this assay. A study of cytokine release in human lung epithelial cells after exposure to swine dust and LPS found that LPS and swine dust were strong inducers of IL-8 release from the cells as well as from alveolar macrophages, whereas §-1,3-D-glucan was only a weak stimulant of cytokine release [15].

As a simple, relatively non-invasive way to test individual responsiveness to inhaled organic dust components a so-called whole blood assay[21] (WBA) might be used, in which whole fresh blood samples are incubated for 18-24 hours with stimulating agents, and cytokine release is measured in the supernatant. In this study we measured the cytokine release in healthy farmers in a WBA with as stimulants LPS, §-1,3-D-glucan and an extract of *Aspergillus*.

Materials and Methods

Study Subjects

From a study of young Danish farmers we enrolled *46 male* healthy farmers without respiratory symptoms in the study, in order to facilitate comparison between groups. The demographic characteristics of the farmers are presented in table 1. During field visits in 96-97 and 97-98 these 18 and 28 farmers respectively were interviewed and clinically examined.

Blood Samples

A blood sample was collected in a pyrogen free EDTA glass tube and sent to the laboratory within 8-10 hours and kept in a refrigerator at 5°C till next morning for the WBA.

Exposures

Of each blood sample, 0.9 ml aliquots were added to 1,5 ml Eppendorf tubes, mixed with 0.1 ml of stimulatory agents in RPMI, vortexed and then incubated overnight at 37°C. After the incubation the tubes were centrifuged at 10,000 rpm for 3 minutes, and the supernatants were collected, and stored in tubes at -80° until analysis, figure A. To measure spontaneous cytokine release, a control tube was included with 0.1 ml RPMI without any stimulants.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:197-200 (1999)
National Cotton Council, Memphis TN

As stimulatory agents we used LPS from E. coli, serotype 055:B5 (Bie & Berntsen, DK; FLUKA 62 326), at 10, 0.5 and 0.001 μg/ml, β-1,3-D-Glucan (Curdlan; from WAKO NL), at 250, 0.5 and 0.001 ug/ml, and an extract of Aspergillus *fumigatus* (ALK-Abello, DK; Ref# 315352), at 250, 0.5 and 0.001 ug/ml.

Effect Measures

The cytokines TNF , IL1, IL6 and IL8 were measured with a sandwich ELISA (QUANTIKINE) from R&D (Abingdon, UK). All samples were diluted 1:1.25, and tested in duplicate and the mean of two readings is reported here. The coefficient of variation between the two measurements was for all samples < 10% and the optical density differed < 0.05 between duplicates.

Statistics

Tabulation, graphical analysis and Mann-Whitney's tests were carried out with SPSS statistical package [17]. For categorised data, χ^2 test was performed.

Results

The median ages of the 10 smokers and 36 non-smokers were 23.0 and 23.5 years. These median values were not significantly different (table 1).

Spontaneous cytokine release in tubes incubated with RPMI alone was low, and not different between smokers and non-smokers. The lowest concentrations of LPS (0.001 μg/ml) and β-1,3-D-glucan (0.50 and 0.001 μg/ml) and none of the Aspergillus extracts concentrations produced significantly increased cytokine release above background values. These results were therefore excluded from the rest of the analyses. Thus we further only compared cytokine release induced by LPS 10 and 0.5 μg/ml, and by β-1,3-D-glucan (250 μg/ml).

TNFα release was significantly higher in non-smokers compared to smokers for all three incubations. The median concentrations were between 626 and 1503 pg/ml for non-smokers and between 213 and 577 pg/ml for smokers (table 3).

IL-1β release was also higher in non-smokers compared to smokers for all three incubations but the difference was only significant for LPS (10 μg/ml). We found a borderline significant difference for LPS (0.5 μg/ml) and β-1,3-D-glucan. The median concentrations were between 1763 and 3014 pg/ml for non-smokers and between 724 and 1110 pg/ml for smokers (table 4).

IL6 release, as well, was higher in non-smokers compared to smokers for all three incubations and this difference was only significant for LPS (0.5 μg/ml). The median concentrations were between 2995 and 5350 pg/ml for non-smokers and between 1072 and 3233 pg/ml for smokers, (table 5).

