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Original article

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Chemical and in vitro toxicologic characterization of wintertime and springtime urban-air particles with an aerodynamic diameter below 10 µm in Helsinki

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Objectives The chemical composition and toxicity of wintertime urban-air particulate matter with an aerodynamic diameter of <10 µm (PM_{10}), derived mostly from long-range transport and local combustion sources, were compared with those of springtime PM_{10} derived mostly from the resuspension of road dust.

Methods Water-soluble ions and elements and polycyclic aromatic hydrocarbons (PAH) were analyzed from seasonally pooled PM_{10} samples collected at a busy traffic site in Helsinki in 1999. These PM_{10} samples were also tested for cytotoxicity [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide test] and the production of proinflammatory cytokines [tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6)] and nitric oxide (NO) in the mouse macrophage cell line RAW 264.7. Their oxidative capacity and the associated DNA (deoxyribonucleic acid) damage were investigated by electron paramagnetic resonance and the formation of 8-hydroxy-2´-deoxyguanosine (8-OH-DG) in isolated calf thymus DNA, respectively.

Results The late wintertime and springtime PM_{10} had similar compositions of water-soluble ions and elements, but the winter PM_{10} had a higher content of PAH. The spring PM_{10} was a much more potent inducer of TNF-α and IL-6 production than the winter PM_{10} was, but there were no consistent differences in cytotoxic potency. In contrast, the winter PM_{10} was a significantly more potent inducer of NO production and 8-OH-DG formation. The large cytokine responses to the spring PM_{10} were caused by its insoluble fraction and largely inhibited by the endotoxin antagonist polymyxin B. The transition metal chelator deferoxamine did not modify the proinflammatory or cytotoxic responses to the PM_{10} samples.

Conclusions The toxicity profile of urban-air PM_{10} changed with season in a subarctic climate. Particulate-bound endotoxin from soil gram-negative bacteria is suggested as a highly proinflammatory constituent of springtime resuspended road dust.

Key terms chemical composition, cytokines, high-volume particulate sampling, inflammation, metals, mouse macrophage, nitric oxide, oxidative deoxyribonucleic acid damage, polycyclic aromatic hydrocarbons, resuspension.

Mass concentrations of urban-air particulate matter with an aerodynamic diameter of <10 µm (PM_{10}) have been associated with a broad spectrum of adverse health outcomes in susceptible population groups such as children, asthmatics, and elderly persons with chronic cardiorespiratory diseases (1, 2). Even the generally low PM_{10} concentrations in Finnish cities have been significantly associated with a variety of health outcomes in epidemiologic studies. In Helsinki, increased respiratory mortality has been found (3). In Kuopio, declines in morning peak expiratory flow (PEF) among asthmatic children (4) have been reported, as have an increased risk

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of cough (5) and reduced biweekly forced vital capacity (FVC) (6) among schoolchildren with chronic respiratory symptoms.

Springtime resuspended dust episodes are an exception to the generally low PM_{10} concentrations in Finnish cities until the sand spread on roads during winter, and other road dust, is removed in cleaning campaigns (7, 8). During dry weather, wind and turbulence caused by busy traffic increase the resuspension of road dust, and the 24-hour average PM_{10} concentration frequently exceeds the European Union limit (50 $\mu g/m^3$) and the national guideline value (70 $\mu g/m^3$). However, there is controversy about the health significance of these PM_{10} peaks, because, in some Finnish epidemiologic studies, health outcomes have been associated not only with the PM_{10} concentration, but also as consistently or even more consistently with the black smoke concentration (4, 6). This finding suggests the importance of combustion sources. Moreover, in some other studies, the inclusion of springtime resuspended dust episodes in the statistical analyses has even led to paradoxical positive associations between PM_{10} concentration and lung function in adults with asthma (9) or schoolchildren with chronic respiratory symptoms (10).

In a previous toxicologic study (11), we have shown that a PM_{10} sample collected during a strong springtime episode of resuspended dust in Helsinki was a much more potent inducer of proinflammatory cytokine production than a PM_{10} sample collected at the same site during the wintertime. The higher potency of the springtime PM_{10} sample was hypothesized, on the basis of previous studies, to be due to a higher content of bioavailable iron (12, 13), surface complexed iron on silica dust (14), or soluble or insoluble gram-negative bacterial lipopolysaccharide (LPS) endotoxin (15, 16).

The purpose of our present study was to compare wintertime and springtime PM_{10} samples in a systematic manner. We pooled samples from the two seasons of the same PM sampling campaign as in our previous toxicologic study (11) using predetermined selection criteria in order to get larger masses of PM_{10} from the winter period with low resuspension of road dust and the spring period with medium and high resuspension of road dust. The specific objectives of the study were to compare the winter and spring PM_{10} samples in Helsinki with regard to (i) the composition of water-soluble ions and elements and polycyclic aromatic hydrocarbons (PAH), (ii) proinflammatory, cytotoxic and oxidative potency, (iii) proinflammatory and cytotoxic activity associated with the water-soluble and insoluble PM_{10} fractions, and (iv) modification of the PM_{10}-induced proinflammatory and cytotoxic effects by an endotoxin antagonist (polymyxin B) and transition metal chelator (deferoxamine). The proinflammatory and cytotoxic responses were investigated in the mouse macrophage cell line RAW264.7, while the oxidative effect was tested in isolated calf thymus DNA (deoxyribonucleic acid).

Material and methods

Sampling and chemical characterization of PM_{10} in ambient air

Sampling site and equipment. The sampling campaign was done at a relatively busy traffic site in Vallila, 2.8 kilometers northeast of downtown Helsinki, Finland, between 5 March and 17 May 1999. The high-volume, low cutoff inertial impactor (HVLI) apparatus consisted of a standard PM_{10} inlet (Andersen G1200, Village of Cleves, OH, USA), a slit impactor, and a blower (Y-Laite, Lahti, Finland). The HVLI slit impactor was developed by the Environmental Science and Engineering Program of the Harvard University, Boston, MA, USA (17). The 280-mm-long slit operated on an air flow of 1100 l/minute and a pressure drop of 250 mbar, and it had a lower PM size cut-off at approximately 0.12 µm. The intake of sample air was placed at a height of 3.5 meters and a distance of 15 meters from the nearest street, where the average daily traffic was about 13 000 vehicles.

