

Human Alveolar Macrophage Phagocytic Function is Impaired by Aggregates of Ultrafine Carbon Particles

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Alveolar macrophages (AM) were collected by bronchoalveolar lavage from healthy volunteers. The AM were loaded with small masses (0.03–3 µg/10⁶ AM) of ultrafine carbon particle aggregates. The phagocytic activity of the cells was studied 20 h after the loading. Fluorescein-labeled silica particles (3 µm) were used as test particles and the attachment and ingestion processes were studied separately. In some experiments, AM were incubated with interferon-γ (IFN-γ) for 20 h before and during the test of phagocytic activity and during measurement of oxidative metabolism. The ingested carbon particles induced a dose-related impairment of both the attachment and the ingestion processes with a marked impairment down to a carbon particle dose around 0.2 µg/10⁶ AM. Such levels should reasonably occur after inhalation of existing concentrations of urban air particles, which to a considerable extent consist of aggregates of ultrafine particles with a carbon skeleton. Incubation with IFN-γ (12.5 U/ml) also induced significant impairments in both the attachment and the ingestion processes. Loading with carbon further aggravated the effect of IFN-γ. In contrast to earlier studies in rat AM, IFN-γ did not impair the oxidative metabolism at rest in these human AM; instead the oxidative metabolism was increased. This difference was due to a difference between rat and human AM and not between rat and human IFN-γ. Our results suggest that ingested environmental particles in AM, e.g., after an episode of high particle concentration, may impair phagocytic capacity of the cells, especially after infections that induce an increased production of IFN-γ. Consequently, there might be a risk for additional infections. Moreover,

inhaled particles not phagocytized by AM might damage the lung tissue. © 2001 Academic Press

Key Words: particulate matter; ultrafine particles; alveolar macrophages; phagocytosis; oxidative metabolism; interferon-γ; infections; carbon.

INTRODUCTION

Health effects by ambient air particles have attracted increased interest in recent years. A large number of epidemiological studies have shown a correlation between rather moderate levels of particles in the ambient air and acute effects such as mortality in heart and lung diseases and chronic lung morbidity (EPA, 1996; Pope *et al.*, 1995; Brunekreef *et al.*, 1995; Areskoug *et al.*, 2000). There are, however, no experimental data that can explain these effects (Areskoug *et al.*, 2000), although long-term exposure to rather innocuous particles such as carbon particles and titanium oxide particles in concentrations down to a few mg/m³ have caused pathological changes, including tumors, in the rat lung (EPA, 1996; Heinrich *et al.*, 1995; Nikula *et al.*, 1995).

Alveolar macrophages (AM) might play an important role in the effects seen in the epidemiological studies. AM protect the lungs by phagocytizing viable and nonviable particles deposited in the lungs. Ambient air particles differ widely in size, water solubility, and chemical composition. However, a considerable fraction of the particles is produced by combustion processes, including, for example, diesel exhaust particles, and these particles have a carbonaceous core. As the mechanical clearance of particles from the alveolar region in humans is extremely slow, the halftime being about 5 years (Philipson *et al.*, 1996), an accumulation of such particles must

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occur within the AM. In a previous study we found that small ingested amounts ($\sim 1 \mu\text{g}/10^6$ AM) of ultrafine carbon particle aggregates markedly impaired the phagocytic activity in rat AM (Lundborg *et al.*, 1999). During episodes with high particle concentrations in the ambient air, the mass of ingested particles in AM will be further increased and might impair the phagocytic capacity of the AM. Therefore there might be an increased risk of infections and damage to other lung cells.

The main purpose of the present experiments was to study the effect of ingested ultrafine carbon particles on phagocytosis by human AM. We investigated the relationship between carbon load in human AM and phagocytic function. Human AM, loaded and not loaded with carbon particles, were also incubated with interferon- γ (IFN- γ), because in an earlier study with rat AM we found an impaired phagocytic function and a reduced oxidative metabolism at rest, following long-term (24 h or longer) incubation with IFN- γ (Lundborg *et al.*, 1999). The results in rat AM was unexpected as IFN- γ has a fundamental role in activating white blood cells, including macrophages. The production is enhanced by infections by microorganisms or exposure to other antigens (Baron *et al.*, 1991; Curfs *et al.*, 1997).

MATERIAL AND METHODS

Human Volunteers and Bronchoalveolar Lavage (BAL)

Twelve nonsmoking healthy volunteers (mean age 30 years, range 18–49), 9 females (mean age 32 years, range 18–49) and 3 males (mean age 26 years, range 21–34), participated in the study. All had a normal chest X ray. The study had the approval of the local ethics committee and all subjects gave informed consent.

After premedication with morphine-hyoscine (Morfin-Scopolamin, Pharmacia & Upjohn, Stockholm, Sweden) and local anesthesia with lignocaine (Xylocain, Astra, Södertälje, Sweden) a flexible fiberoptic bronchoscope (BF Type 4B2, Olympus, Tokyo, Japan) was wedged in a subsegmental bronchus of the right middle lobe. Sterile saline solution at 37°C was instilled in five aliquots of 50 ml and gently suctioned back. The fluid was collected in a siliconized plastic bottle kept on ice. The recovered volume was measured and data was presented as percentage of instilled volume.

