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Inter- and intraindividual variation of endotoxin- and β(1→3)-glucan-induced cytokine responses in a whole blood assay

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Inflammatory airway responses to bioaerosols and to their active compounds, such as endotoxin and β(1→3)-glucan, vary between individuals. These differences may be explained by variation in cytokine responsiveness, which can be assessed by *in vitro* stimulation tests with isolated blood leukocytes or lung macrophages. In large-scale population studies, *ex vivo* induced cytokine production may also be tested with a more simple 'whole blood assay' (WBA). However, applicability of a WBA to characterize a subject's responsiveness depends largely on its reproducibility. This study was conducted to: 1) assess the within- and between-subject variability in cytokine production in a WBA after stimulation with endotoxin or β(1→3)-glucan; and 2) to determine under which conditions this test is most discriminating between subjects and most reproducible within subjects.

Blood was collected from 14 healthy volunteers, of whom 10 also participated on a second occasion. Each blood sample was used in two WBA tests; the first WBA was initiated two hours and the second 26 hours after venapuncture. The WBA test itself comprised overnight incubation with serial dilutions of endotoxin [lipopolysaccharide (LPS)] and curdlan (a β(1→3)-glucan), after which blood cell supernatant was collected. Interleukin(IL)-1β, IL6, IL8 and tumor necrosis factor α (TNFα) were determined in the supernatant.

In all individuals, a dose-dependent production of cytokines was observed for both LPS and curdlan. For all cytokines, variation between subjects was higher than within subjects, and this was most pronounced for IL1β and IL6. There was moderate-to-high correlation in the induced release of all four cytokines, and between cytokine release induced by LPS or curdlan. Optimal stimulation concentrations were 6.25 and 12.5 ng/mL for endotoxin and 12 500 and 25 000 ng/mL for curdlan. Cytokine production in WBA initiated 26 hours after venapuncture showed lower between-subject and larger within-subject variance, thus favoring an early initiation of the assay.

In conclusion, measuring endotoxin- or glucan-induced cytokine production in a WBA initiated within two hours after venapuncture appears to be an effective method to determine a person's cytokine responsiveness, at least in healthy naive subjects. *Toxicology and Industrial Health* 2002; **18:** 15–27.

Key words: *cytokines; endotoxin; β(1→3)-glucan; individual susceptibility; whole blood assay*

Introduction

Bioaerosol exposure is common in many domestic and occupational environments and is associated with acute and chronic respiratory disorders (Björnsonn *et al.*, 1995; Husman, 1996; Rylander and Jacobs, 1997). Several microbial agents in these bioaerosols may contribute to the associated health

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effects. Best-known and most studied are bacterial endotoxins and fungal $\beta(1\rightarrow3)$-glucans, both proinflammatory microbial cell-wall components. Both substances have been shown, in domestic (Douwes *et al.*, 2000b; Michel *et al.*, 1991; 1992; 1996) and occupational (Douwes *et al.*, 2000a; Eduard *et al.*, 2001; Rylander, 1997; Rylander *et al.*, 1992; Thorn *et al.*, 1998) environments, to be associated with the presence and development of airway diseases or an aggravation of already present respiratory symptoms, especially in asthmatics.

The exact underlying pathogenic mechanisms behind bioaerosol-induced symptoms are not yet known. A nonallergic neutrophil-mediated airway inflammation induced by inhaled microbial components, such as endotoxin and $\beta(1\rightarrow3)$-glucan, most likely accounts for the subsequent symptomatic inflammatory reactions, like enhanced mucus production, bronchoconstriction, cough, phlegm, and wheezing. Reed and Milton (2001) have recently reviewed these endotoxin-induced inflammatory processes and emphasized the important role of cytokines, chemokines, adhesion molecules, and other inflammatory mediators in the recruitment and activation of neutrophils. The cytokines interleukin(IL)-1β, IL6, IL8, and tumor necrosis factor α (TNFα) play a key role in these processes and are produced both by airway epithelial cells and alveolar macrophages directly upon stimulation by the inhaled agents, and by infiltrating white blood cells during further amplification of the inflammatory response (Fogelmark *et al.*, 1994; Michel, 1997; Sandstrom *et al.*, 1992; Ulmer, 1997; Wang *et al.*, 1996).

Human experimental exposure studies with purified endotoxin have demonstrated marked interperson differences in endotoxin-induced airflow obstruction, measured as a reduction in forced expiratory volume in one second (FEV_1; Kline *et al.*, 1999; Michel *et al.*, 1989). Similar interindividual variation has been observed at workplaces where not all exposed workers develop symptoms and the severity of symptoms differs from person-to-person even at more or less similar exposure levels (Castellan *et al.*, 1984; Jacobs *et al.*, 1993). Given the key role of cytokine production, variations in the release of cytokines may very well explain these interindividual differences in bioaerosol-related responses. Two recent experimental studies have shown that increased production of inflammatory mediators by *in vitro* stimulated peripheral blood monocytes and alveolar macrophages was positively associated with the endotoxin- or cotton dust-induced decline in FEV_1 (Beijer *et al.*, 1995; Kline *et al.*, 1999).

These results indicate that *in vitro* release of inflammatory mediators by blood monocytes or alveolar macrophages may indeed be used as a measure of individual susceptibility for bioaerosol-induced airway inflammation. Assessment of this individual susceptibility might be of importance as a modifying factor in population studies exploring bioaerosol-related respiratory health effects. The tests using isolated leukocytes or alveolar macrophages may, however, be too laborious for large-scale population studies, where a more simple and inexpensive approach would be needed. A whole blood assay (WBA), measuring the *ex vivo* induced cytokine release in whole blood, may be that simple alternative approach, since a WBA requires no prior processing of the blood before use in the assay.

Similar WBAs have been used in various recent studies (Kline *et al.*, 1999; Magnan *et al.*, 2000; Sigsgaard *et al.*, 2000) but there is little information on the reproducibility of the assay. When assessing individual susceptibility, it is important that between-subject, or interindividual, variability in the test results is relatively large and within-subject, or intraindividual, variability is relatively small. To our knowledge only a few studies have addressed this issue for the WBA (van der Linden *et al.*, 1998; Wilson *et al.*, 1991; Yaqoob *et al.*, 1999), reporting a larger between-subject than a within-subject variation, thus favoring the use of a WBA to determine individual susceptibility. However, only a limited number of lipopolysaccharide (LPS) concentrations were studied, and the within- and between-subject variations were either not quantified (Yaqoob *et al.*, 1999) or intraindividual variation was assessed in other subjects than those studied for interindividual variation (van der Linden *et al.*, 1998; Wilson *et al.*, 1991). In the present study, we thus investigated the reproducibility of the *ex vivo* induced cytokine release in a WBA, with the aim to optimize the WBA for use in future population studies. We determined the between- and within-subject variance of induced IL1β, IL6,

IL8, and TNFα release upon stimulation with a wide range of concentrations of either LPS or curdlan, a β(1→3)-glucan. We determined optimal stimulation concentrations based on the inter- and intraindividual variance of the test system responses. For practical reasons it may be difficult in actual field studies to initiate the WBA on the day of blood collection; we thus considered whether the WBA may also be performed with shipped and/or stored blood. We, therefore, also investigated whether storage for 26 hours of the collected blood prior to initiating the WBA affected the outcomes.