No significant differences in IL8 release were seen between non-smokers and smokers. These results were however complicated by an unexplained finding: significantly higher concentrations were found in the samples from 96-97 compared to 97-98 for all incubations except RPMI. The associated variance at population level thus prevented a meaningful comparison of smokers and non-smokers (table 6).

Discussion

The results in our WBA confirms the pro-inflammatory potency of several known organic dust components, particularly LPS and glucans. The mechanism of induced cytokine production probably involves an antibody-independent interaction with cell surface receptors that can occur in all healthy, non-sensitised subjects. No cytokine release was induced with Aspergillus fumigatus extracts, which may indicate that Aspergillus-induced inflammation only occurs in specifically sensitised individuals, but this should be further investigated.

The release of cytokines is similar for LPS 0.5 μg/ml and for 250 μg/ml β-1,3-D-glucan, while LPS (10μg/ml) gave much stronger, and β-1,3-D-glucan (0.5 μg/ml) no measurable responses. This suggests that LPS is the most potent inducer of cytokines in our assay having 250/0.5 = 500 times the potency of β-1,3-D-glucan on a weight basis in healthy young males. More extensive dose-response studies, however, should be performed to confirm this finding. Moreover, the β-1,3-D-glucan preparations are not very well standardised and it is far from clear whether its molecules can fully participate in the reaction system. Thus it is a matter of debate whether 1 ng of LPS might at all be compared with 1 ng of any glucan preparation.

Smoking is generally assumed to be associated with chronic inflammatory reactivity in the airways. However, recent studies have revealed that smokers may have lower concentrations of cytokines in BAL fluid, in contrast to an elevated cell concentration [18]. In the same study smoking was found to attenuate in vitro cytokine release from BAL-macrophages. The latter finding was confirmed in a study from Sweden. Moreover, in that study NO_2 exposure further attenuated the spontaneous as well as the LPS induced release of cytokines in smokers, but not in non-smokers [20].

The results from this study show that smoking habits may strongly attenuate in vitro cytokine release from blood cells in a WBA. The cytokine release in the present study was in non-smokers from 1.5 times higher for IL-6 induced by LPS (0.5 μg/ml) to 4.6 times higher for TNFα induced by β-1,3-D-glucan. The results for IL-8 however, are not unequivocal since we have contradictory findings in the two study years, so the conclusion only holds for IL-1β IL-6 and TNFα.

This study focuses on the non-IgE mediated pathway of cytokine release. However, the results mirror what we have shown earlier in cotton workers namely that smokers have a lower concentration of IgG antibodies in serum compared to non-smokers [10,19,21].

The fact that there are significant differences between IL8 release in the two consecutive years emphasises that the inference of these results has to be taken with caution.

Conclusion

This study shows that both LPS and β-1,3-D-glucan can induce cytokine release in a whole blood assay, but that LPS seems to be a more potent pro-inflammatory agent. Smoking appeared to be associated with significantly lower responses for both stimulatory agents, and for various cytokines, and therefore can be a strong confounder in studies of the release of proinflammatory cytokines in a WBA.

Table 1. The age of the study persons.

	N	Median	Percentiles 25%	75%
Non-smokers	36	23.0	22.0	23.8
Smokers	10	23.5	23.0	24.0
P Mann-Whitney		0.17		

Table 2 . The final concentrations of exposures in the whole blood assay.

Exposure per ml	I	II	III
LPS	10 μg	0.5 μg	0.001 μg
β-1,3-D-glucan	250 μg	0.5 μg	0.001 μg
A. fumigatus	250 μg	0.5 μg	0.001 μg

Table 3. TNFα concentrations pg/ml in the supernatant according to smoking. Median (25-75% percentiles)

Exposure	Non-smokers	Smokers	p*
LPS 10 μg /ml	1503 (1024-2665)	577 (301-940)	.003
LPS 0.5 μg /ml	626[a] (357-1180)	229[a] (17-375)	.002
β-1,3-D-glucan 250 μg /ml	972[ab] (453-2703)	213 (73-481)	.010
RPMI	8[abc] (3-38)	3[abc] (3-14)	.202

