Pyrogenic effect of respirable road dust particles

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Abstract. Because pyrogenic (fever-inducing) compounds on ambient particles may play an important role for particle toxicity, simple methods to measure pyrogens on particles are needed. Here we have used a modified in vitro pyrogen test (IPT) to study the release of interleukin 1β (IL-1β) in whole human blood exposed to respirable road-dust particles (RRDP). Road dusts were collected from the roadside at six different streets in three Swedish cities and particles with a diameter less than 10 µm (RRDP) were prepared by a water sedimentation procedure followed by lyophilisation. RRDP (200 µl of 1–10⁶ ng/ml) were mixed with 50 µl whole blood and incubated at 37 ºC overnight before IL-1β was analysed with chemiluminescence ELISA in 384-well plates. Endotoxin (lipopolysaccharide from Salmonella minnesota), zymosan B and Curdlan (β-1,3-glucan) were used as positive controls. All RRDP samples had a pyrogenic effect and the most active sample produced 1.6 times more IL-1β than the least active. This formation was of the same magnitude as in samples with 10 ng LPS/ml and was larger than that evoked by zymosan B and Curdlan (by mass basis). The method was sensitive enough to determine formation of IL-1β in mixtures with 10 ng RRDP/ml or 0.01 ng LPS/ml. The endotoxin inhibitor, polymyxin B (10 µg/ml), strongly reduced the RRDP-induced formation of IL-1β at 1 µg RRDP/ml (around 80% inhibition), but had only marginal or no effects at higher RRDP-concentrations (10 and 100 µg/ml). In summary, all RRDP tested had a clear pyrogen effect in this in vitro model. Endotoxin on the particles but also other factors contributed to the pyrogenic effect. As opposed to the limulus amebocyte lysate (LAL) assay (which measures endotoxin alone), IPT measures a broad range of pyrogens that may be present on particulate matter. The IPT method thus affords a simple, sensitive and quantitative determination of the total pyrogenic potential of ambient particles.

1. Introduction
It is now widely acknowledged that exposure to particulate air pollution is associated with respiratory and cardiovascular morbidity and mortality [1, 2]. For example, epidemiological studies have shown that exposure to fine particulate air pollution is associated with the incidence of cardiovascular disease and death among postmenopausal women [3]. Out of various environmental factors, ultrafine particles, PM₁₀ and carbon dioxide were significantly associated with out-of-hospital coronary deaths in Rome, Italy [4]. One important type of particulate air pollution is respirable road dust particles (RRDP). These are heterogeneous by nature and built up by materials that have their origin in mechanical wear processes in the tire-road interface or from vehicle break, combustion-generated material or from organic materials from plant and soil [5]. The frequent use of studded tires during wintertime in the Nordic countries results in an enhanced formation of wear-induced road particles but due to the wet climate there are generally low concentrations of airborne respirable particulate...
matter during winter. However, when the roads become dryer in March and April the level of RRDP in the air rises considerably and may often exceed 50 µg/m³ [6].

After inhalation and deposition in the lung, ambient particles can interact with a variety of lung cells and may also have systemic effects [7, 8, 9, 10]. These interactions eventually lead to a release of various cytokines with ensuing inflammatory reaction and tissue injury. There is presently much interest in the factors that may cause such a cytokine release. One possibility is the presence of surface-bound pyrogenic (fever-inducing) substances on the particles, such as bacterial endotoxin (lipopolysaccharide, LPS, from Gram-negative bacteria), lipoteichoic acid (from Gram-positive bacteria), peptidoglycan (from bacterial cell walls), fungal spores, and viral pathogens. Because the presence of pyrogens may determine particle toxicity, simple methods to measure pyrogens on particles are needed. The most commonly used method for measuring endotoxin is the *limulus* amebocyte lysate (LAL) assay [11]. This is a sensitive assay for endotoxin but does not detect other pyrogenic agents. A broader spectrum of pyrogens can be detected with the *in vitro* pyrogen test (IPT) which is a model where the sample is mixed with a small volume of human whole blood, incubated overnight, and the levels of the cytokine interleukin-1 beta (IL-1β) are determined with ELISA-technique [12]. This method has previously been successfully used to study air-borne pyrogens from samples collected on membrane filters [13] and immune-stimulating compounds on surfaces [14]. IPT has been proposed as an alternative to the animal-based LAL-test and the rabbit pyrogen test [15], which both are used to test pyrogens in pharmaceutical products but have several shortcomings. The IPT (which uses human cells) has also been validated against these two animal-based techniques and found to be sensitive, accurate and cost efficient [16].

In the present work, we have used the IPT to measure pyrogenic compounds on RRDP collected at six different locations in three Swedish cities during wintertime. The pyrogenic effect of the RRDP samples was also compared with that of lipopolysaccharide (LPS), zymosan B and β-glucan.