Methods

Study design

Peripheral blood was collected after informed consent was given from 14 healthy adult volunteers from our own department (seven men and seven women). Ten of the 14 participants were able and willing to provide blood at a second occasion, with a one-to-four-week(s) interval between both occasions. On each occasion two WBA tests were performed with a donor's blood sample. Approximately half of the blood was used to initiate a WBA test within two hours after venapuncture, and the other half was stored on melting ice until the next day, when a second WBA test was initiated approximately 26 hours after venapuncture. Blood collection was performed on four days in each of two consecutive months, with a maximum of six individuals per day.

Whole blood assay

Approximately 30 mL peripheral blood per subject was collected by venapuncture in sterile EDTA tubes (K_3-EDTA,Vacutainer™; Becton Dickinson, Plymouth, UK), and subsequently transferred into a sterile 50-mL tube (Greiner Labortechnik BV, Alphen aan de Rijn, the Netherlands), gently mixed and stored on melting ice until further processing.

Half of the blood was immediately transferred into 16 sterile tissue-culture tubes (Greiner Labortechnik BV, Alphen aan de Rijn, the Netherlands), containing 900 νL each. Aliquots of 100 νL of serially diluted stimulatory agents in RPMI 1640 (Gibco Life Technologies, Paisley, UK), or corresponding control solutions (see next section) were added and mixed with the blood. All handling took about two hours, thus WBA was initiated two hours after blood collection. The blood was incubated for 18 hours at 37°C, 5% CO_2, 96% relative humidity, after which 0.5 mL of cold RPMI was added to the tubes and mixed. Tubes were centrifuged for 10 minutes at 1000 *g*, and cell-free supernatants were collected, transferred to polypropylene vials (Sarstedt B.V., Etten-Leur, the Netherlands) and stored at −20°C until cytokine analysis. The entire WBA procedure was repeated the next day with the remaining half of the blood. Total leukocyte counts were performed by means of a hemocytometer (Bürker-Türk counting chamber, Assisant, Fritzens, Austria) and differential cell counts were performed on blood smears stained with May-Grünwald-Giemsa by means of light microscopy, both on fresh blood and on blood stored for 26 hours.

Stimulatory agents

A 100-νL aliquot of stimulatory agents or control solutions (diluents of stimulatory agents) were added to the 900-νL aliquots of whole blood. Stimulatory agents were serial dilutions of endotoxin (LPS from *E. coli* 055:B5; Fluka Biochemika, Buchs, Germany) or β(1→3)-glucan (curdlan; Wako Pure Chemical Industries Ltd, Osaka, Japan). Stock solutions of LPS (1 mg/mL in pyrogen-free water) and curdlan (10 mg/mL in 0.05 M NaOH in pyrogen-free water) were prepared and serially diluted in RPMI to generate final concentrations in the test tubes of 100, 50, 25, 12.5, 6.25, and 3.13 ng/mL for LPS and 100 000, 50 000, 25 000, 12 500, 6250, and 3130 ng/mL for curdlan, respectively. Incubations with control solutions were included in order to determine background cytokine release. RPMI served as a control for LPS stimulation. Since curdlan was dissolved in 0.05 M NaOH in pyrogen-free water (Douwes *et al.*, 1996) prior to serial dilution in RPMI, a 10-fold dilution of 0.05 M NaOH in RPMI served as a control for curdlan stimulation. All controls were tested in duplicate in every WBA, thus with fresh and stored blood from each person.

Cytokine analyses

Levels of IL1β, IL6, IL8, and TNFα in the supernatant were measured by enzyme immuno assays (EIA; Biosource, Biosource Europe S.A., Fleurus, Belgium), using recombinant human cytokines provided by the manufacturer as calibration standards. Supernatants were tested in dilutions of 5-, 15-, and 45-fold. Cytokine concentrations in the supernatant were calculated as the average of values obtained at those dilutions, where values were within the range of the standard curve. Duplicate testing of a random sample of 12 supernatants on different days revealed a mean coefficient of variation (CV) of 15% for IL1β, 16% for IL6, and 19% for IL8. CV values could not be determined for TNFα analysis since not enough sample was available. The limits of detection of the cytokine EIAs were 34 pg/mL for IL1β, 14 pg/mL for IL6, 5 pg/mL for IL8, and 95 pg/mL for TNFα. Supernatants with cytokine levels below the limit of detection were assigned a value of two-thirds the detection limit.

Statistical analyses

Data were analysed using SAS statistical software (version 6.12; SAS Institute, Cary, NC, USA). For each cytokine, net induced cytokine production was calculated by adjusting – by subtraction – for the corresponding nonstimulated control. Net induced cytokine production was regarded nondetectable when adjusted levels were below or equal to zero. This occurred in 4.8% of the IL1β tests, 11.7% of IL6 tests, 1.9% of IL8 tests, and 8.6% of TNFαtests; in those cases a value of two-thirds the lowest of the detectable concentrations was assigned. Percentages of difference (%D) of cytokine levels of WBA initiated at two hours and at 26 hours after venapuncture were calculated to evaluate changes due to storage prior to initiating the WBA, with

$$\%D = ((X_{26} - X_2)/((X_{26} + X_2)/2)) * 100\%$$

X being the cytokine concentration. Inter- and intraindividual variances were determined using natural log (ln) transformed net induced cytokine levels to ensure a normal distribution of the residuals in an analysis of variance (ANOVA by PROC NESTED). Pearson correlation coefficients were determined on ln-transformed cytokine production levels and ln-transformed monocyte levels (PROC CORR). Comparisons were considered significant at $P < 0.05$.