*: Mann-Whitney
a: p < 0.05 group vs. LPS 10 μg /ml Wilcoxon Test
b: p < 0.05 group vs. LPS 0.5 μg /ml Wilcoxon Test
c: p < 0.05 group vs. GLU 250 μg /ml Wilcoxon Test

Table 4. IL1β concentrations pg/ml in the supernatant according to smoking. Median (25-75% percentiles)

Exposure	Non-smokers	Smokers	p*
LPS 10 μg /ml	3014 (1801-5469)	926 (442-3003)	.026
LPS 0.5 μg /ml	1763[a] (524-2629)	724 (93-1562)	.079
β-1,3-D-glucan 250 μg /ml	1856[a] (1088-2897)	1110 (161-2198)	.083
RPMI	28[abc] (5-245)	11[abc] (5-133)	.554

*: Mann-Whitney
a: p < 0.05 group vs. LPS 10 μg /ml Wilcoxon Test
b: p < 0.05 group vs. LPS 0.5 μg /ml Wilcoxon Test
c: p < 0.05 group vs. GLU 250 μg /ml Wilcoxon Test

able 5. IL6 concentrations pg/ml in the supernatant according to smoking. Median (25-75% percentiles)

Exposure	Non-smokers	Smokers	p*
LPS 10 μg /ml	5350 (2380-10422)	3233 (2339-4913)	.150
LPS 0.5 μg /ml	3443[a] (1578-11403)	2268[a] (661-2537)	.048
β-1,3-D-glucan 250 μg /ml	2995[a] (976-10964)	1072[b] (414-2093)	.140
RPMI	27[abc] (8-201)	3[abc] (1-120)	.119

*: Mann-Whitney
a: p < 0.05 group vs. LPS 10 μg /ml Wilcoxon Test
b: p < 0.05 group vs. LPS 0.5 μg /ml Wilcoxon Test
c: p < 0.05 group vs. GLU 250 μg /ml Wilcoxon Test

Table 6. IL8 concentrations pg/ml in the supernatant according to smoking. Median (25-75% percentiles)

Exposure	Non-smokers	Smokers	p*
LPS 10 μg /ml	682 (7-33310)	543 (7-2161)	.492
LPS 0.5 μg /ml	400[a] (7-23337)	551 (164-3116)	.815
β-1,3-D-glucan 250 μg /ml	1483 (136-99349)	426 (111-1697)	.289
RPMI	750[abc] (38-1662)	925 (438-1865)	.319

*: Mann-Whitney
a: p < 0.05 group vs. LPS 10 μg /ml Wilcoxon Test
b: p < 0.05 group vs. LPS 0.5 μg /ml Wilcoxon Test
c: p < 0.05 group vs. GLU 250 μg /ml Wilcoxon Test

Acknowledgements

This study was supported by the Danish Medical Research Council, the Danish Agricultural Research Council, The Health Foundation and the P.C.Pedersens fond. The authors wish to thank the students and staff of all the farming schools for their eminent support and enthusiasm.

References

1. Larsson KA, Eklund AG, Hansson L-O, Isaksson B-M, Malmberg PO. Swine dust causes intensive airways inflammation in healthy subjects. Am J Respir Crit Care Med 1994; 150:973-977.
2. Malmberg P, Larsson KA. Acute exposure to swine dust causes bronchial hyperresponsiveness in healthy subjects. Eur Respir J 1993; 6:400-404.
3. Iversen M, Dahl R, Jensen EJ, Korsgaard J, Hallas T. Lung function and bronchial reactivity in farmers. Thorax 1989; 44:645-649.
4. Manfreda J, Holford-Strevens V, Cheang M, Warren CPW. Acute symptoms following exposure to grain dust in farming. Environmental Health Perspectives 1986; 66:73-80.