### 2. Materials and Methods

#### 2.1. Preparation of respirable road dust particles (RRDP)

Road surface-samples were collected from six different streets in three different Swedish cities during February 2007. All sites had similar surroundings with residential areas and greenery but with varying traffic intensity and mean annual concentrations of PM$_{10}$ (table 1). There were no factories, animal facilities or other similar potential sources of endotoxin in the vicinity of the sites. The crude samples were transferred into plastic bags and kept at 4 °C and used within one week. To prepare a fraction of respirable particles the material was mixed with Milli-Q water to a slurry in a polypropylene tube (1 kg road course-sample per litre of pyrogen free water). After a short agitation, 800 ml slurry was poured into a 1 l sterile glass flask (Schott) that was left in room temperature for 18 hours. The upper phase was then carefully decanted into a 2 l sterile round-bottom flask where it was frozen and lyophilized. The dry fraction of RRDP was then transferred to a pyrogen-free glass vial. A scanning electron microscope micrograph of a typical sample is shown in figure 1.
Table 1. Road dust samples used in the study.

| Sample | Street (city)a | Coordinatesb | PM$_{10}$ concentrationc,d (µg/m$^3$) | Daily traffic intensityd (x 10$^3$ cars per day) |
|--------|----------------|---------------|--------------------------------------|-----------------------------------------------|
| 1      | N. Tanneforsvägen (Li) | 58° 24' 08.86 N, 15° 38' 54.54 E | 23.2                                | 15.7                                         |
| 2      | Drottninggatan (Li) | 58° 24' 29.81 N, 15° 37' 15.86 E | 25.2                                | 12.2                                         |
| 3      | Östra promenaden (No) | 58° 35' 28.78 N, 16° 11' 51.08 E | 28.4                                | 24.7                                         |
| 4      | G. Tanneforsvägen (Li) | 58° 24' 29.31 N, 15° 38' 48.47 E | 23.7                                | 15.3                                         |
| 5      | Ålidbacken (Um) | 63° 48' 50.10 N, 20° 18' 18.07 E | No data                            | 7.7                                          |
| 6      | Söderleden (Li) | 58° 23' 41.48 N, 15° 38' 51.22 E | 24.4                                | 10.5                                         |

a (Li) = Linköping, (No) = Norrköping, (Um) = Umeå
b Coordinates from Google Earth
c Mean annual
d Information given by respective municipality.

Figure 1. Scanning electron micrograph of one of the RRDP samples examined for pyrogenic effect (no. 6).
2.2. In vitro Pyrogen Test (IPT)

Blood was drawn from the forearm of healthy volunteers (personal in the laboratory) into heparinised tubes (BD Vacutainer, Plymouth, UK) and kept at room temperature until use within one hour. Heparin tubes could sometimes be contaminated by endotoxins [17]. Although this was not a problem in the present investigation, it is necessary that a batch of endotoxin-free tubes is tried out and used. Samples of RRDP (1 – 10⁶ ng/ml), LPS (10⁻⁴ - 10⁻² ng/ml from Salmonella minnesota, Sigma L-6261), zymosan B (10⁻¹ to 10⁷ ng/ml, Sigma Z-4250), or Curdlan (10⁻¹ to 10⁵ ng/ml, β-1-3-glucan from Wako Pure Chemical Industries Ltd) were diluted in 0.9 % NaCl solution (Ecotainer® 0.9 % NaCl, B. Braun Melsungen, Germany). To examine if particles per se caused formation of IL-1β, experiments were performed in which well-characterised polystyrene particles (1 µg/ml in NaCl-solution) were incubated with whole blood as described above (uniform microspheres, PA02N, polystyrene particles with a mean diameter of 0.15 µm from Bang Laboratories Inc., Fishers, IN, USA). In some experiments the endotoxin inhibitor, polymyxin B, was used (1-10 µg/ml, Sigma P0972). RRDP (1, 10 or 100 µg/ml or LPS (1 ng/ml) were then treated with polymyxin B for 1 hour and incubated together with whole blood as described below. Each sample (200 µl) was mixed with 50 µl whole blood in wells of a 96-well plate (Polypropylene, Round bottom plate, Nunc A/S, Roskilde, Denmark; no: 267245) and incubated covered with a lid for 18 hours in a cell culture chamber (37 °C and 5% CO₂). Samples were then transferred to 1.5 ml Eppendorf tubes and centrifuged at 10000 x g for 3 minutes before 40 µl of the supernatant was pipetted in duplicate into anti IL-1β precoated wells on a 384-well plate (Maxisorp, Nalge Nunc International, Rochester, USA; no: 460372).