Results

Background cytokine levels were low and similar for control tests with either RPMI or NaOH (Table 1). IL1β and IL6 remained below the limit of detection (34 pg/mL for IL1β and 14 pg/mL for IL6) in nearly all control cultures. Background TNFα levels were slightly but not significantly higher in the WBA initiated 26 hours after venapuncture compared with two hours after venapuncture, and the number of control tests with detectable levels of TNFα (above 95 pg/mL) increased. IL8 was detectable in all control cultures (above 5 pg/mL) and these background IL8 levels were significantly higher when WBA was initiated

Table 1. Median and range of background cytokine concentrations (in pg/mL) in WBAs initiated within two hours and within 26 hours after venapuncture.

	Cytokine	Initiation two hours			Initiation 26 hours		
		Nd/N[a]	Median	Min–max	Nd/N	Median	Min–max
RPMI	IL1β	2/48	22.8	22.8–68.0	1/35	22.8	22.8–45.2
	IL6	2/48	9.3	9.3–29.5	2/36	9.3	9.3–16.2
	IL8	48/48	23.4	11.7–71.5	36/36	45.3***	22.3–92.1
	TNFα	22/48	63.5	63.5–629.0	21/36	107.1	63.5–1010.4
NaOH	IL1β	2/48	22.8	22.8–71.7	2/45	22.8	22.8–55.6
	IL6	2/48	9.3	9.3–32.1	3/45	9.3	9.3–28.3
	IL8	48/48	23.4	6.8–74.4	45/45	41.7***	19.7–978.5
	TNFα	20/48	63.5	63.5–696.9	30/45	119.0	63.5–603.5

*** $P < 0.001$ Wilcoxon test, within two hours versus within 26 hours after venapuncture.
[a] Number of samples with detectable cytokine concentration/total number of samples.

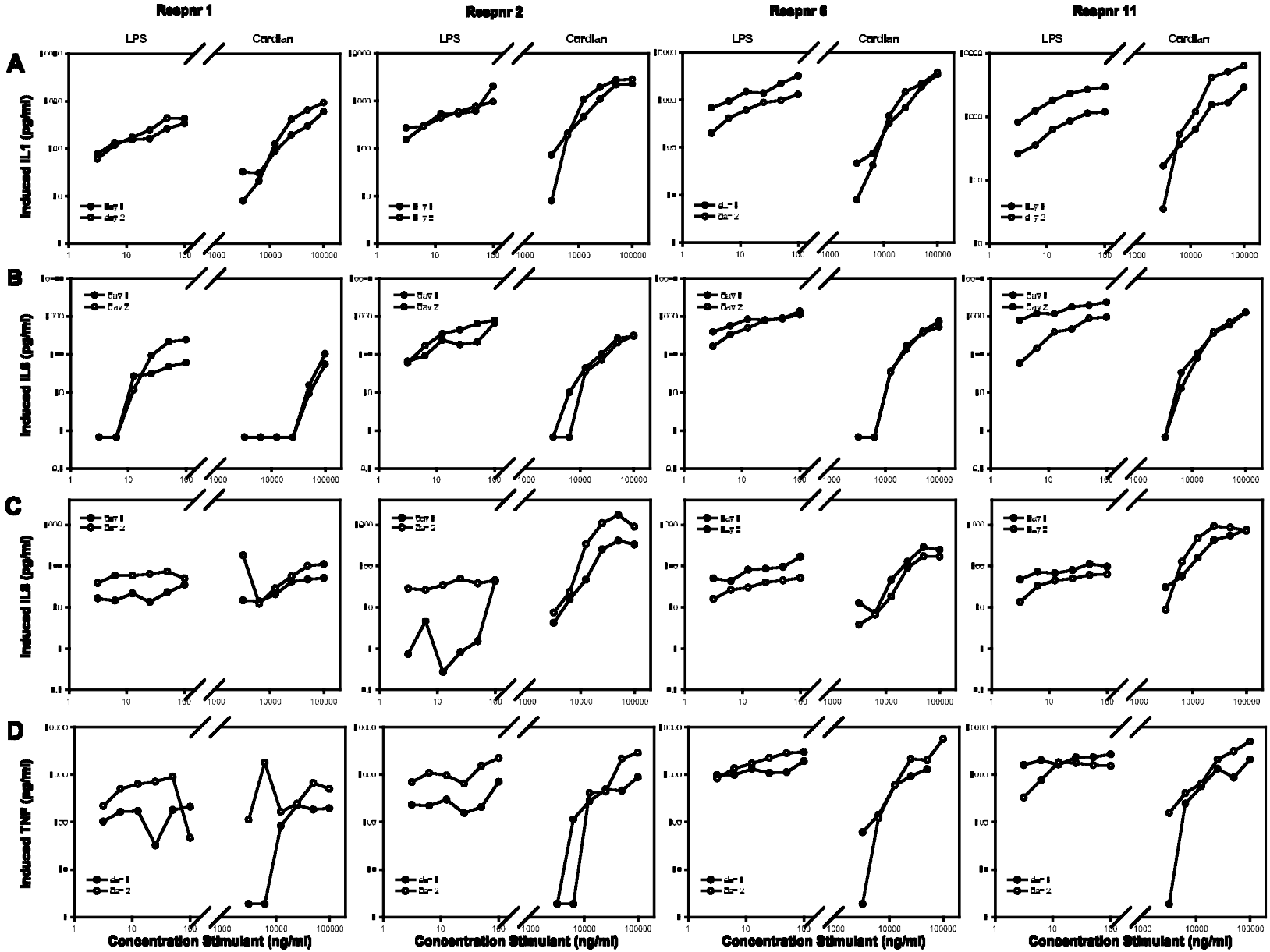

Figure 1. Test day-to-day variation in *ex vivo* induced IL1β (A), IL6 (B), IL8 (C), and TNFα (D) cytokine production in whole blood of four subjects selected as examples to represent the total range of cytokine production and variability

26 hours after blood collection (Wilcoxon test $P < 0.001$ for both RPMI and NaOH).

Stimulation with LPS or curdlan resulted in detectable levels of all four cytokines in almost all supernatants (IL6: 89%, TNFα: 94%, IL1β: 95%, and IL8: 100%). Net induced IL1β and IL6 levels (i.e., IL production corrected for background concentrations) were up to 100 times higher than the background levels, whereas TNFα and IL8 levels showed a much less pronounced increase (Table 1, Figures 1 and 2). Endotoxin- and curdlan-induced production of IL1β and TNFα were lower in WBAs initiated 26 hours after venapuncture compared with two hours after collection. The median percentage of difference was −61% for IL1 and −19% for TNFa (both $P < 0.05$). In contrast, IL6 and IL8 showed a higher net induced production when the WBA was initiated 26 hours after collection. For these cytokines, the median percentage of difference was +6% for IL6 and +105% for IL8 (both $P < 0.05$). *Ex vivo* net cytokine production in fresh and stored blood of the same donor were significantly positively correlated for both stimulatory agents and all stimulant concentrations (overall Pearson correlation coefficient: 0.58 for IL1β, 0.78 for IL6, 0.26 for IL8, and 0.61 for TNFα; $P < 0.001$).