Sampling procedure and PM extraction. Ambient air PM_{10} was collected in 2- to 7-day periods on high-capacity polyurethane foam (PUF) (Merryweather Foam, Barbarton, OH, USA), which was cut into a millimeter size of 320 × 6.4 × 6.4. The PUF strips were cleaned before use in four successive baths with 15-minute sonications, two in water and two in ethanol. The collected PM_{10} samples were extracted from the PUF substrate into pure methanol by sonication for 60 minutes. The suspensions formed from the selected samples were pooled into three separate PM_{10} categories (winter, spring I, spring II). Each of these three pooled suspensions was divided into several tubes for chemical analyses and toxicity testing, and methanol was subsequently evaporated in vacuum at room temperature.

PM_{10} sample pooling. The original PM_{10} samples were pooled into the three categories in order to get sufficiently large masses of the same PM material for both chemical analyses and toxicologic tests. As shown in table 1, the selection was based on the ambient air $PM_{2.5}$:PM_{10} concentration ratio during each sampling period; this procedure was motivated by a rationale based on the recent source apportionment studies on coarse particles ($PM_{10-2.5}$, aerodynamic diameter between 10 and 2.5 µm) and fine particles ($PM_{2.5}$, aerodynamic diameter <2.5 µm) in Helsinki (18, 19): (i) the winter PM_{10} concentration consisting mostly of a $PM_{2.5}$ subfraction derived

Table 1. Criteria for pooling the urban-air PM_{10} samples, collected in 2- to 7-day sampling periods, into three categories on the basis of the ambient-air $PM_{2.5}$:PM_{10} concentration ratio (continuous air quality monitoring data) during each sampling period. (PM_{10} and $PM_{2.5}$ = particles with aerodynamic diameters of <10 µm and <2.5 µm, respectively)

PM category	$PM_{2.5}$:PM_{10} ratio		Contribution of resuspended PM
	Mean	Range	
Winter PM_{10}	0.77	0.73–0.80	Low resuspension
Spring I PM_{10}	0.55	0.47–0.66	Medium resuspension
Spring II PM_{10}	0.36	0.28–0.41	High resuspension

from long-range transport and local combustion sources plus, to a small extent, $PM_{10–2.5}$ from resuspended road dust (three samples with a high $PM_{2.5}$:PM_{10} ratio), (ii) the spring I PM_{10} concentrations consisting of $PM_{2.5}$ derived from its main sources plus, to a moderate extent, $PM_{10–2.5}$ from resuspended road dust (four samples with a medium $PM_{2.5}$:PM_{10} ratio), and (iii) the spring II PM_{10} concentrations, consisting of $PM_{2.5}$ derived from its main sources plus, to a large extent, $PM_{10–2.5}$ from resuspended road dust (five samples with a low $PM_{2.5}$:PM_{10} ratio).

Inorganic analyses. Water-soluble ions were extracted from the dry pooled PM_{10} samples with deionized water (Millipore Alpha-Q) and analyzed by ion chromatography (IC) (DX 500, Dionex Corporation, Sunnyvale, USA). Correspondingly, water-soluble elements were extracted with 0.08 M nitric acid (stabilizes the extraction procedure) and analyzed by inductively coupled plasma mass spectrometry (ICP/MS) (Sciex Elan 6000, Perkin-Elmer Corporation, Norwalk, USA). Both methods have been described in more detail by Pakkanen et al (18).

Polycyclic aromatic hydrocarbon analysis. The soxhlet extractions of PAH from the dry pooled PM_{10} samples were made with dichloromethane. A total of 31 PAH was subsequently detected using a gas chromatograph–mass spectrometer single-ion monitoring (GCMS-SIM) technique [Hewlitt Packard 5890 GC with a HP5970B series mass selective detector (Agilent Technologies, Germany)] (20). The sum of all the detected PAH and that of the genotoxic PAH were calculated, the latter being defined according to a classification of the International Programme on Chemical Safety (IPCS) of the World Health Organization (21).

Routine air quality monitoring. Meteorological parameters (temperature, relative humidity, rain time, wind direction and speed) were monitored at a Kallio site that was 1.7 km southwest of the PM sampling site in Vallila. A series of air quality parameters was monitored at the sampling site as a part of the monitoring network of the Helsinki Metropolitan Area Council (YTV). The continuous measurement of nitrogen oxides (NO, NO_2) was based on chemiluminescence, sulfur dioxide (SO_2) on ultraviolet fluorescence, and carbon monoxide (CO) on infrared absorption. The ambient air PM pollution was characterized by continuous monitoring of $PM_{2.5}$ and PM_{10} concentrations with the beta attenuation method (Eberline FH 62 I-R, Erlangen, Germany).

Toxicologic tests for proinflammatory activity and cytotoxicity

Chemicals. A mixture of RPMI (Roswell Park Memorial Institute) medium 1640, L-glutamine and penicillin-streptomycin (PNS) antibiotic and also fetal bovine serum (FBS, North America) were obtained from Gibco Laboratory (Paisley, United Kingdom). Sulfanilamide, naphthylethylenediamine dihydro-chloride, 5-bromo-4-chloro-3-idolylphosphate/nitrobluetetra-zolium (BCIP/NBT), gram-negative bacterial LPS (from *Escherichia coli* serotype 0111:B4), polymyxin B sulfate (LPS antagonist), deferoxamine-mesylate (metal chelator), and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) came from Sigma (St Louis, MO, USA). The rat anti-mouse tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) monoclonal captured antibodies were obtained from Pharmingen (San Diego, CA, USA).

Macrophage cell line. The mouse macrophage cell line RAW264.7 was obtained from American Type Tissue Collection (Rockville, MD, USA) and grown on six well plates at 37°C and 5% carbon dioxide, containing RPMI medium supplemented with 10% heat-inactivated FBS, 1% L-glutamine, and a 1% PNS antibiotic mixture (ie, complete RPMI). In the in vitro experiments, the RAW 264.7 cells were diluted to 10^6 cells/ml and dispensed to six well plates with 2 ml/well. The cells were allowed to adhere, and the nonadherent cells were washed away with complete RPMI medium. Fresh medium containing PM_{10} or corresponding virtual doses of the blank was added to the cells.