The fluid was carefully stirred and 20 ml was then immediately sucked up and strained through a double layer of Dacron nets. After centrifugation at 400g for 5 min at $+4^\circ\text{C}$, the supernatant was

discarded. The cells were resuspended in Hanks' balanced salt solution, and a total cell count was performed in a Bürker hemocytometer. The viability was evaluated by means of excluding trypan blue-colored cells. A differential cell count was performed after the preparation of centrifugal smears in a Cytospin 2 apparatus (Shandon, Runcorn, UK) at 500 rpm for 3 min. Cells were stained according to May-Grünwald Giemsa and 500 cells were counted. The data of the cells were adjusted according to the total recovered BAL fluid. The recovered cell suspension was centrifuged for 10 min at 300g and the resulting cell pellet was resuspended in Hepes-buffered Medium 199, pH 7.4 (GIBCO, Paisley, Scotland) with 10% human serum (AB, Rh⁺, Blood-central, Karolinska Hospital, Stockholm, Sweden), 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (complete medium).

Animals and Lung Lavage

AM were obtained by lavage from six healthy rats (Charles River, Uppsala, Sweden) weighing 250–300g. Two rats were used in the phagocytosis study with washed and unwashed carbon particles and four rats were used in the oxidative metabolism study. The rats were sacrificed by an overdose of sodium pentobarbital. The lungs were excised and lavaged eight times (in total 40 ml) with Hanks' balanced salt solution (without Ca^{2+} and Mg^{2+} , pH 7.4, 37°C) using brief massage. Around 30 ml lavage fluid, containing $10\text{--}15 \times 10^6$ cells, was obtained. More than 90% of the cells were estimated to be AM as determined from typical macrophage morphology by light microscopy. The cells were washed once by a 10-min, 300g centrifugation at room temperature and the resulting cell pellet was resuspended in Hepes-buffered Medium 199, pH 7.4 (GIBCO, Paisley, Scotland), with 10% inactivated calf serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (complete medium). The number of cells was counted in a Bürker hemocytometer.

Preparation of Carbon Particle Suspension

Aggregates of ultrafine carbon powder (Günther Wayner, Hannover, Germany) were used. A stable suspension of carbon particles was obtained by suspending the powder in Medium 199 (with 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin) and by sonicating for 3 h with short breaks every 20 min for vortexing. The size of the carbon particles was measured using a transmission electron microscope (Jeol 100S, Jeol Ltd, Tokyo, Japan). The

carbon particles occurred as aggregates with a mean diameter of 0.17 ± 0.08 (\pm SD) μm . The size of the primary particles was 0.044 ± 0.01 μm . The mass concentration of carbon particles in suspension was quantified by measuring the optical density at 800 nm with a spectrophotometer (Shimadzu, UV-160A). There was a linear relationship between mass concentration of carbon particles and optical density (Fig. 1) and the sonicated suspension was stable for at least 24 h. The data in the figure are based on measurements on samples obtained by dilution from a stem suspension. The SD in percentage of the mean was for 1 $\mu\text{g/ml}$ 17%, for 3 $\mu\text{g/ml}$ 6.6%, for 10 $\mu\text{g/ml}$ 2.5%, and for 20 $\mu\text{g/ml}$ 0.9%.

In order to examine whether some organic compounds, which might be adsorbed to the carbon particles, could be responsible for the impairment of phagocytic activity found in the study by Lundborg *et al.* (1999), the carbon powder was washed by a Soxhlet extraction. About 500 mg carbon powder was wrapped in a filter paper and refluxed for 24 h with 500 ml toluene in a Soxhlet apparatus. The toluene extract was then analyzed on a gas chromatograph (HP 5890, Little Falls, DE) with a mass selective detector (5972AMSD, Palo Alto, CA) and a HP-5MS column (film thickness 0.25 μm , length 30 m, i.d. 0.25 mm). No organic compounds were found to be extracted from the carbon particles. This carbon is later referred to as "washed carbon."

Loading of AM with Carbon Particles

In the study of phagocytosis of test particles, 1 ml of carbon particles in complete medium was added to test tubes with and without 1×10^6 AM. In the study of oxidative metabolism, 2 ml of carbon particles in complete medium was added to test tubes either

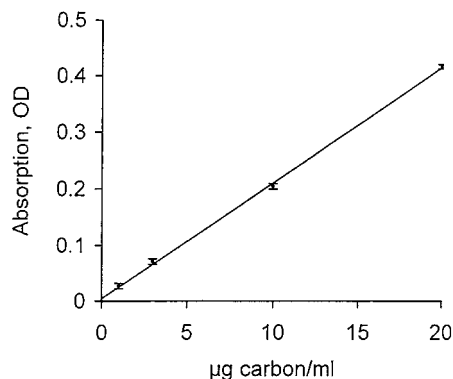


FIG. 1. Relationship between the concentration of suspended carbon particles and optical density (OD) at 800 nm. Bars show \pm SD, $n = 6-8$.

with or without 2×10^6 AM. The samples were placed in a shaking water bath at 37°C for time periods ranging from 2 to 6 h (see below) in order to obtain AM with attached and ingested carbon particles. The tubes were then centrifuged at $225g$ for 10 min. The supernatants from the tubes with and without AM were measured in the spectrophotometer and the difference in carbon particle concentration was taken as a measure of attached and ingested particles by AM. Centrifugation of the carbon particle suspension in tubes without AM had only a small effect on the mass concentration of suspended particles, less than 1%. New complete medium was added to the precipitated cells and in order to remove remaining free carbon particles additional washing was performed. To ensure that all carbon particles attached to the AM would be ingested, new complete medium was then added to the AM. The suspension was added to culture dishes (Falcon, 35×10 mm) with coverglasses and incubated at 37°C with 5% CO_2 in air and 80% relative humidity for about 20 h. Control AM were treated in exactly the same way from lavage to the end of the phagocytosis and oxidative metabolism experiments, only leaving out the carbon particles.