Intraindividual reproducibility of cytokine responsiveness was investigated by repeating the whole procedure, with two complete WBAs initiated at two hours and 26 hours, one to four weeks after the first test. Individual dose–response curves showed that, in general, the WBA performed with blood from the same individual on two different occasions produced similar dose–response curves for both stimulatory agents (Figure 1). This indicates little day-to-day or intraindividual variation in WBA responses. On the other hand, marked differences were found between dose–response curves of different subjects (Figure 2). Figures 1

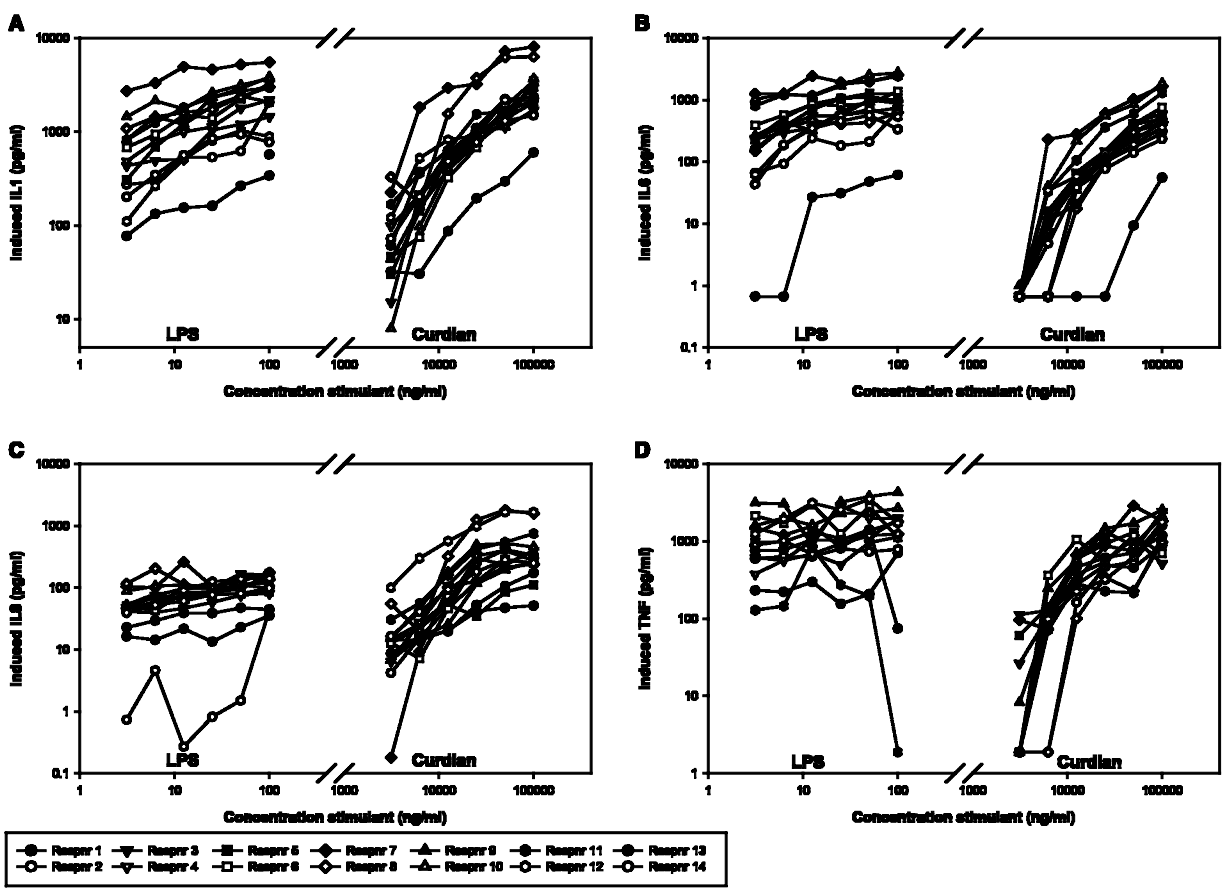

Figure 2. Individual dose–response curves of LPS- and curdlan-induced IL1β (A), IL6 (B), IL8 (C), and TNFα (D) cytokine production in whole blood expressed in pg/mL for 14 subjects at the first day of blood collection, when stimulation was initiated two hours after venapuncture

Table 2. Inter- and intraindividual variance of induced IL1β, IL6, IL8, and TNFα production in WBAs initiated within two hours after blood collection ($n = 10$).

Agent	Concentration (ng/mL)	IL1β		IL6		IL8		TNFα	
		Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra
LPS	3.125	0.38	0.47	3.25***	0.67	0.35	0.94	Ne[a]	0.62
LPS	6.25	0.45*	0.25	4.02***	0.38	0.37	0.38	0.16	0.30
LPS	12.5	0.44**	0.15	1.27***	0.14	0.40	1.39	0.08	0.56
LPS	25	0.45**	0.15	0.75**	0.24	0.23	1.19	0.67*	0.65
LPS	50	0.36**	0.17	0.61**	0.25	0.38	0.72	0.16	0.52
LPS	100	0.36**	0.18	0.55**	0.22	0.17*	0.15	0.70	0.78
Curdlan	3125	0.88[2]	0.87	0.00	0.47	0.19	0.74	Ne	3.50
Curdlan	6250	0.59**	0.16	2.36**	0.87	0.45*	0.35	Ne	4.65
Curdlan	12500	0.46**	0.12	2.05***	0.13	0.50	0.58	Ne	1.72
Curdlan	25000	0.37**	0.16	3.34***	0.07	0.79**	0.39	0.23*	0.18
Curdlan	50000	0.29*	0.18	1.29***	0.06	0.55*	0.33	Ne	0.59
Curdlan	100000	0.24**	0.12	0.63***	0.08	0.72***	0.14	0.36*[b]	0.29

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ F-test significance interperson variance.
[a] Ne = not estimable; the interperson variance component could not be estimated as the total variance was almost completely determined by intraperson variance.
[b] $n = 9$.

and 2 further show that, per nanogram, LPS is approximately 1000 times more potent a stimulator than curdlan.