In vitro exposures of macrophages to PM_{10}. The pooled PM_{10} samples were prepared daily for each experiment by suspension in RPMI medium. The suspension was thoroughly mixed in an ultrasonic water bath (30 minutes) before being applied to the cells. The macrophages were exposed for 24 hours to five mass doses (15, 50, 150, 500 and 1000 µg per milliliter of RPMI in the well) prepared from each of the three pooled PM_{10} samples. After incubation for 24 hours (37°C, 5% carbon dioxide), the cell culture media were collected, centrifuged, and analyzed for nitrite (NO production), and the remaining culture media were stored at –80°C for later analyses of cytokines. Subsequently, the viability of exposed cells was determined as described later in this article.

So that the effects of the water-soluble PM fraction could be separated from those of the insoluble

PM fraction, the PM_{10} samples were thoroughly mixed with RPMI medium in an ultrasonic water bath (30 minutes), and the suspensions were centrifuged at 10 000 revolutions/minute for 10 minutes. Thereafter, the supernatants were removed, and the washed pellets were resuspended in RPMI medium. The RAW 264.7 macrophages were separately exposed to the RPMI-containing PM supernatants and the RPMI-resuspended insoluble PM pellets corresponding to total PM_{10} mass doses of 150 and 500 μg/ml of RPMI in a well. In order to assess the contribution of LPS-structured endotoxins from soil gram-negative bacteria or that of chelatable metals (eg, iron, copper) to the cellular responses, we incubated the PM_{10} mass doses of 150 and 500 μg/ml overnight at 37°C and 5% carbon dioxide with either polymyxin B sulfate (20 μg/ml) (LPS antagonist) or deferoxamine-mesylate (0.1 mM) (metal chelator). All the experiments were repeated at least four times in duplicate at each dose level.

We used LPS (2.5 ng/ml) from *Escherichia coli* as a positive control on every experiment day in order to check the overall responsiveness of the RAW 264.7 macrophages. As this dose increased the production of proinflammatory cytokines with comparable efficacy to the largest mass doses prepared from the PM_{10} samples, it was used also for screening an effective dose of polymyxin B to block endogenous LPS.

Biochemical markers of inflammation. In order to assess the proinflammatory activation of macrophages, the productions of TNF-α and IL-6 were analyzed by a "sandwich-type" enzyme immunoassay (ELISA) using a commercial ELISA kit. The NO production was analyzed spectrophotometrically as the stable metabolite nitrite according to the Griess method (22). More-detailed descriptions of the analytical procedures have been published by Hälinen et al (23).

Cell viability. The viability of cultured macrophages after incubation with PM_{10} or blank solutions was assessed by using the spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test to detect functioning mitochondria (24). In some cell experiments, the portion of living macrophages was also calculated in a hemocytometer after staining with Trypan Blue solution.

Oxidative capacity and DNA damage. The oxidant activity and acellular oxidative DNA damage by PM_{10} were measured as previously described by Shi et al (25, 26). Hydrogen peroxide was added to the PM suspensions (200 μg/ml), and the production of reactive hydroxyl (OH) radicals was detected by a specific spin-trap (DMPO) and electron paramagnetic resonance (EPR). The formation of the ·OH-specific DNA lesion, 8-hydroxy-2´-deoxyguanosine (8-OH-DG), was investigated in isolated calf thymus DNA by using an immunodotblot assay. Freshly prepared suspensions of PM_{10} (125, 500 and 2000 μg/ml) were incubated for 90 minutes at 37°C with 50 μg of calf thymus DNA dissolved in tris-hydrochloric acid (10 mM, pH 8.0) and hydrogen peroxide (1 mM). On each experiment day, DNA incubated without PM_{10} and hydrogen peroxide, as well as DNA incubated with 0.1 mM ferrous sulfate and 1 mM hydrogen peroxide, were included as negative and positive controls, respectively. Immunodot blots prepared according to the standard procedure were analyzed by computer-assisted densitometry scanning (Gel Doc 2000, BioRad, Milan, Italy) and expressed relative to the density of the negative controls.

Quality assurance and quality control of the PM_{10} samples

A blank sample was made for the chemical analyses and toxicologic tests by pooling methanol extracts from two original field blanks (ie, clean PUF strips fitted into HVLI without airflow). The pooled blank sample was used as an internal control for PM_{10} sampling and sample handling procedures in the same way as the actual pooled PM_{10} samples. Its chemical constituents and toxic potency in the test systems were negligible.

Statistical analysis

A one-way analysis of variance (ANOVA) and Fisher's test were used in the analysis of the dose relationships in toxic responses and the differences between the three pooled PM_{10} samples. The same tests were used also in the analysis of differences in air quality and meteorology between the corresponding PM_{10} sampling periods. In all the statistical analyses, P-values of <0.05 were regarded as significant.

Results

Ambient-air quality and PM_{10} constituents

Routine air quality data. The air quality during the three PM_{10} sampling periods is summarized in table 2. The winter period was characterized by a low temperature, high relative humidity, and a high $PM_{2.5}$:PM_{10} ratio. In contrast, the spring II period had the lowest relative humidity, rain time, and $PM_{2.5}$:PM_{10} ratio. The levels of all the pollutants originating mainly from combustion sources (SO_2, NO_2, CO, $PM_{2.5}$) were lowest in the spring II period, whereas the PM_{10} concentrations in this period and the winter period were similar.

Water-soluble inorganic constituents of PM_{10}. All three pooled PM_{10} samples showed similar contents of water-soluble ions and elements (table 3). There were only a

Table 2. Hourly measured ambient-air quality at the sampling site and meteorological parameters during three study periods. (PM_{10} and $PM_{2.5}$ = particles with aerodynamic diameters of <10 µm and <2.5 µm, respectively)

	Winter		Spring I		Spring II	
	Mean	SD	Mean	SD	Mean	SD
Sulfur dioxide (µg/m³)	5.1	1.8	4.2	1.2	2.9	1.7
Nitrogen dioxide (µg/m³)	30.8	7.6	33.6	6.9	24.5	6.7
Carbon monoxide (mg/m³)	0.4	0.1	0.4	0.1	0.3[b]	0.1
Ozone (µg/m³)	61.3	7.3	48.8	14.7	58.3	7.0
PM_{10} (µg/m³)	18.6	10.1	28.0	5.5	20.3	2.4
$PM_{2.5}$ (µg/m³)	14.2	7.8	15.7	4.7	7.3[b]	1.2
Temperature (°C)	–0.6	2.2	4.3[a]	1.5	4.3[a]	1.5
Relative humidity (%)	91	4	87	5	65[a,b]	11
Rain (% of time)	12	6	18	12	2[b]	3

[a] Significantly different from the winter period (P<0.05).
[b] Significantly different from the spring I period (P<0.05).