The ELISA was performed with antibodies and standards from Diaclone (Human IL-1β Eli-pair, Diaclone, Besancon, France; nr: 851-610-010). The protocol from the manufacturer was followed with the exception that 384-well polystyrene plates were used instead of clear 96-well plates and chemiluminescence instead of absorbance to quantify the signal. These modifications made it possible to use less sample and reagents and also resulted in an improved sensitivity and a larger span in which the standard curve was linear. In brief, 40 µl coating antibody in 0.05M carbonate buffer pH 9.6 was added to each well and the plate was covered with adhesive tape and incubated in a refrigerator over night. After two washings with 100 µl washing solution (phosphate buffered saline with 0.05 % Tween 20), 100 µl of 5 % bovine serum albumin (BSA) in phosphate buffered saline was added and the plate was incubated for 2 hour at room temperature (RT) under agitation (200 r/min). After two additional washings with 100 µl washing solution, 40 µl of sample, standard, or blank and 40 µl of biotinylated detection antibody (diluted in PBS with 1 % BSA) were added to wells in duplicate and the plate were incubated for 3 h at RT under agitation (200 r/min). In each washing step the plate was tapped firmly on sheets of paper. After three washings with 100 µl washing solution/well, 40 µl Streptavidin-HRP (in PBS with 0.1 % Tween 20 and 1 % BSA) was added to each well. The plate was incubated for 20 min in RT and under agitation (200 r/min) before each well was washed three times with 100 µl washing solution and two times with 100 µl water. Finally 40 µl ECL plus (diluted 1+3 with MilliQ-water) was added to each well (Western Blotting Detection System, no; RPN 2132, GE Healthcare, Little Chalfont, UK). The plate was then incubated for 15 min in darkness before the chemiluminescence was read in a plate reader (Lumistar, BMG labtechnologies, Offenburg, Germany). The coefficient of variation (CV) when blood from one person was exposed for 1 ng LPS /ml and incubated in nine wells and then analysed as separate samples in duplicate was 7 % (n=9). When one blood sample was incubated with 1 ng LPS /ml in 12 wells and then pooled and analysed as 12 different samples in duplicate the CV was 6 % (n=12).
3. Results
The pyrogenic effect of RRDP (sample no. 6), LPS, zymosan B and Curdlan is shown in figure 2. The lowest concentration of RRDP that caused a detectable IL-1\(\beta\) formation was 10 ng RRDP/ml. At higher RRDP concentrations, the IL-1\(\beta\) formation increased in a dose-dependent way up to 1 mg RRDP/ml. On a mass basis, the RRDP sample was more pyrogenic than zymosan B and Curdlan but less pyrogenic than LPS. An elevated IL-1\(\beta\) formation was determined at LPS concentrations as low as 0.01 ng/ml, and the levels were increased in mixtures up to 1 ng LPS/ml where after the dose-response curve was flattened out. The dose-response curve for LPS did therefore appeared different from that of RRDP, zymosan B and Curdlan (figure 2).

To examine if pure particulate matter \textit{per se} caused formation of IL-1\(\beta\), whole blood was stimulated with 0.15 \(\mu\)m polystyrene particles. The formation of IL-1\(\beta\) in samples with polystyrene particles (1 \(\mu\)g/ml) was found to be around 40 pg IL-1\(\beta\)/ml which was just over the background and only 3 % of that caused by the same concentration of RRDP.

As shown in figure 3, the LPS inhibitor, polymyxin B, largely abolished the pyrogenic effect of RRDP at low concentrations (1 \(\mu\)g/ml). Here, the IL-1\(\beta\) level in samples with 10 \(\mu\)g polymyxin B/ml was reduced by 82 % compared to that in samples without polymyxin B. Higher concentrations of polymyxin B did not reduce the IL-1\(\beta\) formation any further (not shown in the figure). On the other hand, at higher concentrations of RRDP (10 \(\mu\)g/ml), polymyxin B (10 \(\mu\)g/ml) reduced the IL-1\(\beta\) formation by only 37% and in samples containing 100 \(\mu\)g RRDP/ml it had no influence on the IL-1\(\beta\) formation (figure 3). The formation of IL-1\(\beta\) by LPS (1 ng/ml) was completely abolished by all tested concentrations of polymyxin B.
Figure 3. The influence of the endotoxin inhibitor, polymyxin B, on the formation of IL-1β in whole blood exposed to different concentrations of RRDP (sample no. 6). Results are presented as mean ± SEM. Further details are given in Materials & Methods.

Figure 4 shows the IL-1β formation from the six RRDP samples collected at different locations. All the samples had a pyrogenic effect and the most active (no. 6) produced 1.6 times more IL-1β than the least active. Samples 3, 4, 5 and 6 caused significantly more IL-1β than samples 1 and 2. The presence of polymyxin B (10 µg/ml) reduced the IL-1β formation substantially (81-90%) in all the RRDP samples. No correlation was found between the formation of IL-1β and the mean annual PM$_{10}$ or daily traffic intensity at the sample sites (table). The formation of IL-1β by LPS (10 ng/ml) was reduced by 94% in the presence of polymyxin B.