The results of these two (initiation at two and 24 hours) twice repeated experiments on 10 subjects were further analysed to calculate the inter- and intraindividual variances of induced cytokine responses for both stimulating agents and at all stimulant concentrations (Table 2). For the WBAs initiated two hours after blood collection, the between-subject variance was usually larger than the within-subject variance, especially for induced IL1β and IL6 production. For these cytokines, a significant interindividual variance was demonstrated at nearly all stimulant concentrations (F-test, $P < 0.05$). Interperson variance of induced IL8 and TNFα production was significant at only a few stimulant concentrations, and was often lower than the intraperson variance (Table 2). Overall, the highest interperson variance and the highest ratios between inter- and intraindividual variance were found at stimulant concentrations of 6.25 and 12.5 ng/mL for LPS, and at 12.5 and 25 νg/mL for curdlan. Thus, these concentrations were judged optimal based on the observed inter- and intraindividual variances (Table 2) and the criterion that induced responses should, in most individuals, exceed the detection limit of the cytokine assay. The same statistical analyses were performed for the results of the WBAs initiated 26 hours after blood collection. Although similar trends were found as in Table 2, there were higher intraindividual variances, and as a result, much less significant interindividual variances (summarized in Table 3).

Correlations between induced productions of the different cytokines were determined (Table 4) to assess whether interindividual differences in cytokine responses showed a similar pattern for all determined cytokines. These correlations are presented in Table 4 for selected optimal stimulation concentrations of 6.25 and 12.5 ng/mL for LPS and 12.5 and 25 νg/mL for curdlan. To avoid autocorrelation in statistical analyses due to repeated measurements, we included in these analyses only data obtained from each subject's first WBA test session. A moderate-to-high positive correlation between the different cytokines was found when WBA was initiated two hours after blood collection (Table 4; Pearson's r from 0.54 to 0.99; $P < 0.05$).

Table 3. Range in inter- and intraindividual variance of induced IL1β, IL6, IL8, and TNFα production in WBAs initiated 26 hours after blood collection ($n = 10$).

Cytokine	Range in variance	
	Interindividuals	Intraindividuals
IL1β	Ne[a]–0.37	0.51–1.95
IL6	Ne–1.74	0.08–4.86
IL8	Ne–3.18	0.29–2.96
TNFα	Ne–1.99	0.21–3.95

[a] Ne = not estimable; the interperson variance component could not be estimated as the total variance was almost completely determined by intraperson variance.

Table 4. Pearson correlation coefficients between induced IL1β, IL6, IL8, and TNFα production in the WBA initiated two hours after venapuncture and at the first day of blood collection ($n = 14$).

Stimulating agent	Concentration (ng/mL)	IL1β versus			IL6 versus		IL8 versus
		IL6	IL8	TNFα	IL8	TNFα	TNFα
LPS	6.25	0.80[a]***	0.71[a]**	0.89[a]***	0.59*	0.71**	0.58*
LPS	12.5	0.92[a]***	0.48[a]	0.88[a]***	0.45	0.83***	0.58*
Curdlan	12500	0.66*	0.54*	0.65*	0.45	0.66*	0.60*
Curdlan	25000	0.73**	0.52	0.48	0.39	0.59*	0.29

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
[a] $n = 13$.

Correlations were slightly less pronounced for curdlan-induced than for endotoxin-induced responses. Induced cytokine production in WBAs initiated 26 hours after venapuncture showed weaker correlation, and in case of curdlan-induced responses no correlation between different cytokines was observed (data not shown).

To identify whether a high response induced by LPS also implicates a high response to curdlan, correlations between endotoxin- and glucan-induced cytokine productions were determined (Table 5). For the WBA initiated at two hours, the positive correlation between LPS- and curdlan-induced production of IL1β, IL6, and TNFα was moderate-to-strong (Table 5; range in correlation coefficients 0.54–0.99; $P < 0.05$), whereas the production of IL8 induced by either LPS or glucan showed much less correlation (Table 5; correlation coefficient range 0.10–0.56). Furthermore, levels of different cytokines produced in the same WBA test were moderately to highly correlated between IL1β, IL6, and TNFα (Table 5; correlation coefficient > 0.6; $P < 0.05$), whereas IL8 levels were generally not correlated with those of any other cytokine (Table 5; $P > 0.05$). When the WBA was initiated 26 hours after blood collection, LPS- and glucan-induced cytokine responses were only significantly correlated for corresponding cytokines, and no correlations between different cytokines could be demonstrated (data not shown).

Since it might be of interest to know whether background cytokine levels are associated with LPS- or curdlan-induced cytokine production, this relation was assessed for IL8, the only cytokine that showed detectable background levels in all tests. There was no significant correlation between background and LPS-induced IL8 concentrations (correlation coefficients: 0.43 for LPS 6.25 ng/mL and 0.32 for LPS 12.5 ng/mL), whereas curdlan-induced IL8 production showed a moderate positive correlation with background levels, with correlation coefficients of 0.58 and 0.56 (both $P < 0.05$) for curdlan at 12500 ng/mL and 25000 ng/mL, respectively.

Table 5. Pearson correlation coefficients between LPS- and glucan-induced IL1β, IL6, IL8, and TNFα production in the WBA initiated two hours after venapuncture, and at the first day of blood collection ($n = 14$).

Stimulating agent	Concentration (ng/mL)	Cytokine	Curdlan 12500 ng/mL				Curdlan 25000 ng/mL			
			IL1β	IL6	IL8	TNFα	IL1β	IL6	IL8	TNFα
LPS	6.25	IL1β	0.61[a]*	0.71[a]**	0.23[a]	0.63[a]*	0.69[a]**	0.76[a]**	0.20[a]	0.63[a]*
		IL6	0.68**	0.91***	0.38	0.66*	0.71**	0.97***	0.33	0.61*
		IL8	0.52	0.44	0.56*	0.50	0.54*	0.48	0.41	0.44
		TNFα	0.39	0.53	0.03	0.54*	0.49	0.64*	0.05	0.65*
LPS	12.5	IL1β	0.72[a]**	0.78[a]**	0.31[a]	0.77[a]**	0.77[a]**	0.84[a]***	0.19[a]	0.64[a]*
		IL6	0.72**	0.89***	0.36	0.78**	0.72**	0.94***	0.21	0.61*
		IL8	0.34	0.27	0.35	0.35	0.25	0.28	0.10	0.23
		TNFα	0.73**	0.56*	0.24	0.65*	0.66**	0.67**	0.08	0.38

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
[a] $n = 13$.