Table 3. Water-soluble ionic and elemental constituents in the three pooled PM_{10} samples. (winter = low resuspension, spring I = medium resuspension, spring II = high resuspension)

Ion or element	Winter (µg/mg PM_{10})	Spring I (µg/mg PM_{10})	Spring II (µg/mg PM_{10})
Chlorine ion	4.6	10.6	19.1
Nitrate ion	153.2	141.7	99.4
Sulfate ion	161.3	111.7	194.8
Sodium ion	14.9	11.6	25.0
Ammonium ion	99.5	83.8	92.4
Potassium ion	1.9	2.3	1.7
Magnesium ion	2.2	1.5	3.8
Calcium ion	2.3	3.0	2.5
Aluminum	1.85	1.53	1.82
Cadmium	0.03	0.00	0.00
Chromium	0.03	0.02	0.03
Copper	0.23	0.19	0.28
Manganese	0.28	0.19	0.27
Nickel	0.11	0.09	0.11
Lead	0.39	0.18	0.12
Vanadium	0.34	0.36	0.32
Arsenic	0.07	0.02	0.02
Iron	4.31	4.66	5.83
Zinc	1.28	0.55	0.77

few clear trends associated with the change in season from winter to spring, such as increasing chlorine ion and sodium ion contents, and decreasing nitrate ion, lead, zinc, and arsenic contents.

Polycyclic aromatic hydrocarbon contents of PM_{10}. The concentrations of 31 measured PAH in the three pooled PM_{10} samples are shown in table 4. There was a clear trend towards a decreasing concentration of most PAH from winter to spring. The total PAH concentrations in the spring I PM_{10} samples and spring II PM_{10} samples were 21% and 33% lower than that in the winter PM_{10} sample. Correspondingly, the sums of genotoxic PAH in the spring I PM_{10} and spring II PM_{10} were 23% and 34% lower than that in the winter PM_{10}. The benzo[a]pyrene contents were similar in all three pooled PM_{10} samples.

Table 4. Polycyclic aromatic hydrocarbon (PAH) contents in the three pooled PM_{10} samples. (winter = low resuspension, spring I = medium resuspension, spring II = high resuspension)

Polycyclic aromatic hydrocarbon	Winter (ng/mg PM_{10})	Spring I (ng/mg PM_{10})	Spring II (ng/mg PM_{10})
Naphthalene	1.5	1.4	1.6
2-Methylnaphthalene	0.7	0.8	0.9
1-Methylnaphthalene	0.3	0.4	0.3
Biphenyl	0.0	0.6	0.0
Acenaphthene	0.0	0.0	0.0
Fluorene	0.7	3.8	0.4
3-Methylbiphenyl	1.3	0.8	1.7
Dibenzofurane	1.9	2.3	4.6
Dibenzothiophene	0.0	0.2	0.2
Phenanthrene	15.3	7.8	7.9
Anthracene	1.0	0.6	0.7
2-Methylanthracene	3.0	2.5	3.0
1-Methylphenanthrene [a]	2.5	1.8	2.1
2-Phenylnaphthalene	4.0	2.5	2.3
Fluoranthene [a]	44.1	28.7	26.2
Pyrene	41.8	28.7	26.9
Benzo[a]fluorene	4.5	3.4	3.2
Benzo[b]fluorene	2.7	2.0	1.9
Benzo[b]naphto[2,1-d]thiophene	1.7	2.7	1.3
Benzo[b]naphto[1,2-d]thiophene	0.3	0.7	0.3
Benz[a]anthracene [a]	15.4	12.4	10.3
Chrycene/triphenylene [a]	19.1	15.8	12.3
Benzo[b]fluoranthene [a]	32.2	23.9	18.8
Benzo[k]fluoranthene [a]	13.5	10.1	8.1
Benzo[e]pyrene [a]	21.0	17.1	9.9
Benzo[a]pyrene [a]	14.4	13.5	15.7
Perylene [a]	2.1	2.0	7.9
Indeno[1,2,3-cd]pyrene [a]	21.5	16.6	11.5
Benzo[g,h,i]perylene [a]	24.4	20.1	16.1
Dibenzo[a,h]anthracene [a]	1.3	1.5	0.7
Coronene [a]	10.8	8.3	7.1
Sum of all PAH	303	233	204
Sum of genotoxic PAH [a]	212	163	140

[a] Genotoxic PAH as defined by WHO/IPCS (21).

Proinflammatory activity and cytotoxicity of PM_{10}

Dose-dependency of the responses. Figure 1 shows the dose-related effects of the three pooled PM_{10} samples on TNF-α, IL-6, and NO production and cell viability in the RAW 264.7 macrophages. There were dose-dependent increases in TNF-α production, but the responses to spring I PM_{10} and spring II PM_{10} at the different dose levels were two to five times as large as those in response to the winter PM_{10} (figure 1A). There were no significant differences in TNF-α production between the spring I PM_{10} and spring II PM_{10} samples.