Figure 4. Formation of IL-1β in diluted whole blood incubated for 18 h with six different RRDP samples. RRDP (1 µg/ml) or LPS were incubated without (filled bars) or with (open bars) the endotoxin inhibitor, polymyxin B (10 µg/ml). Results are presented as mean ± SEM. Further details are given in Materials & Methods. *** = p < 0.001 compared with RRDP sample number 1.
4. Discussion

In this modified IPT, we used only 50 µl whole blood per sample and measured IL-1β with chemiluminescence ELISA in 384-well plates. This enabled a sensitive analysis of a large number of samples simultaneously. Increased levels of IL-1β were detected at RRDP concentrations as low as 10 µg/ml, which was three orders of magnitude lower than that of LPS but three orders of magnitude higher than that of zymosan and Curdlan, respectively (figure 2). The dose-response curve for RRDP was different from that of LPS: with increasing LPS concentrations, the IL-1β-formation increased up to a concentration of 1 ng LPS/ml and was then flattened out while no such effect could be seen with increasing concentrations of RRDP (up to 1 mg/ml). The presence of the endotoxin inhibitor, polymyxin B, resulted in a dose-related reduction in IL-1β formation in mixtures that contained 1 µg RRDP/ml (figure 3). It is likely, therefore, that endotoxin played a significant role for the in vitro pyrogenic effect of the RRDP.

Endotoxin can bind to particle surfaces [18, 19] and the levels in urban ambient PM2.5 particulate samples have been found to be higher in spring and summer than in winter [20]. A rather big week to week fluctuation in the endotoxin concentration has been found in particulate air samples collected from the same place, while a good correlation has been found between endotoxin levels on particles collected at the same time in two cities that lay 80 km apart [21] or at 40 different sites in and around Munich [22]. Previous studies have indicated an important role of endotoxin in the formation of cytokines (IL-6 and TNF-α) by cultured macrophages exposed to ambient particles [6]. Moreover, particles collected in the spring was found to cause more cytokine formation than particles collected in the winter, and it was suggested that this difference was mainly due to differences in endotoxin content [6]. Aside from endotoxin, there are a number of other cytokine-inducing compounds in bacteria [23], and β-glucans [24], fungal spores [25] and viral pathogens [26] can also act as pyrogens. It cannot be precluded that such non-bacterial pyrogens could contribute to the pyrogenic effects of RRDP as seen in the present study.

A number of epidemiological studies have shown that individuals exposed to high levels of particulate air pollution have an increased risk of acute cardiovascular events [27]. It is possible that this increased risk is the result of a particle-evoked inflammation in the lung with systemic cardiovascular involvement, or due to an extrapulmonary translocation of particles into the circulation with subsequent pro-atherosclerotic effects [28]. For the moment, it is not clear to what extent ambient ultrafine particles can be translocated into the bloodstream in humans. In rats, radiolabeled polystyrene particles with a diameter of 54.4 nm that had been instilled in the lung were found to enter into the circulation. The translocation was rather small, however, but increased significantly in animals that had been pre-treated with LPS, implicating that pulmonary inflammation enhances the extrapulmonary translocation of nanoparticles [29]. Nemmar and co-workers have found that carbon nanoparticles labelled with technetium-99 translocate from the lung into the circulation in humans [30] but this finding has not been confirmed by other investigators [31, 9, 32]. Thus, it is presently under debate if a systemic translocation of ultrafine carbon particles takes place in healthy human subjects and more research has to be conducted in order to elucidate this [32]. Yet, as mentioned above, animal studies have shown that pretreatment with LPS enhances the translocation of polystyrene nanoparticles [29] and it is possible that the presence of LPS (or other pyrogens) on RRDP might enhance their transfer into the circulation in humans. This is yet another reason why simple methods to measure pyrogens on RRDP might be useful. It should also be pointed out that endotoxin per se has great potential as a proinflammatory mediator of atherosclerosis [34] and that RRDP might act as a vector for the transfer of endotoxin into the circulation.

In summary, our results show that IPT can be used to measure the pyrogenic potential of ambient particulate matter. All RRDP tested had a clear pyrogen effect in this in vitro model. Endotoxin on the particles but also other factors contributed to the pyrogenic effect. As opposed to the limulus amebocyte lysate (LAL) assay (which measures endotoxin alone), IPT measures a broad range of pyrogens that may be present on particulate matter. The IPT method thus affords a simple, sensitive and quantitative determination of the total pyrogenic potential of ambient particles.
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