Table 6. Pearson correlation coefficients between induced IL1β, IL6, IL8, and TNFα production and monocyte counts in the WBA initiated two and 26 hours after venapuncture and at the first day of blood collection ($n = 14$).

Stimulating agent	Concentration (ng/mL)	Initiation two hours				Initiation 26 hours			
		Monocyte count versus				Monocyte count versus			
		IL1β	IL6	IL8	TNFα	IL1β	IL6	IL8	TNFα
LPS	6.25	0.42[a]	0.24	0.14	0.38	−0.05[b]	−0.14[b]	−0.08[b]	−0.35[b]
LPS	12.5	0.41[a]	0.37	0.25	0.51	0.04[b]	−0.23[b]	−0.14[b]	−0.48[b]
Curdlan	12500	0.41	0.22	0.05	0.12	0.31[c]	−0.20[c]	0.03[c]	−0.43[c]
Curdlan	25000	0.28	0.25	−0.01	−0.14	0.30[c]	−0.25[c]	−0.12[c]	−0.41[c]

[a] $n = 13$, [b] $n = 12$, [c] n = 11.

Total and differential white blood cell counts were carried out to investigate whether the interperson variation in cytokine production was due to different number of blood cells. Cell numbers and differentials were all within the normal range. No significant correlation was observed between the monocyte counts and the cytokine levels produced after stimulation (Table 6; correlation coefficients ranging from −0.48 to 0.42; $P > 0.05$).

Discussion

In the present study we evaluated whether *ex vivo* cytokine responses induced by endotoxin or a β(1→3)-glucan (curdlan) in a WBA can be used to reproducibly measure an individual's cytokine responsiveness. We showed systematic interindividual differences in induced production of IL1β and IL6 and to a lesser extent in production of IL8 and TNFα. These interindividual differences were larger than the variation within individuals over a one-to-four-week period. As interindividual variation was relatively large and the intraindividual variation was relatively small, we conclude that such responses in the WBA could serve as a measure of a person's cytokine responsiveness. Furthermore, the induced production of the different cytokines showed a moderate-to-high correlation, and the responses to endotoxin and to glucan were also correlated (Tables 4 and 5). Thus, if one is a high responder for a particular cytokine, one is also a high responder for the other cytokines, and having a high response to LPS suggests a high response to curdlan will also occur.

Previous studies found considerable interperson differences in LPS-induced cytokine responses, with little intraindividual variation in the responses over a time period of 30 days up to six weeks (van der Linden *et al.*, 1998; Wilson *et al.*, 1991; Yaqoob *et al.*, 1999). However, in these studies only a limited number of LPS concentrations were tested. Moreover, inter- and intraindividual variations were not quantified (Yaqoob *et al.*, 1999) or when quantified, day-to-day variation was assessed in subjects other than those studied for between-subject variation (Wilson *et al.*, 1991). The present study and the studies by van der Linden *et al.* (1998) and Yaqoob *et al.* (1999) suggest that measuring cytokine responsiveness could be useful for determining a person's individual characteristic: 'being a high or low cytokine producer'. It should be noted that these studies, as well as our own study, were applied in normal healthy populations and a time interval of only up to four weeks was considered. So far, no data exist on reproducibility of induced cytokine responses in a WBA over a longer time period. In one study, data have been reported regarding the reproducibility of induced cytokine production by isolated peripheral blood monocytes over a five-year period (Schins and Borm, 1995). Schins and Borm studied a cohort of coal mine workers in which spontaneous, LPS and coal dust-induced TNFα production were significantly different after a five-year follow-up period. It thus remains to be seen whether induced cytokine responsiveness is a consistent characteristic over longer time than the one-tofour-week period studied.

Several factors may determine a person's cytokine responsiveness. First, it could be genetically

determined. Studies regarding the genetics of endotoxin-induced symptoms, lung function decrease or inflammatory processes have been performed in mice as well as in humans. Endotoxin susceptibility in mice and humans was, at least partially, genetically determined involving mutations in the Toll-like receptor-4 and Toll-like receptor-2 genes (Medzhitov *et al.*, 1997; Poltorak *et al.*, 1998; Schwartz *et al.*, 1994). In humans, genes encoding for the TNFα or the LPS-receptor CD14 have been suggested to be related to variation in endotoxin-induced symptoms (Baldini *et al.*, 1999; Mira *et al.*, 1999). To our knowledge, only one study has assessed underlying genetic factors of cytokine responsiveness in humans, and showed that endotoxin-induced TNFα and IL10 production in whole blood was strongly genetically determined (Westendorp *et al.*, 1997b). The same investigators also showed that low TNFα and high IL10 responsiveness was a predictor of familial susceptibility for fatal meningococcal disease, which emphasizes the clinical relevance of cytokine responsiveness (Westendorp *et al.*, 1997a).

Two possible other important factors that may qualitatively or quantitatively modulate inflammatory responses are external exposure to proinflammatory substances, or bacterial colonization during infectious diseases. The effects of high and prolonged airborne exposure to organic dust, including exposure to LPS and glucans, should be taken into account. It is well established that repeated exposure to endotoxins may result in reduced symptom rates and a smaller cross-shift decline in lung function (Castellan *et al.*, 1984). This could be due to a downregulation of cytokine responses. In a recent study, mice were tolerized to endotoxin by repeated injection and then exposed to swine barn dust by inhalation (personal cpmmunication P.S. Thorne). Animals tolerized to LPS and exposed to swine dust had lower responses in lung lavage neutrophilia and cytokine response, and significantly reduced responses in the WBA. This study demonstrated that the WBA is a useful biomarker of inhalation exposure and may be used as a measure of susceptibility to organic dust-induced lung inflammation. Besides downregulation, upregulation due to occupational dust exposure has also been suggested, e.g., by Schins and Borm (1995). They showed increased TNFα release from blood monocytes in (retired) coal mine workers compared with healthy controls, although within the group of mine workers TNFα release was not correlated with markers of exposure. No data are available on the effects of internal microbial exposure due to infectious diseases on the cytokine responsiveness.