Incubation with IFN- γ

In the effect studies of IFN- γ on phagocytosis of the silica particles and oxidative metabolism, IFN- γ was present during the loading of the AM with the carbon particles and during the entire experimental procedure. For the AM not loaded with carbon, IFN- γ was present at corresponding time periods. Whenever new complete medium was added, new IFN- γ was also added. In the experiment with the human AM, human IFN- γ (Nova Kemi AB, Stockholm, Sweden) was used and in the experiment with rats recombinant rat IFN- γ (Nova Kemi AB) was used.

Assay of Phagocytosis

Spherical particles of amorphous silica (Spherisorb S 3, NH 2, Phase Separations Ltd, Queens Ferry, Clwyd, UK) labeled with fluorescein isothiocyanate (FITC) (Nyberg *et al.*, 1996) were used as test particles in the studies of phagocytosis. The size of the silica particles measured under a light microscope (Viscopan projection microscope, Reichert, Austria) was 3.2 ± 0.4 μm (mean \pm SD). A modification of the method described by Hed (1977) was used for the assessment of phagocytic function.

Phagocytic ability of rat AM with and without ingested washed and unwashed carbon particles was studied in four samples from two rats. Phagocytic activity of human AM loaded with carbon particles in various amounts was studied in AM from six volunteers. From another four volunteers, phagocytic activity was studied in AM loaded with one carbon particle concentration and incubated with two IFN- γ -concentrations (12.5 and 50 U/ml). Loading time for the rat AM was 6 h and for the human AM 2–4 h. After loading, following washing procedure and the last centrifugation of the cell suspension, 2 ml of complete medium was added to the cell precipitate in each test tube. The suspensions were added to culture dishes (Falcon, 35 \times 10 mm) containing coverglasses and then incubated at 37°C with 5% CO₂ in air and 80% relative humidity. After 20 h, the medium was exchanged and 1 ml of complete medium containing FITC-labeled silica particles (10 \times 10⁶/ml) was added to the cells (1 \times 10⁶/ml). After incubation for 30 min, the slides were placed in ice-cold Ringer acetate (to interrupt the phagocytosis) and unattached particles were rinsed off. The cells were then stained with trypan blue (0.4 mg/ml) for 30 s. The number of particles ingested by and attached to AM were then directly counted in a Zeiss microscope, which permits examination in both visible light and UV light (fluorescence). When the microscope is turned on the visible light mode, the attached particles are clearly visible by the trypan blue staining and the ingested ones are not. When the microscope is switched over to the UV light mode, only the ingested particles are seen, as trypan blue quenches the fluorescence from the attached particles. In each sample 100 consecutive macrophages were scored. The parameters “accumulated attachment” and “ingested fraction” were introduced in order to differentiate between the attachment and the ingestion processes. The accumulated attachment is the sum of the numbers of attached and ingested particles per alveolar macrophage. As all ingested particles must have been attached to the AM, the accumulated attachment is the integrated number of particles attached to the AM during the time of the test. Therefore, this parameter is a measure of the attachment process. The ingested fraction is the number of ingested particles per alveolar macrophage divided by the accumulated attachment; i.e., the ingested particles are related to the integrated number of attached particles. Thus, this parameter represents a measure of the ingestion process and is rather independent on the accumulation process.

TABLE 1
Phagocytic Activity of AM Loaded with Either Washed or Unwashed Carbon Particles Studied in Four Samples from Two Rats

Sample	AM ^a	AM + washed carbon particles	AM + unwashed carbon particles
Ingested particles per AM			
1	2.8	1.9	2.1
2	2.8	2.0	2.3
3	1.6	0.6	0.5
4	1.6	0.5	0.5
Mean	2.2	1.3	1.4
SD	0.7	0.8	1.0
Accumulated attachment, particles per AM			
1	5.8	4.7	5.1
2	6.0	5.2	5.5
3	3.3	2.4	2.6
4	3.5	2.5	2.7
Mean	4.7	3.7	4.0
SD	1.4	1.5	1.5
Ingested fraction			
1	0.48	0.39	0.41
2	0.46	0.39	0.42
3	0.49	0.24	0.20
4	0.46	0.20	0.18
Mean	0.47	0.31	0.30
SD	0.01	0.10	0.13

Note. FITC-labeled silica particles were used as test particles and the period of phagocytosis was 30 min.

^aNot loaded with carbon particles.

Assay of Oxidative Metabolism

The oxidative metabolism (“spontaneous” metabolism with no stimulation by agents like zymozan) of AM was measured by using the ability of the produced superoxide to reduce yellow nitroblue tetrazolium (NBT) to blue formazan, according to the method described by Jarstrand *et al.* (1978).

