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Alveolar Macrophage Interaction with Air Pollution Particulates

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We applied flow cytometric analysis to characterize the *in vitro* response of alveolar macrophages (AM) to air pollution particulates. Normal hamster AM were incubated with varying concentrations of residual oil fly ash (ROFA) or concentrated ambient air particulates (CAP). We found a dose-dependent increase in AM-associated right angle light scatter (RAS) after uptake of ROFA (e.g., mean channel number 149.4 ± 6.5 , 102.5 ± 4.1 , 75.8 ± 3.5 , and 61.0 ± 4.6 at 200, 100, 50, and 25 mg/ml, respectively) or CAP. A role for scavenger-type receptors (SR) in AM uptake of components of ROFA and CAP was identified by marked inhibition of RAS increases in AM pretreated with the specific SR inhibitor polyinosinic acid. We combined measurement of particle uptake (RAS) with flow cytometric analysis of intracellular oxidation of dichlorofluorescein. Both ROFA and CAP caused a dose-related intracellular oxidant stress within AM, comparable to that seen with phorbol myristate acetate (PMA) (e.g., fold increase over control, 6.6 ± 0.4 , 3.6 ± 0.4 , 4.6 ± 0.5 , 200 mg/ml ROFA, 100 mg/ml ROFA, and 10^{-7} M PMA, respectively). We conclude that flow cytometry of RAS increases provides a useful relative measurement of AM uptake of complex particulates within ROFA and CAP. Both ROFA and CAP cause substantial intracellular oxidant stress within AM, which may contribute to subsequent cell activation and production of proinflammatory mediators. — *Environ Health Perspect* 105(Suppl 5):1191–1195 (1997)

Key words: alveolar macrophage, flow cytometry, particles, phagocytosis, oxidant stress, scavenger receptor

Introduction

Alveolar macrophages (AM) ingest and remove inhaled particulates from the lung (1). The health effects of inhaled particulates range from minimal changes to profound acute or chronic injury (2). Recent epidemiologic data have identified an important association between levels of respirable ambient particulates and morbidity and mortality in the population (3–5). The precise mechanism of these effects by particles and the role of AM remain to be identified.

We applied flow cytometric analysis to characterize the *in vitro* response of AM to air pollution particulates. In previous studies of AM interaction with well-defined,

homogeneous particles (latex beads, monotypic TiO_2 or SiO_2), we established multiparameter methods to measure both uptake by AM and intracellular oxidant responses [e.g., respiratory burst following uptake of pathogenic SiO_2 but not inert TiO_2 (6–8)]. This approach identified scavenger-type receptors as important in the AM binding of unopsonized particles such as latex beads, TiO_2 or quartz (9).

In the studies reported here, our purpose was to determine the utility of this approach for *in vitro* study of AM interaction with two particulates of heterogeneous composition relevant to experimental analysis of air pollution particulates: residual oil fly ash

(ROFA) particulate collected from a power plant furnace and concentrated ambient air particles (CAP) collected by the Harvard concentrator as described (10). We wanted to determine specifically if flow cytometry could provide quantitation of AM uptake and intracellular oxidant response to these heterogeneous particulates. We also investigated the role of scavenger-type receptors in AM binding of these particle samples.

Materials and Methods

Particles

ROFA particles collected from a local power plant were generously provided by J. Godleski (Harvard School of Public Health, Boston, MA) (11). Particles were collected from an oil-burning power plant in the Boston area. These particles were gathered at the electrostatic filter (the final step in environmental controls at the plant) and stored in our laboratory. Known quantities of ROFA were resuspended in sterile balanced salt solution (BSS⁺) for use. ROFA suspensions were sonicated for 1 min in a bath sonicator before use. The size profile of the suspended particles by light scatter analysis resembled that of TiO_2 particles, with mean diameter of approximately 1 μm . CAP of respirable size (0.1–2.5 μm) were collected onto preweighed filters (Teflo, 47 mm diameter, 2- μm pore size; Gelman Sciences, Ann Arbor, MI) by the Harvard concentrator (10). Portions of filters were cut into small strips, placed into sterile, deionized water, and sonicated (1 \times 3 min bath sonication, followed by 3 \times 1 min probe sonication) to release particulates. After sonication, filter pieces were removed and the resulting suspension of particles was used as a stock solution for experiments with AM. In some cases, aliquots were passed through preweighed Millipore filters to re-collect and weigh the particulate actually resuspended from the original filter. All reagents not otherwise specified were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Isolation