5. Donham KJ, Reynolds SJ, Whitten P, Merchant J, Burmeister L, Popendorf WJ. Respiratory dysfunction in swine production facility workers: Dose-response relationships of environmental exposures and pulmonary function. Am J Ind Med 1995; 27:405-418.
6. Chan-Yeung M, Dimich-Ward H, Enarson DA, Kennedy SM. Five Cross-sectional Studies of Grain Elevator Workers. American Journal of Epidemiology 1992; 136, no. 10:1269-1279.
7. Sigsgaard T, Abell A, Jensen LD. Work related symptoms and lung function measurements in paper mill workers exposed to recycled water. Occ Hyg 1994; 1:177-189.
8. Rylander R, Schilling RSF, Pickering CAC, Rooke GB, Dempsey AN, Jacobs JJ. Effects after acute and chronic exposure to cotton dust: the Manchester criteria. Br J Ind Med 1987; 44:9-10.
9. Rylander R, Haglind P, Lundholm M. Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom. Am Rev Respir Dis 1985; 131:209-213.
10. Sigsgaard T, Pedersen OF, Juul S, Gravesen S. Respiratory disorders and atopy in cotton wool and other textile mill workers in Denmark. Am J Ind Med 1992; 22:163-184.
11. Wang Z, Larsson KA, Palmberg L, Malmberg P, Larsson P, Larsson L. Inhalation of swine dust induces cytokine release in the upper and lower airways. Eur Respir J 1997; 10:381-387.
12. Borm PJ, Schins RP, Derhaag TJ, Kant I, Jorna TH. Cross-shift changes in blood inflammatory markers occur in the absence of airway obstruction in workers exposed to grain dust. Chest 1996; 1078-1085.
13. Jagielo PJ, Thorne PS, Watt JL, Fress KL, Quinn TJ, Schwartz DA. Grain dust and endotoxin inhalation challenges produce similar inflammatory response in normal subjects. Chest 1996; 110:263-270.
14. Roepstorff V, Sigsgaard T. Cytotoxic effect of organic dust extracts from different working environments, an in vitro assay. AAEM 1997; 4:195-201.
15. Dunoyer-Geindre, S., F. Ludi, T. Perez, N. Terki, P. I. Gumowski, and J. P. Girard. 1992. PAF acether release on antigenic challenge. A method for the investigation of drug allergic reactions. Allergy 47:50-54.
16. Palmberg L, Larsson BM, Malmberg P, Larsson KA. Induction of IL-8 production in human alveolar macrophages and bronchial epithelial cells in vitro by swine dust. Thorax 1998; 53:260-264.
17. Norusis MJ/SPSS INC. SPSS/PC+ V2.0 Base manual for the IBM PC/XT/AT and PS/2. SPSS INC., 1988:
18. McCrea KA, Ensor JE, Nall K, Bleecker ER, Hasday JD. Altered cytokine regulation in the lungs of cigarette smokers. Am J Respir Crit Care Med 1998; 150:696-703.
19. Dandrea T, Tu B, Blomberg A, et al. Differential inhibition of inflammatory cytokine release from cultured macrophages from smokers and non-smokers by NO2. Hum Exp Toxicol 1997; 16:577-588.
20. Kjærgaard SK, Pedersen OF, Frydenberg M, Sch¢nheyder H, Andersen P, Bonde GJ. Respiratory diseases and lung function in a tobacco industry. Archives of Environmental Health 1989; 1:164-170.
21. Sigsgaard T, Gravesen S. Molds and endotoxins isolated from the air of two Danish cotton mills, a wool mill, and a mmf mill, and the relation to serum IgG. Proceedings XV cotton conf. Memphis, Tennessee, 1991; 7:209-212.

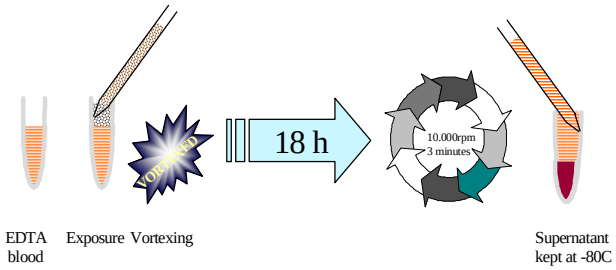

Figure A. Overview of the whole blood assay.