The oxidative metabolism was studied in AM from four rats and five volunteers. After 20 h of incubation with or without IFN- γ (12.5 and 50 U/ml) in a shaking 37°C water bath, the cell suspensions were centrifuged at 300g for 10 min. Complete medium was added and the suspension was again centrifuged. One milliliter of complete medium was added to the cell precipitate in each tube and the cell suspension was added to culture dishes (Falcon, 35 \times 10 mm) containing coverglasses. Incubation was performed at 37°C with 5% CO₂ in air and 80% relative humidity for about 20 h. The reaction was stopped after 1 h by adding 1 ml of 0.5% HCl to each culture dish. With a “rubber policeman” we scraped off the cells and the cell suspension was collected in test tubes. The tubes

were centrifuged at 2500 *g* for 30 min. One milliliter dimethylsulfoxide was added to the precipitate in each tube to dissolve the produced formazan and the tubes were sonicated for 30 min. The absorption of the solution from each tube was measured in a spectrophotometer (Shimadzu, UV-160A) at wavelength 572 nm.

Statistical Analysis

A one-way or two-way repeated-measures analysis of variance was used. If significant differences were obtained, detailed comparisons between groups were performed by the paired, two-tailed *t* test. Level of significance was 0.05.

RESULTS

Phagocytic Activity of Rat AM Loaded with Unwashed and Washed Carbon Particles

Table 1 shows a comparison of the phagocytic activity of rat AM loaded with either washed or unwashed carbon particles with that of AM not loaded with carbon. In all samples, preexposure to ultrafine carbon particles reduced ingested particles per alveolar macrophages, the accumulated attachment, and the ingested fraction. However, the values for all phagocytic parameters were highly similar for washed and unwashed carbon particles. Table 2 shows that the uptake of both washed and unwashed carbon particles by the AM was around 1 $\mu\text{g}/10^6$ AM. Because the values were highly similar for washed and unwashed carbon particles, unwashed carbon particles were used in the experiments with human AM.

Characteristics of the BAL Fluid from the Volunteers

Table 3 shows the characteristics of the BAL fluid from all the 12 nonsmoking volunteers. The BAL findings regarding the recovery and the viability

TABLE 2
Uptake of Washed and Unwashed Carbon Particles by AM

Sample	20 $\mu\text{g}/\text{ml}$ of washed carbon particles		20 $\mu\text{g}/\text{ml}$ of unwashed carbon particles	
	% reduct. of OD	$\mu\text{g}/10^6$ AM	% reduct. of OD	$\mu\text{g}/10^6$ AM
1	5	1.0	5	1.0
2	4	0.8	4	0.8
3	9	1.8	9	1.8
4	8	1.6	10	2.0
Mean	7	1.3	7	1.4
SD	2	0.5	3	0.6

Note. Four samples in two rats were studied. The amounts of carbon taken up by the AM were estimated by measurements of optical density (OD) of the carbon particle suspension (20 $\mu\text{g}/\text{ml}$) before the AM (10^6 AM/ml) were added and after the AM were removed by centrifugation.

of the cells, the total cell count, the cell concentration, and the proportion of the various cells are well in accordance with earlier findings (Cherniack, 1990).

Phagocytic Activity of Human AM Loaded with Various Amounts of Carbon

Table 4 presents the levels of ingested particles, accumulated attachment, and ingested fraction of silica particles in human AM preexposed to the ultrafine carbon particles at concentrations of 0, 0.1, 1, 3, and 10 μg carbon/ml. There were significant reductions in all parameters of phagocytosis for concentrations of ultrafine carbon of 1 $\mu\text{g}/\text{ml}$ and higher. Table 5 presents the uptake of carbon by AM at various carbon particles concentrations. Because the concentrations of 0.1 and 1.0 μg carbon/ml were too low for detecting any difference in the optical density before and after incubation with

TABLE 3
General Characteristics of the BAL Fluid Recruited from 12 Nonsmoking Healthy Volunteers

	Recovery (%)	Viability (%)	Total cell count ($\times 10^6$)	Cell conc ($\times 10^6$)	Ma (%)	Ly (%)	PMN (%)	Eos (%)	Mast/10 vf
Median	80	92	21	110	88	10	2	0	1
Iq range	(77-81)	(90-97)	(15-33)	(78-170)	(82-93)	(6-16)	(1-2)	(0-0)	(1-5)
Mean	77	93	25	129	87	11	2	0	3
SD	(10)	(4)	(14)	(64)	(8)	(7)	(1)	(0)	(4)

Note. Data are given as both medians with interquartile ranges and mean values with SD. Recovery, percentage recovered fluid of instilled volume; Viability, percentage viable of total cells; Total cell count, total number of cells recovered; Cell conc, calculated cell concentration in the recovered volume; Ma, macrophages; Ly, lymphocytes; PMN, polymorphonuclear neutrophils; Eos, eosinophils, Mast, mast cells/10 visual fields (vf) with a magnification of 16 times.