The production of IL-6 did not increase at all with winter PM_{10}, whereas spring I and II PM_{10} induced

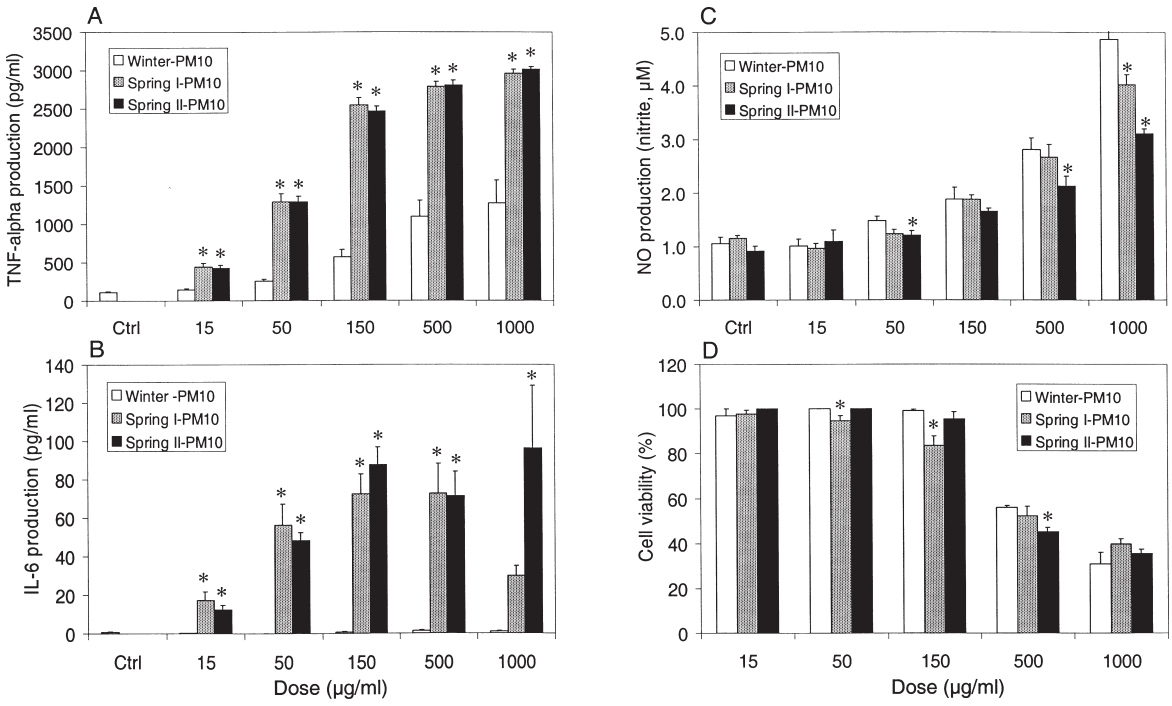

Figure 1. Dose-dependency of tumor necrosis factor alpha (TNF-α) (A), interleukin 6 (IL-6) (B), and nitric oxide (NO, measured as nitrite) production (C) and the reduction in cell viability (D) caused by urban-air particulate matter with an aerodynamic diameter of <10 µm (PM_{10}) in winter and spring (I-PM_{10} and II-PM_{10}) in the mouse macrophage cell line RAW 264.7. The means and standard errors of the means are shown for three separate experiments in duplicate (N=6). Both of the spring PM_{10} samples caused clearcut cytokine production even at low, noncytotoxic doses. [* = statistically significant difference (P<0.05, Fisher's test) from winter PM_{10}]

similar, statistically significant increases. However, the dose-dependencies of the IL-6 responses to the springtime PM_{10} samples were bell-shaped or irregular (figure 1B), probably due to a reduced cell viability with the two largest PM_{10} doses.

All three of the pooled PM_{10} samples induced modest dose-dependent NO production. There was a tendency, especially at the two highest dose levels, for the winter PM_{10} to induce a somewhat higher NO production than the spring I and II PM_{10} (figure 1C).

There were no consistent potency differences between the three pooled PM_{10} samples in causing a dose-dependent decrease in cell viability. The mean portion of alive macrophages after exposure to the largest mass dose (1000 µg/ml) was 31% with winter PM_{10}, 40% with spring I PM_{10}, and 35% with spring II PM_{10} (figure 1D).

Separation of the responses into water-soluble and water-insoluble PM_{10} fractions. Figure 2 shows the TNF-α and IL-6 productions and the cell viability in RAW 264.7 macrophages as a result of exposure to the whole suspensions of winter PM_{10} and spring I PM_{10}, and their water-soluble and water-insoluble fractions, corresponding to the total PM_{10} mass doses of 150 and 500 µg/ml of RPMI in the well. Nearly all of the proinflammatory activity (as assessed by cytokine production) and a large part of the cytotoxicity (MTT test) were associated with the insoluble PM_{10} fractions. This finding applied to both the winter PM_{10} and the spring I PM_{10}, although the pattern of the cytokine responses was more pronounced with the latter due to the higher proinflammatory potency. Actually, the IL-6 production caused by the insoluble fraction of spring I PM_{10} was significantly larger than the response to the corresponding whole PM_{10} suspension (figure 2). NO production at the selected PM_{10} doses was negligible, and, therefore, the responses to the water-soluble and insoluble PM fractions could not be separated (data not shown).

Inhibition of responses by polymyxin B and deferoxamine. The antagonism of the PM_{10}-induced proinflammatory and cytotoxic responses by polymyxin B (LPS antagonist) and deferoxamine (metal chelator) are shown in figure 3. Polymyxin B abolished the IL-6 production induced by spring I PM_{10}, and there was also a tendency towards a smaller TNF-α production with both the winter PM_{10} and spring I PM_{10}. Deferoxamine did not modify the PM_{10}-induced TNF-α or IL-6 production. Neither polymyxin B nor deferoxamine antagonized the PM_{10}-induced decreases in the viability of RAW 264.7 macrophages. In fact, deferoxamine alone was cytotoxic (data not shown).