Another objective of our study was to identify optimal conditions for the WBA as a test to distinguish low and high cytokine responders in population studies. We evaluated the inter- and intraindividual variance of the WBA at various concentrations of LPS and glucans, and assessed whether storage of blood prior to initiating the WBA affected variability and magnitude of the response. Interindividual variation differed with type and concentration of the stimulant, as shown previously by others (van der Linden *et al.*, 1998; Yaqoob *et al.*, 1999). We concluded that optimal stimulation concentrations were 6.25 or 12.5 ng/mL for endotoxin and 12500 or 25000 ng/mL for curdlan. This choice was based upon the ratio of inter- and intraindividual variances and the criterion that at these concentrations only a small minority of tests would show cytokine levels below the detection limit of the cytokine assay. Since individual dose–response curves (Figure 2) were more or less parallel but at different absolute levels, the observed interindividual variation was largely due to different maximum levels of cytokine responses, and less dependent on differences in the slope of the dose–response curves. Therefore, the chosen optimal concentrations may not be highly critical and similar results may be obtained at slightly higher or lower stimulant concentrations.

Ratios of inter- versus intraindividual variance were in general much higher for IL1β and IL6 than for IL8 and TNFα (Table 2), and correlations between responses induced with different concentrations of LPS and glucans were also generally higher for the first two cytokines (Table 4). Apparently, IL1β and IL6 production in our WBA was less subject to intraindividual changes in responsiveness and/or to measurement error, because of the larger differences between background and induced levels of IL1β and IL6. Thus, it might be cost effective to assess only induced IL1β and IL6 production to distinguish high and low responders in larger population studies when the costs of cytokine analyses may be critical. This

conclusion should, however, be interpreted with caution, since it may strongly depend on the specific protocol for the WBA, including the duration of the culture period after initiation of the WBA. A limited number of kinetic studies of the WBA revealed that TNFα and IL6 levels peaked at four to 10 hours after initiation of the culture followed by stabilization or a decrease of their concentration, whereas IL8 levels reached a plateau after six to 12 hours followed by another rise in concentration during continued stimulation (DeForge and Remick, 1991; Wilson *et al.*, 1991).

We showed a moderate to good correlation between cytokine responses in the WBA initiated two hours after venapuncture and the WBA initiated 26 hours after venapuncture. However, storage of blood decreased interindividual variance and increased intraindividual variance of induced cytokine production, and negatively impacted the reliability of the WBA as a method to determine individual cytokine responsiveness. So far, studies determining the effect of blood storage on WBA responses only studied the effect on the level of the response, showing a decrease in TNFα and/or IL10 production with storage of blood (Borm *et al.*, 1996; van der Linden *et al.*, 1998). Based upon these findings, it was also suggested to perform the WBA as soon as possible after venapuncture.

It should be noted that these conclusions regarding optimal WBA conditions – particularly those dealing with the choice of stimulant concentrations and of cytokines to be measured – apply to a sample of healthy volunteers. Optimal conditions may be different when applying WBA in exposed populations or other populations with possibly modified inflammatory responses, like patients with infectious disease or acute or chronic inflammatory disorders.

We conclude that endotoxin- or curdlan-induced cytokine production in the WBA can be used to estimate a person's cytokine responsiveness in population studies. The WBA works best if initiated within two hours after blood collection, and optimal stimulation concentrations are 6.25–12.5 ng/mL for endotoxin and 12 500–25 000 ng/mL for curdlan in healthy nonexposed controls. It still needs to be verified in humans whether this cytokine responsiveness is a reliable indicator of the individual's susceptibility to develop symptoms upon organic dust exposure. Experimental exposure chamber studies in humans have indicated that *in vitro* release of inflammatory mediators by blood monocytes or alveolar macrophages may be used as a measure of individual susceptibility for bioaerosol induced airway inflammation (Beijer *et al.*, 1995; Kline *et al.*, 1999). Further studies in occupationally exposed populations, before, during, and after bioaerosol exposure are required to assess whether cytokine responsiveness measured with the WBA is associated with the risk of work-related airway diseases.

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References

Baldini, M., Lohman, I.C., Halonen, M., Erickson, R.P., Holt, P.G. and Martinez, F.D. 1999: A polymorphism in the 5′ flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. *American Journal of Respiratory and Cell Molecular Biology* 20, 976–83.

Beijer, L., Jacobs, R.R., Boehlecke, B., Andersson, B. and Rylander, R. 1995: Monocyte responsiveness and a T-cell subtype predict the effects induced by cotton dust exposure. *American Journal of Respiratory and Critical Care Medicine* 152, 1215–20.

Björnsonn, E., Norbäck, D., Janson, C., Windström, J., Palmgren, U., Ström, G. and Boman, G. 1995: Asthmatic symptoms and indoor levels of micro-organisms and house dust mites. *Allergy* 25, 423–31.

Borm, P.A., Schins, R.F., Derhaag, J.M., Kant, I. and Jorna, T.M. 1996: Cross-shift changes in blood inflammatory markers occur in the absence of airway obstruction in workers exposed to grain dust. *Chest* 109, 1078–85.

Castellan, R.M., Olenchock, S.A., Hankinson, J.L., Millner, P.D., Cocke, J.B., Bragg, C.K., Perkins, H.H., Jr. and Jacobs, R.R. 1984: Acute bronchoconstriction induced by cotton dust: dose-related responses to endotoxin and other dust factors. *Annals of Internal Medicine* 101, 157–63.

DeForge, L.E. and Remick, D.G. 1991: Kinetics of TNF, IL-6, and IL-8 gene expression in LPS-stimulated human whole

blood. *Biochemical and Biophysical Research Communications* 174, 18–24.

Douwes, J., Doekes, G., Montijn, R., Heederik, D. and Brunekreef, B. 1996: Measurement of β(1→3)-glucans in the occupational and home environment with an inhibition enzyme immunoassay. *Applied and Environmental Microbiology* 62, 3176–82.

Douwes, J., Wouters, I., Dubbeld, H., van Zwieten, I., Steerenberg, P., Doekes, G. and Heederik, D. 2000a: Upper airway inflammation assessed by nasal lavage in compost workers: a relation with bio-aerosol exposure. *American Journal of Industrial Medicine* 37, 459–68.

Douwes, J., Zuidhof, A., Doekes, G., van der Zec, S.C., Wouters, I., Boezen, M.H. and Brunckreef, B. 2000b: (1→3)-beta-D-glucan and endotoxin in house dust and peak flow variability in children. *American Journal of Respiratory and Critical Care Medicine* 162, 1348–54.

Eduard, W., Douwes, J., Mehl, R., Heederik, D. and Melbostad, E. 2001: Short term exposure to airborne microbial agents during farm work: exposure-response relations with eye and respiratory symptoms. *Occupational and Environmental Medicine* 58, 113–18.

Fogelmark, B., Sjostrand, M. and Rylander, R. 1994: Pulmonary inflammation induced by repeated inhalations of β(1→3)–D-glucan and endotoxin. *International Journal of Experimental Pathology* 75, 85–90.