TABLE 4

Phagocytic Activity of Human AM after Loading with Carbon Particles at Carbon Concentrations of 0, 0.1, 1, 3, and 10 µg Carbon/ml

	0 µg Volunteer carbon/ml	0.1 µg carbon/ml	1 µg carbon/ml	3 µg carbon/ml	10 µg carbon/ml
Ingested silica particles per AM					
1	4.3	— ^a	2.6	2.3	2.2
2	1.3	1.4	1.0	0.8	0.6
3	3.7	1.8	1.5	1.1	0.6
4	5.2	4.0	1.7	1.1	0.7
5	3.1	2.9	1.6	1.0	0.3
6	3.7	3.6	2.0	0.9	0.3
Mean	3.6	2.7	1.7**	1.2**	0.8**
SD	1.3	1.1	0.5	0.6	0.7
Accumulated attachment of silica particles, particles per AM					
1	7.0	— ^a	5.8	4.8	4.4
2	4.7	4.8	4.2	3.8	3.5
3	6.5	5.6	4.3	3.4	3.3
4	15.0	12.2	9.2	8.5	6.7
5	7.1	6.6	5.3	3.9	1.8
6	6.8	6.7	4.8	5.1	1.7
Mean	7.9	7.2	5.6*	4.9*	3.6**
SD	3.6	2.9	1.9	1.9	1.9
Ingested fraction of silica particles					
1	0.61	— ^a	0.44	0.48	0.49
2	0.28	0.29	0.24	0.22	0.17
3	0.57	0.33	0.34	0.32	0.18
4	0.36	0.33	0.19	0.13	0.10
5	0.44	0.43	0.30	0.26	0.19
6	0.55	0.54	0.42	0.16	0.17
Mean	0.47	0.38	0.32**	0.26**	0.22**
SD	0.13	0.10	0.10	0.13	0.14

Note. FITC-labeled silica particles were used as test particles and the period of phagocytosis was 30 min.

^aNot tested.

**P* < 0.05 compared with controls (two-tailed, paired *t* test).

***P* < 0.01 compared with controls (two-tailed, paired *t* test).

AM, the same average percentage reduction was assumed for these concentrations as for the 3 µg/ml carbon concentration. In Fig. 2, the values of ingested particles, accumulated attachment, and the ingested fraction expressed as percentage of controls (0 µg carbon/ml) were plotted against the mean values of the mass of carbon taken up by 10⁶ AM (shown in Table 5).

Phagocytic Activity of Human AM Incubated with IFN-γ and Loaded with Carbon

Table 6 shows the phagocytic activity of human AM incubated with 0, 12.5, or 50 U IFN-γ/ml

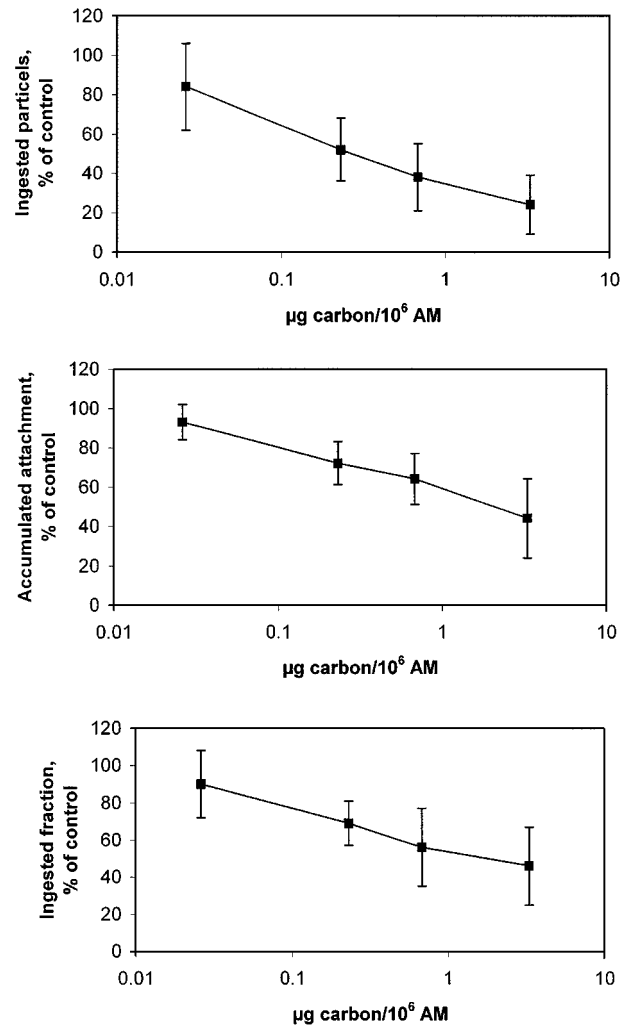


FIG. 2. Relationships between the masses of ingested carbon particles per 10⁶ AM and ingested silica particles, accumulated attachment of silica particles, and ingested fraction of silica particles. All three parameters of phagocytosis are expressed as percentage of AM not loaded with carbon.

for about 20 h before and during the test of phagocytosis. AM both loaded and not loaded with carbon were incubated with IFN-γ (loading time, 2–4 h; loading concentration, 10 µg/ml). Two-way analyses of variance show significant reductions in all three parameters of phagocytosis both for incubation with IFN-γ and for carbon load, i.e., for the number of ingested particles (*P* < 0.05 for carbon and *P* < 0.01 for IFN-γ), for the accumulated attachment (*P* < 0.05 for carbon and *P* < 0.01 for IFN-γ), and for the ingested fraction (*P* < 0.01 for carbon and IFN-γ). Table 7 shows that uptake of carbon by the human AM is fairly similar whether these have been incubated with IFN-γ or not.