Oxidative capacity and DNA damage by PM_{10}. Figure 4 shows the dose-dependent effects of the three pooled PM_{10} samples on hydroxyl-radical production, measured

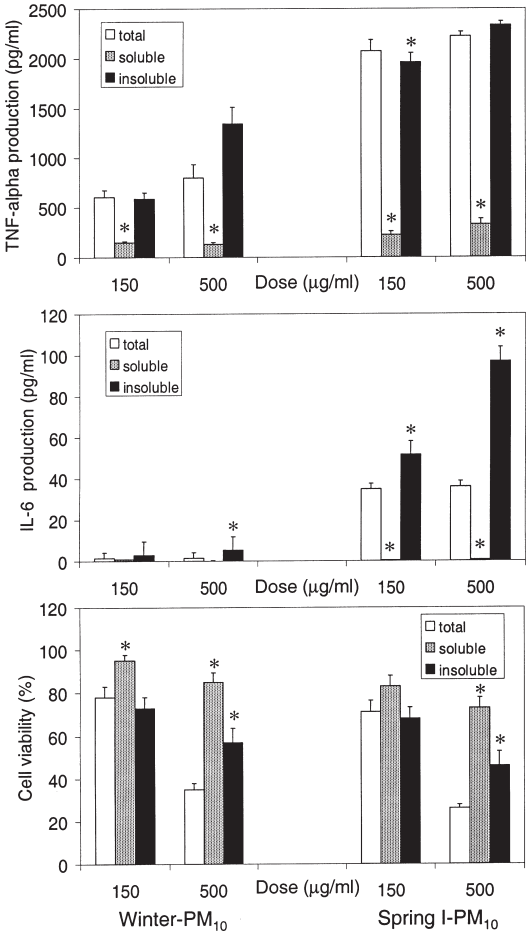

Figure 2. Tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) production and the change in cell viability in response to the whole suspension, and the water-soluble and water-insoluble fractions, of urban-air particulate matter with an aerodynamic diameter of <10 µm (PM_{10}) in winter and spring (I-PM_{10}) at two dose levels (150 and 500 µg/ml) in mouse RAW264.7 macrophages. The means and standard errors of the means are shown for three separate experiments in duplicate (N=6). [* = statistically significant difference (P<0.05, Fisher's test) from the respective whole PM_{10} suspension]

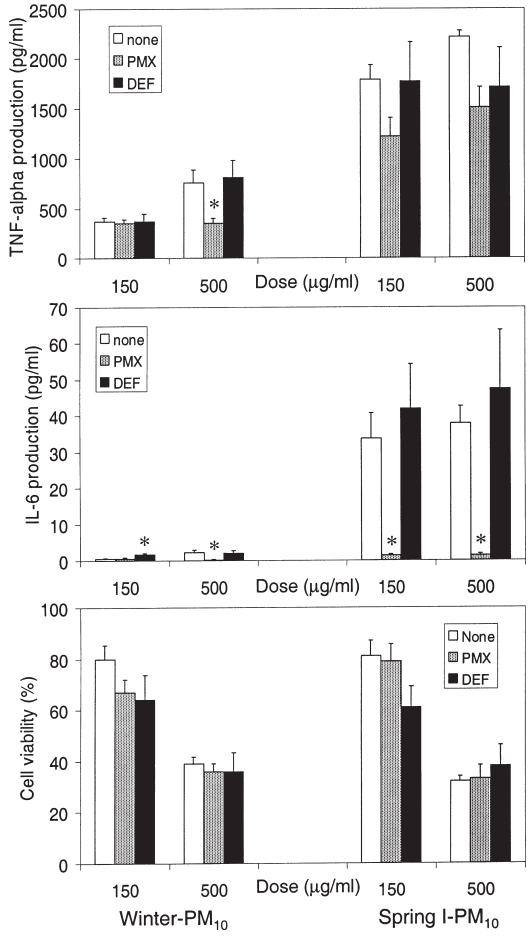

Figure 3. Inhibition of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) production induced by winter and spring (I-PM_{10}) urban-air particulate matter with an aerodynamic diameter of <10 µm (PM_{10}) (150 and 500 µg/ml) and the change in cell viability, by polymyxin B (20 µg/ml) (endotoxin antagonist) or deferoxamine (0.1 mM) (metal chelator) in mouse RAW264.7 macrophages. The means and standard errors of the means are shown for three separate experiments in duplicate (N=6). [* = statistically significant difference (P<0.05, Fisher's test) from the respective PM_{10} suspension alone]

as the EPR signal and 8-OH-DG formation in isolated calf thymus DNA. Spring II PM_{10} was the most potent inducer of hydroxyl radical production, followed by winter PM_{10} and spring I PM_{10}. With regard to 8-OH-DG formation, there was a tendency at all three dose levels for winter PM_{10} to produce somewhat higher responses than spring I PM_{10} or spring II PM_{10}.

Discussion

Our study showed few differences between the pooled winter PM_{10} sample and the two pooled spring PM_{10} samples with regard to their composition of water-soluble ions and elements, but the winter PM_{10} had the highest content of PAH. The spring PM_{10} samples were much more potent inducers of proinflammatory cytokine production (TNF-α, IL-6) than the winter PM_{10}, while there was no consistent difference in the cytotoxic potency between the three pooled PM_{10} samples. In contrast, winter PM_{10} was a significantly more potent inducer of NO production and oxidative DNA damage than either of the two spring PM_{10} samples. Nearly all of the proinflammatory cytokine production and cytotoxicity was associated with the insoluble PM_{10} fraction in both seasons. The endotoxin antagonist polymyxin B abolished the IL-6 production caused by spring PM_{10}, but it did not modify the simultaneous cytotoxicity. The metal chelator deferoxamine did not modify either the PM_{10}-induced cytokine production or the cytotoxicity. Thus the toxicity profile of urban-air PM_{10} changed with season, while the cytokine production and cytotoxicity caused by PM_{10} seemed to be mediated via different

particulate-bound constituents, even during the same season.

Ambient-air quality and PM_{10} constituents

The spring II period represented typical resuspended road dust episodes of that time of year in Helsinki, characterized by low relative humidity and rain and a large portion of $PM_{10-2.5}$ in PM_{10} (table 2). Weak and strong winds and turbulence caused by busy traffic are factors that enhance resuspension and increase the urban air PM_{10} concentration (8). The levels of gaseous pollutants (SO_2, NO_2, CO) and $PM_{2.5}$ were the lowest in the spring II period, probably because of the smaller contribution of local combustion sources (fewer cold starts of vehicle engines, less energy production, fewer days with weak winds and temperature inversion) and long-range transported pollutants to the urban air quality in this period than in the winter or spring I period.