Husman, T. 1996: Health-effects of indoor-air microorganisms. *Scandinavian Journal of Work, Environment and Health* 22, 5–13.

Jacobs, R.R., Boehlecke, B., van Hage, H.M. and Rylander, R. 1993: Bronchial reactivity, atopy, and airway response to cotton dust. *American Review of Respiratory Diseases* 148, 19–24.

Kline, J.K., Cowden, D., Hunninghake, G.W., Schutte, B.C., Watt, J.L., Wohlford-Lenane, C.L., Powers, L.S., Jones, M.P. and Schwartz, D.A. 1999: Variable airway responsiveness to inhaled lipopolysaccharide. *American Journal of Respiratory and Critical Care Medicine* 160, 297–303.

Magnan, A.O., Mely, L.G., Camilla, C.A., Badier, M.M., Montero-Julian, F.A., Guillot, C.M., Casano, B.B., Prato, S.J., Fert, V., Bongrand, P. and Vervloet, D. 2000: Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma. *American Journal of Respiratory and Critical Care Medicine* 161, 1790–96.

Medzhitov, R., Preton-Hurlburt, P. and Janeway, C.A., Jr. 1997: A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388, 394–97.

Michel, O. 1997: Human challenge studies with endotoxins. *International Journal of Occupational and Environmental Health* 3, S18–S25.

Michel, O., Duchateau, J. and Sergysels, R. 1989: Effect of inhaled endotoxin on bronchial reactivity in asthmatics and normal subjects. *Journal of Applied Physiology* 66, 1059–64.

Michel, O., Ginnani, R., Duchateau, J., Vertongen, F., Le Bon, B. and Sergysels, R. 1991: Domestic endotoxin exposure and clinical severity of asthma. *Clinical and Experimental Allergy* 21, 441–48.

Michel, O., Ginnani, R., Le Bon, B., Content, J., Duchateau, J. and Sergysels, R. 1992: Inflammatory response to acute inhalation of endotoxin in asthmatic patients. *American Review of Respiratory Diseases* 146, 352–57.

Michel, O., Kips, J., Duchateau, J., Vertongen, F., Robert, L. and Coller, H. 1996: Severity of asthma is related to endotoxin in house dust. *American Journal of Respiratory and Critical Care Medicine* 154, 1641–46.

Mira, J.P., Cariou, A., Grall, F., Delclaux, C., Losser, M.R., Heshmati, F., Cheval, C., Monchi, M., Teboul, J.L., Riche, F., Leleu, G., Arbibe, L., Mignon, A., Delpeeh, M. and Dhainaut, J.F. 1999: Association of TNF2, a TNF-alpha promoter polymorphism, with septic shock susceptibility and mortality: a multicenter study. *Journal of the American Medical Association* 282, 561–68.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B. 1998: Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282, 2085–88.

Reed, C.E. and Milton, D.K. 2001: Endotoxin-stimulated innate immunitiy: a contributing factor for asthma. *The Journal of Allergy and Clinical Immunology* 108, 157–66.

Rylander, R. 1997: Investigations of the relationship between disease and airborne (1→3)-beta-D-glucan in buildings. *Mediators of Inflammation* 6, 275–77.

Rylander, R. and Jacobs, R.R. 1997: Endotoxins in the environment: a criteria document. *International Journal of Occupational and Environmental Health* 3, S1–S48.

Rylander, R., Persson, K., Goto, H, Yuasa, K. and Tanaku, S. 1992: Airborne β(1-3)-glucan may be related to symptoms in sick buildings. *Indoor Environment* 1, 263–67.

Sandstrom, T., Biermer, L. and Rylander, R. 1992: Lipopolysaccharide (LPS) inhalation in healthy subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid. *European Respiratory Journal* 5, 992–96.

Schins, R.P. and Borm, P.J. 1995: Epidemiological evaluation of release of monocyte TNF-alpha as an exposure and effect marker in pneumoconiosis: a five year follow up study of coal workers. *Occupational and Environmental Medicine* 52, 441–50.

Schwartz, D.A., Thorne, P.S., Jagielo, P.J., White, G.E., Bleuer, S.A. and Frees, K.L. 1994: Endotoxin responsiveness and grain dust-induced inflammation in the lower respiratory tract. *American Journal of Physiology – Lung Cellular and Molecular Physiology* 267, L609-L17.

Sigsgaard, T., Bonefeld-Jørgensen, E.C., Kjaergaard, S.K., Mamas, S. and Pedersen, O.F. 2000: Cytokine release from the nasal mucosa and whole blood after experimental exposures to organic dusts. *European Respiratory Journal* 16, 140–45.

Thorn, J., Beijer, L. and Rylander, R. 1998: Airways inflammation and glucan exposure among household waste

collectors. *American Journal of Industrial Medicine* 33, 463–70.

Ulmer, A.J. 1997: Biochemistry and cell biology of endotoxins. *International Journal of Occupational and Environmental Health* 3, S8–S17.

van der Linden, M.W., Huizinga, T.W., Stocken, D.J., Sturk, A. and Westendorp, R.G. 1998: Determination of tumour necrosis factor-alpha and interleukin-10 production in a whole blood stimulation system: assessment of laboratory error and individual variation. *Journal of Immunological Methods* 218, 63–71.

Wang, Z., Malmberg, P., Larsson, P., Larsson, B.M. and Larsson, K. 1996: Time course of interleukin-6 and tumor necrosis factor-alpha increase in serum following inhalation of swine dust. *American Journal of Respiratory and Critical Care Medicine* 153, 147–52.

Westendorp, R.G., Langermans, J.A., Huizinga, T.W., Elouali, A.H., Verweij, C.L., Boomsma, D.I. and Vandenbrouke, J.P. 1997a: Genetic influence on cytokine production and fatal meningococcal disease. *Lancet* 349, 170–73.

Westendorp, R.G., Langermans, J.A., Huizinga, T.W., Verweij, C.L. and Sturk, A. 1997b: Genetic influence on cytokine production in meningococcal disease. *Lancet* 349, 1912–13.

Wilson, B.M., Severn, A., Rapson, N.T., Chana, J. and Hopkins, P. 1991: A convenient human whole blood culture system for studying the regulation of tumour necrosis factor release by bacterial lipopolysaccharide. *Journal of Immunological Methods* 139, 233–40.

Yaqoob, P., Newsholme, E.A. and Calder, P.C. 1999: Comparison of cytokine production in cultures of whole human blood and purified mononuclear cells. *Cytokine* 11, 600–605.