TABLE 5
Uptake of Carbon by Human AM from Various Concentrations of Carbon Particles

Volunteer	0.1 µg carbon/ml		1 µg carbon/ml		3 µg carbon/ml		10 µg carbon/ml	
	% red. of OD ^a	µg per 10 ⁶ AM	% red. of OD ^a	µg per 10 ⁶ AM	% red. of OD	µg per 10 ⁶ AM	% red. of OD	µg per 10 ⁶ AM
1	— ^b	— ^b	8	0.08	8	0.24	20	2.0
2					25	0.75	32	3.2
3					19	0.57	27	2.7
4					21	0.63	28	2.8
5					44	1.32	63	6.3
6					19	0.57	28	2.8
Mean	26	0.026	23	0.23	23	0.68	33	3.3
SD					12	0.36	15	1.5

Note. The amount of uptake of carbon was estimated by measurements of optical density (OD) before addition of the AM and after removal of the AM by centrifugation.

^aThe concentrations of 0.1 and 1.0 µg carbon/ml were too low for measurements of the percentage difference in OD. The same percentage uptake was therefore used as for the 3 µg/ml concentration. For the 1 µg/ml concentration it was based on all six volunteers and for the 0.1 µg/ml concentration it was based on Volunteers 2–6.

^bVolunteer 1 was not tested with 0.1 µg carbon/ml.

Oxygen Metabolism in Resting Human and Rat AM Incubated with IFN-γ

Table 8 shows the amount of formazan reduced from NBT by superoxide anions in resting human AM, incubated with human IFN-γ at concentrations 0, 12.5, or 50 U/ml. One-way analysis of variance shows a significant increase in the oxygen metabolism ($P < 0.01$). As this result was opposite to our earlier experiment with rat AM (Lundborg *et al.*, 1999), we incubated AM from two of the volunteers with rat IFN-γ. Also rat IFN-γ induced an increase in oxygen metabolism (Table 9). Table 10 shows oxygen metabolism of rat AM incubated with two concentrations of rat and human IFN-γ. Both types of IFN-γ induced a significant reduction in oxidative metabolism at rest in rat AM (analysis of variance, $P < 0.01$).

DISCUSSION

In the present article, we washed carbon particles using the Soxhlet method and compared the phagocytic activity of rat AM loaded with either washed or unwashed particles. The result indicates that the impaired phagocytic activity was caused by the ingested carbon particles and not any organic compounds released from these particles.

The studies of phagocytic activity in the present study showed preexposure to ultrafine carbon particles impaired both the attachment and the inges-

tion processes of human AM, a result that is in agreement with the results of studies on rat AM (Lundborg *et al.*, 1999). In a study with rat AM, an impairment was seen at a mass of carbon particles around 1 µg/10⁶ AM. In the present study with human AM, there was a marked impairment of both the attachment and the ingestion processes already at carbon exposure levels of 0.2 µg/10⁶ AM.

In the study by Lundborg *et al.* (1999), 1 µg carbon particles ingested by 10⁶ rat AM was estimated to approximately correspond to a long-term inhalation of carbon particles at a concentration of about 40 µg/m³. This estimation was based on experiments by Oberdörster *et al.* (1994), who exposed rats to aggregates of ultrafine TiO₂ particles and also measured the load of these particles in the AM. Because we demonstrated an impaired phagocytic activity of human AM *in vitro* already at one-fifth of the load of carbon particles used in our study with rat AM (Lundborg *et al.*, 1999), it is reasonable to believe that inhalation of ambient air particles at moderate levels might impair the phagocytic function of AM in humans. Such particles include diesel exhaust particles, which consist of aggregates of ultrafine carbon particles on which organic compounds are adsorbed.

Earlier studies indicate that fairly small ingested particle masses can impair phagocytic activity in AM. Becker and Soukup (1998) incubated human AM (2×10^5 AM/ml) with urban air particles (100 µg/ml) and tested the phagocytic activity of the AM after incubation times of 18–20 h. They found

TABLE 6

Phagocytic Activity of Human AM Incubated with 12.5 or 50 U IFN- γ for 24 h and Loaded with Carbon

Volunteer	0 U/ml IFN- γ		12.5 U/ml IFN- γ		50 U/ml IFN- γ	
	AM	AM + carbon	AM	AM + carbon	AM	AM + carbon
Ingested particles per AM ^a						
7	3.0	1.7	1.4	1.0	1.1	0.6
8	4.3	1.7	2.7	0.7	0.9	0.4
9	8.1	5.5	5.1	3.1	4.0	2.1
10	9.8	5.1	3.9	3.2	2.6	2.3
Mean	6.3	3.5	3.3	2.0	2.2	1.4
SD	3.2	2.1	1.6	1.4	1.5	1.0
Accumulated attachment, particles per AM ^b						
7	4.4	2.9	2.3	2.6	2.4	1.4
8	9.7	7.0	8.7	3.8	3.3	2.1
9	9.6	7.6	6.8	4.6	5.5	3.2
10	10.5	7.7	6.5	5.3	4.6	4.1
Mean	8.6	6.3	6.1	4.1	3.9	2.7
SD	2.8	2.3	2.7	1.1	1.4	1.2
Ingested fraction ^c						
7	0.68	0.58	0.59	0.37	0.48	0.44
8	0.45	0.24	0.31	0.19	0.26	0.19
9	0.84	0.72	0.74	0.67	0.73	0.65
10	0.93	0.66	0.61	0.62	0.57	0.56
Mean	0.73	0.55	0.56	0.46	0.51	0.46
SD	0.21	0.21	0.18	0.22	0.20	0.20

Note. FITC-labeled silica particles were used as test particles and the period of phagocytosis was 30 min.