The three pooled PM_{10} samples did not differ much with regard to their composition of water-soluble ions and elements (table 3). This result may be partially due to the fact that the PM_{10} sampling campaign took place during late winter and the spring (between 5 March and 17 May 1999) and excluded the coldest winter periods of January and February. However, the lack of difference in water-soluble inorganic constituents between the winter PM_{10} and the spring PM_{10} contrasts with the results of our previous study (11), which used two extreme PM_{10} samples from the same PM sampling campaign that was used in our present study. A PM_{10} sample collected during a strong springtime episode of resuspended dust contained much higher levels of water-soluble soil metals (aluminum, iron) and lower levels of sulfate ion, than a PM_{10} sample collected during a cold winter period. Therefore, another likely influencing factor is the pooling of selected PM_{10} samples per season in our present study, which probably averaged out the extremes in water-soluble soil metal compositions. The seasonal differences in the soil metal composition of PM_{10} might have been shown if we had had a parallel low-volume PM_{10} sample for measurement of the total elemental compositions with ICP-MS, X-ray fluorescence spectrometry, or instrumental neutron activation analysis (7, 8, 18), because soil metals appear primarily as poorly water-soluble salts. However, the highest arsenic and lead (table 3) and PAH (table 4) contents in the winter PM_{10} confirmed a larger contribution of local or distant combustion sources to this period than to the spring I and spring II periods.

Proinflammatory and cytotoxic findings

The RAW 264.7 macrophages showed that the spring I and II PM_{10} were much more potent inducers of TNF-α and IL-6 production than the winter PM_{10}. In contrast, the winter PM_{10} was a slightly more potent inducer of NO production, and there was no clear difference in the cytotoxic potency between the three pooled PM_{10} samples (figure 1). The different patterns in potency between the seasonal PM_{10} samples suggest that the resuspended road dust and its chemical constituents were major contributors to the proinflammatory cytokine responses, while combustion sources and their chemical constituents may have had a role in the relatively small increases in NO production. Cytotoxicity is such a nonspecific endpoint that it could be influenced by multiple biochemical pathways and chemical constituents of PM_{10}. In fact, our findings for winter PM_{10} agree reasonably well with the results of our earlier work (23) on diesel particles, which induced a dose-dependent increase in NO production and a decrease in the viability of the RAW 264.7 macrophages but did not induce TNF-α or IL-6 production.

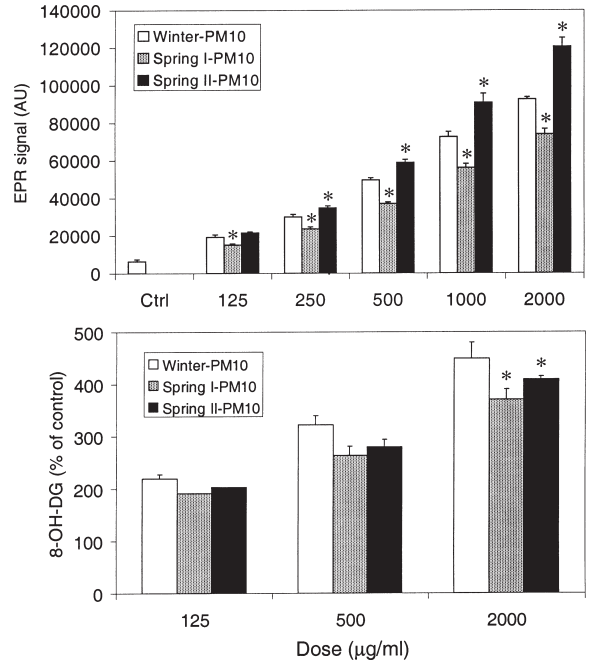

Figure 4. Dose dependency of hydroxyl-radical production [measured as the electron-paramagnetic-resonance (EPR) signal] derived from winter and spring (I-PM_{10}, II-PM_{10}) urban-air particulate matter with an aerodynamic diameter of <10 µm (PM_{10}), as well as the PM_{10}-induced formation of the hydroxyl-radical-specific lesion, 8-hydroxy-2´-deoxyguanosine (8-OH-DG), in isolated calf thymus DNA (deoxyribonucleic acid). The means and standard errors of the means are shown for 4–5 experiments. [* = statistically significant difference ($P<0.05$, Fisher's test) from winter PM_{10}]

Nearly all the proinflammatory cytokine production and cytotoxicity were associated with the insoluble fractions of both the winter and spring PM_{10} samples (figure 2). In fact, the insoluble fraction of spring I PM_{10} induced a significantly larger IL-6 production than the corresponding whole PM_{10} suspension, the finding suggesting that the removed supernatant had contained

some inhibitory constituent(s). Our findings are supported by the results of a previous study by Soukup & Becker (27) on human alveolar macrophages, in which the insoluble fraction of urban-air $PM_{10–2.5}$ from Chapel Hill (NC, USA) was mainly responsible for the entire PM_{10}-induced TNF-α and IL-6 production and apoptosis. Moreover, Imrich et al (28) have shown that the TNF-α production and cytotoxicity induced in nonprimed rat alveolar macrophages by urban-air $PM_{2.5}$ samples from Boston (MA, USA) were largely due to the insoluble PM fraction.

Polymyxin B totally abolished the IL-6 response and partially inhibited the TNF-α response to spring I PM_{10} (figure 3). This finding suggests that LPS-structured endotoxin from soil gram-negative bacteria was mainly responsible for the production of proinflammatory cytokines induced by the resuspended road dust constituents in the RAW 264.7 macrophages. This finding is supported by the results of numerous previous studies conducted on urban-air samples of total suspended particulates from several cities in rat and human alveolar macrophages (15, 16), PM_{10} samples from southeastern Mexico City (Mexico) in mouse J774A.1 monocyte cell line (29), $PM_{10–2.5}$ samples from Chapel Hill (NC, USA) in human alveolar macrophages (27), $PM_{10–2.5}$ and $PM_{2.5}$ samples from central Taiwan in mouse RAW264.7 macrophages (30), and $PM_{2.5}$ samples from Boston (MA, USA) in rat and mouse alveolar macrophages (28, 31). However, our study and the previous studies have not used methodologies that could have directly proved the presence of gram-negative bacteria or their fragments in the poorly water-soluble fraction of urban-air PM samples.

In the present study, TNF-α production was only partially inhibited by polymyxin B (figure 3), and therefore, in addition to endotoxin, some other constituent(s) (possibly inorganic or biological) of the spring PM_{10} induced proinflammatory effects on these cells. One possible factor is simply the platelike shape of some mineral particles like micas in resuspended road dust (32).