^a $P < 0.05$ for carbon, $P < 0.01$ for IFN- γ , $P = 0.072$ for interaction.

^b $P < 0.05$ for carbon, $P < 0.01$ for IFN- γ , $P = 0.052$ for interaction.

^c $P < 0.01$ for carbon; $P < 0.001$ for IFN- γ ; $P = 0.10$ for interaction (two-way ANOVA).

a 50% decrease in the phagocytic activity after incubation with the particles. Rudell *et al.* (1999) exposed healthy volunteers for 1 h to diesel exhaust at a particle concentration of about 300 $\mu\text{g}/\text{m}^3$.

TABLE 7

Uptake of Carbon by Human AM Incubated with IFN- γ for 24 h

Volunteer	μg carbon taken up per 10^6 AM		
	0 U/ml IFN- γ	12.5 U/ml IFN- γ	50 U/ml IFN- γ
7	3.0	3.5	2.7
8	2.2	1.5	1.5
9	3.2	3.4	3.9
10	6.1	5.6	5.1
Mean	3.6	3.5	3.3
SD	1.7	1.7	1.5

Note. Loading time for carbon was 2–4 h and the concentration of particles was 10 $\mu\text{g}/\text{ml}$.

TABLE 8

Amount of Formazan Reduced from NBT by Superoxide Anions in Resting Human AM Incubated with Human IFN- γ

Volunteer	Optical density (arbitrary units)		
	Control	12.5 U/ml IFN- γ	50 U/ml IFN- γ
9	0.15	0.36	0.41
10	1.51	1.77	1.92
6	0.37	0.39	0.42
11	0.47	0.50	0.62
12	0.15	0.31	0.34
Mean	0.53	0.67*	0.74*
SD	0.57	0.62	0.67

* $P < 0.05$ compared to controls (paired two-tailed t test).

Twenty-four hours later, AM were collected by lavage and were found to have an impaired phagocytic function.

IFN- γ has a fundamental role in the activation of leukocytes (Curfs *et al.*, 1997). It has been shown that this cytokine increases the oxidative metabolism in neutrophils, monocytes, and macrophages obtained from the blood (Nathan *et al.*, 1983; Cassatella *et al.*, 1985, 1988; Kowanko and Ferrante, 1987). Lundborg *et al.* (1999) also found a tendency of increased phagocytic activity in rat AM following short-term incubation with IFN- γ (40 min). However, long-term incubation with IFN- γ (28 or 44 h) markedly impaired both the attachment and the ingestion processes of test particles in rat AM (Lundborg *et al.*, 1999), and in the present experiments we found an impaired phagocytic activity also in human AM. In accordance with the experiments with rat AM, carbon loading further impaired phagocytosis after long-term incubation with IFN- γ . However, the reduction of the oxidative metabolism at rest in rat AM after long-term

TABLE 9

Amount of Formazan Reduced from NBT by Superoxide Anions in Resting Human AM Incubated with Human or Rat IFN- γ

Volunteer	Optical density (arbitrary units)				
	Control	12.5 U/ml IFN- γ		50 U/ml IFN- γ	
		Human	Rat	Human	Rat
11	0.47	0.50	0.54	0.62	0.53
12	0.15	0.31	— ^a	0.34	0.21

^aNot tested.

TABLE 10

Amount of Formazan Reduced from NBT by Superoxide Anions in Resting Rat AM Incubated with Either Rat or Human IFN- γ

Optical density (arbitrary units)					
		Rat	Rat	Human	Human
		IFN- γ	IFN- γ	IFN- γ	IFN- γ
Rat	Control	12.5 U/ml	50 U/ml	12.5 U/ml	50 U/ml
1	0.62	0.23	0.35	0.43	0.48
2	0.23	0.11	0.11	0.06	0.05
3	0.55	0.45	0.39	0.53	0.48
4	0.49	0.23	0.18	0.39	0.32
Mean	0.47	0.26*	0.26*	0.35	0.33*
SD	0.17	0.14	0.13	0.20	0.20

* $P < 0.05$ compared with controls (paired two-tailed t test).

incubation with IFN- γ was not seen in the human AM. IFN- γ instead increased the oxidative metabolism. This difference between rat and human AM was apparently not caused by a difference between rat and human IFN- γ but by a difference between rat and human AM.

In conclusion, preexposure loading of human AM with carbon particles markedly impaired both the attachment and the ingestion processes of the test particles, and there was a combined (probably additive) effect of long-term IFN- γ incubation and carbon loading. These data suggest that ingested ambient air particles in AM may impair the phagocytic capacity of these cells especially after an episode of high particle concentration. This impairment should be of particular importance for persons with infections that have induced an increased production of IFN- γ . Consequently, there might be an increased risk for additional infections. Moreover, inhaled particles may not be as efficiently phagocytized by AM, which may lead to further damage of the lung tissue.

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REFERENCES

Areskoug, H., Camner, P., Dahlén, S. E., Låstbom, L., Nyberg, F., Pershagen, G., and Sydbom, A. (2000). Particles in ambient air—A health risk assessment. *Scand. J. Work Environ. Health* (in press).

- Baron, S., Tyring, S. T., Fleischmann, R. W., Coppenhaver, D. H., Niesel, D. W., Klimpel, G. R., Stanton, G. J., and Hughes, T. K. (1991). The interferons. Mechanisms of action and clinical applications. *JAMA* **266**, 1375–1383.
- Becker, S., and Soukup, J. M. (1998). Decreased CD11B expression, phagocytosis, and oxidative burst in urban particulate pollution-exposed human monocytes and alveolar macrophages. *J. Toxicol. Environ. Health A* **55**, 455–477.
- Brunekreef, B., Dockery, D. W., and Krzyzanowski, M. (1995). Epidemiologic studies on short-term effects of low levels of major ambient air pollution components. *Environ. Health Perspect.* **103**(Suppl. 2), 3–13.
- Cassatella, M. A., Bianca, V. D., Berton, G., and Rossi, F. (1985). Activation by gamma interferon of human macrophage capability to produce toxic oxygen molecules is accompanied by decreased Km of the superoxide-generating NADPH oxidase. *Biochem. Biophys. Res. Commun.* **132**, 908–914.
- Cassatella, M. A., Cappelli, R., Bianca, D., Grzeskowiak, M., Dusi, S., and Berton, G. (1988). Interferon-gamma activates human neutrophil oxygen metabolism and exocytosis. *Immunology* **63**, 499–506.
- Cherniack, R. M. (1990). Bronchoalveolar lavage constituents in healthy individuals, idiopathic pulmonary fibrosis, and selected comparison groups. *Am. Rev. Respir. Dis.* **141**, S169–S202.
- Curfs, J. H. A. J., Meis, J. F. G. M., and Hoogkamp-Korstanje, J. A. A. (1997). A primer on cytokines: Sources, receptors, effects, and inducers. *Clin. Microbiol. Rev.* **10**, 742–780.
- Diamond, R. D., Llyman, C. A., and Wysong, D. R. (1991). Disparate effects of interferon- γ and tumor necrosis factor- δ on early neutrophil respiratory burst and fungicidal responses to *Candida albicans* hyphae in vitro. *J. Clin. Invest.* **87**, 711–720.
- EPA. (1996). "Air Quality Criteria for Particulate Matter." US EPA/600/P-95/00 1bF, Research Triangle Park, NC.
- Hed, J. (1977). The extinction of fluorescence by crystal violet and its use to differentiate between attached and ingested microorganisms in phagocytosis. *FEMS Microbiol. Lett.* **1**, 357–361.
- Heinrich, U., Fahst, R., Rittinghausen, S., Creutzenberg, O., Bellman, B., Koch, W., and Leven, K. (1995). Chronic inhalation exposure of Wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide. *Inhal. Toxicol.* **7**, 533–556.
- Jarstrand, C., Lundborg, M., Wiernik, A., and Camner, P. (1978). Alveolar macrophage function in nickel dust exposed rabbits. *Toxicology* **11**, 353–359.
- Kowanko, I. C., and Ferrante, A. (1987). Stimulation of neutrophil respiratory burst and lysosomal enzyme release by human interferon-gamma. *Immunology* **62**, 149–151.
- Lundborg, M., Johansson, A., Låstbom, L., and Camner, P. (1999). Ingested aggregates of ultrafine carbon particles and interferon- γ impair rat alveolar macrophage function. *Environ. Res.* **A 81**, 309–315.
- Nathan, C. F., Murray, H. W., Wiebe, M. E., and Rubin, B. Y. (1983). Identification of interferon- γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med.* **158**, 670–689.
- Nikula, K. J., Snipes, M. B., Barr, E. G., Griffith, W. C., Henderson, R. F., and Mauderly, J. L. (1995). Comparative pulmonary toxicities and carcinogenicities of chronically inhaled diesel exhaust and carbon black in F344 rats. *Fundam. Appl. Toxicol.* **25**, 80–94.

- Nyberg, K., Nessa, K., Johansson, A., Jarstrand, C., and Camner, P. (1996). Alveolar macrophage response to yeasts and to inert particles in humans. *J. Med. Vet. Mycol.* **34**, 11-17.
- Oberdörster, G., Ferin, J., and Lehnert, B. E. (1994). Correlation between particle size, *in vivo* particle persistence, and lung injury. *Environ. Health Perspect.* **102**, 173-179.
- Philipson, K., Falk, R., Gustafsson, J., and Camner, P. (1996). Long-term lung clearance of ¹⁹⁵Au labelled Teflon particles in humans. *Exp. Lung Res.* **22**, 65-83.
- Pope, C. A., III, Bates, D. V., and Raizenne, M. E. (1995). Health effects of particulate air pollution: Time for reassessment. *Environ. Health Perspect.* **103**, 472-480.
- Rudell, B., Blomberg, A., Helleday, R., Ledin, M. C., Lundbäck, B., Stjernberg, N., Hörstedt, P., and Sandström, T. (1999). Bronchoalveolar inflammation after exposure to diesel exhaust: Comparison between unfiltered and particle trap filtered exhaust. *Occup. Environ. Med.* **56**, 527-534.