The cytotoxic effect of spring I PM_{10} was not at all inhibited by polymyxin B. This finding suggests that the endotoxin content in the PM_{10} sample was too low to induce the response well known from exposure to exogenous LPS doses (23). The best support for our finding comes from the study of Soukup & Becker (27) on human alveolar macrophages, in which IL-6 production, but not apoptosis, induced by the insoluble fraction of urban-air $PM_{10–2.5}$ from Chapel Hill (NC, USA), was mainly due to endotoxin. Thus some other (possibly inorganic or biological) constituent(s) in the poorly water-soluble fraction of spring PM_{10} is(are) likely to cause cytotoxicity.

The metal chelator deferoxamine did not modify either the winter or the spring PM_{10}-induced proinflammatory cytokine production or cytotoxicity in RAW 264.7 macrophages (figure 3). Several other authors have also reported that deferoxamine had no effect on the IL-6 production induced by total suspended particulates (16), insoluble $PM_{10–2.5}$ (27), or $PM_{2.5}$ (28).

Oxidative capacity and DNA damage

Our results did not show a consistent seasonal difference in the PM_{10}-derived hydroxyl-radical production (figure 4) as seen with respect to the PM_{10}-induced proinflammatory cytokine production in RAW 264.7 macrophages. The order of potency of the different samples in producing hydroxyl radicals was spring II PM_{10} > winter PM_{10} > spring I PM_{10}. This finding cannot be explained by large differences between the PM_{10} samples in the contents of Fenton-active water-soluble metals (iron, vanadium, copper, chromium, nickel, zinc) (table 3) potentially capable of catalyzing the formation of hydroxyl radicals from hydrogen peroxide. However, Shi et al (25) have previously reported that carbon black PM coated with copper ions had a 3- to 30-fold higher potency to induce hydroxyl-radical production than the other transition metals at different valencies. Moreover, they showed a highly significant correlation between hydroxyl-radical production and the copper content of urban-air PM_{10}. Thus the differences in the copper contents between our PM samples (spring II PM_{10} > winter PM_{10} > spring I PM_{10}) may explain the hydroxyl-radical responses.

Winter PM_{10} was a more potent inducer of 8-OH-DG production in isolated calf thymus DNA than spring I and II PM_{10} (figure 4). This finding suggests that chemical constituents from local combustion sources or long-range transport rather than those from resuspended road dust were responsible for this effect. The 8-OH-DG is a DNA lesion that is specific for hydroxyl radicals formed in the Fenton reaction of transition metals in the presence of hydrogen peroxide (26). As the rank order of potency of the three pooled PM_{10} samples in 8-OH-DG formation differed from that in hydroxyl-radical production, our findings do not suggest a high correlation between the two parameters of our air pollution situations in the same manner as previously reported in the German study by Shi et al (26). Nor do our findings suggest a single causative constituent like copper ions to these effects. In fact, the highest potency of winter PM_{10} to produce 8-OH-DG may be due to its largest content of reactive organic compounds. Squadrito et al (33) have recently hypothesized that the breakdown products of PAH, semiquinone radicals, produce hydrogen peroxide in their continuous redox cycles to be available for the Fenton reaction, but of course we do not know whether the higher PAH content in winter PM_{10} also indicated a higher content of semiquinones or other

reactive organic compounds. Formation of the premutagenic DNA adduct 8-OH-DG by urban-air PM has also been observed in human A549 alveolar type II epithelial cells without any addition of exogenous hydrogen peroxide. This finding suggests that the endogenous production of hydrogen peroxide from activated inflammatory cells is sufficient to induce this lesion (26).

Concluding remarks and implications

The urban-air PM_{10} samples collected in a subarctic climate during wintertime (low resuspendion of road dust) and springtime (high resuspension of road dust) had different proinflammatory and oxidant profiles in the in vitro test systems. The cytotoxicity and proinflammatory activity of both types of PM_{10} in mouse RAW 264.7 macrophages were strongly associated with the insoluble PM fraction, while soluble constituents like transition metals played no major role. The higher proinflammatory potency of springtime PM_{10} seemed to be largely due to particulate-bound endotoxin derived from soil gram-negative bacteria, but endotoxin was not the causative constituent of cytotoxicity.

Our findings raise the interesting clinical hypothesis that the irritative symptoms in the upper and lower respiratory tract frequently complained of by both healthy and asthmatic persons during spring episodes of resuspended road dust are largely due to the endotoxin content of poorly soluble coarse particles. This hypothesis is supported by the results of several clinical studies in which inhaled endotoxin was a potent inducer of acute respiratory symptoms, inflammation, and lung function decrements in both healthy and asthmatic persons (34). In general, it has been very difficult to determine the associations of lung function decrements or symptoms with resuspended PM_{10} from those with PM_{10} from other emission sources (5). This difficulty could be at least partially due to the fact that susceptible population groups like asthmatic persons can sense (reduced visibility, immediate irritation) resuspended dust episodes and they also receive warnings from the media (air quality index, news coverage in springtime) about such episodes, whereas the situation with "normal" combustion-derived or long-range transported PM_{10} is not so evident. On the basis of good information about actual resuspended dust pollution, these self-care educated patients can change their daily activities (visits to city centers, avoidance of walking along the worse polluted streets, timing of maintenance medication, etc) in ways that are good for their asthma but distort epidemiologic analyses. However, in one analysis of lung function data from a 12-week panel study during winter and spring in Kuopio (35), the decrements in the morning PEF among asthmatic schoolchildren were significantly associated with the black smoke values lagged by 2 days, whereas the evening PEF tended to be associated with the same-day and lagged resuspended PM_{10} levels (estimated on the basis of total aluminum content in PM_{10}). Moreover, Gordian et al (36) from Anchorage (Alaska, USA) showed that the 24-hour average PM_{10} concentration of mainly earth crustal material and volcanic ash was significantly associated with same-day and next-day outpatient visits for asthma and upper respiratory illness.

Additional experimental cell and animal studies are needed on the contribution of endotoxin, and possibly some other biological constituents, to the seasonal differences in the proinflammatory and cytotoxic potency of PM_{10}, and its subclasses $PM_{10-2.5}$ and $PM_{2.5}$.

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