Hamster AM were obtained by repeated bronchoalveolar lavage of Syrian golden-hamsters (Charles River Breeding Laboratories, Wilmington, MA) after being euthanized by ip injection. Bronchoalveolar lavage fluid (BAL) was centrifuged at $150 \times g$ for 10 min and resuspended in BSS⁺ containing 124 mM NaCl, 5.8 mM

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Abbreviations used: AM, alveolar macrophage(s); BSS, balanced salt solution; CAP, concentrated ambient air particulate(s); DCF, dichlorofluorescein; PI, polyinosinic acid; PMA, phorbol myristate acetate; RAS, right angle light scatter; ROFA, residual oil fly ash; SR, scavenger-type receptor(s).

KCl, 10 mM dextrose, and 20 mM HEPES containing 0.3 mM CaCl₂, and 1.0 mM MgCl₂; the resulting suspension was then titrated with NaOH to pH 7.4 before use. Cell counts and viability were determined using a hemocytometer and trypan blue dye exclusion. Cells were then adjusted to 0.5×10^6 cells/ml in BSS⁺ for experimental use.

Phagocytosis Assay

Aliquots of hamster AM (0.5 ml, 0.5×10^6 cells/ml) were incubated as suspensions at 37°C in 1.5-ml microfuge tubes on an end-end rotator in the presence or absence of particulates. ROFA particulates were added at concentrations between 0 and 200 mg/ml based on preliminary experiments to determine the concentrations resulting in AM uptake of particles and right angle light scatter (RAS) changes. CAP suspensions were added at stated dilutions. In some experiments, data on the actual particulate mass present was obtained and is provided. After incubation for 30 min with particulates, the samples were removed and propidium iodide was added to measure cell viability. The cells were immediately analyzed by flow cytometry. Viability was greater than 90%. In some experiments, to test for inhibition of particle binding by soluble receptor ligands, aliquots of potential inhibitors were added to AM 5 min before particle addition.

Flow Cytometric Measurement of Particle Uptake by Alveolar Macrophages

Flow cytometry was performed using an Ortho 2150 Cytofluorograph (Ortho Diagnostics, Westwood, MA) equipped with an air-cooled argon laser (488 nm excitation line, 15 mW output) and a Cyclops data acquisition and analysis hardware/software package (Cytomation, Fort Collins, CO). RAS (488 nm) was collected unfiltered at 90° incident to the flow cell. The number of cells analyzed ranged from 3000 to 10000 per sample. Data acquisition and analysis of AM uptake of nonfluorescent environmental particles were measured using the increased RAS signal caused by these granular materials (8). The same photomultiplier gain setting for the RAS parameter was used for all experiments to facilitate interexperiment comparisons. Unbound particles were substantially smaller than AM and were removed from the gated window by adjusting the electronic threshold settings when samples incubated in the presence of particulates were analyzed.

Intracellular Oxidant Production

We used flow cytometric assay of intracellular oxidant production, which relies on the oxidative generation of a fluorescent reporter, dichlorofluorescein (DCF), from a nonfluorescent precursor (DCFH) (6,7).

In experiments using DCFH analysis to compare effects of environmental particulates on AM respiratory burst, AM were preincubated for 5 min with 20 μM DCFH-diacetate (Molecular Probes, Eugene, OR) followed by incubation with aliquots of

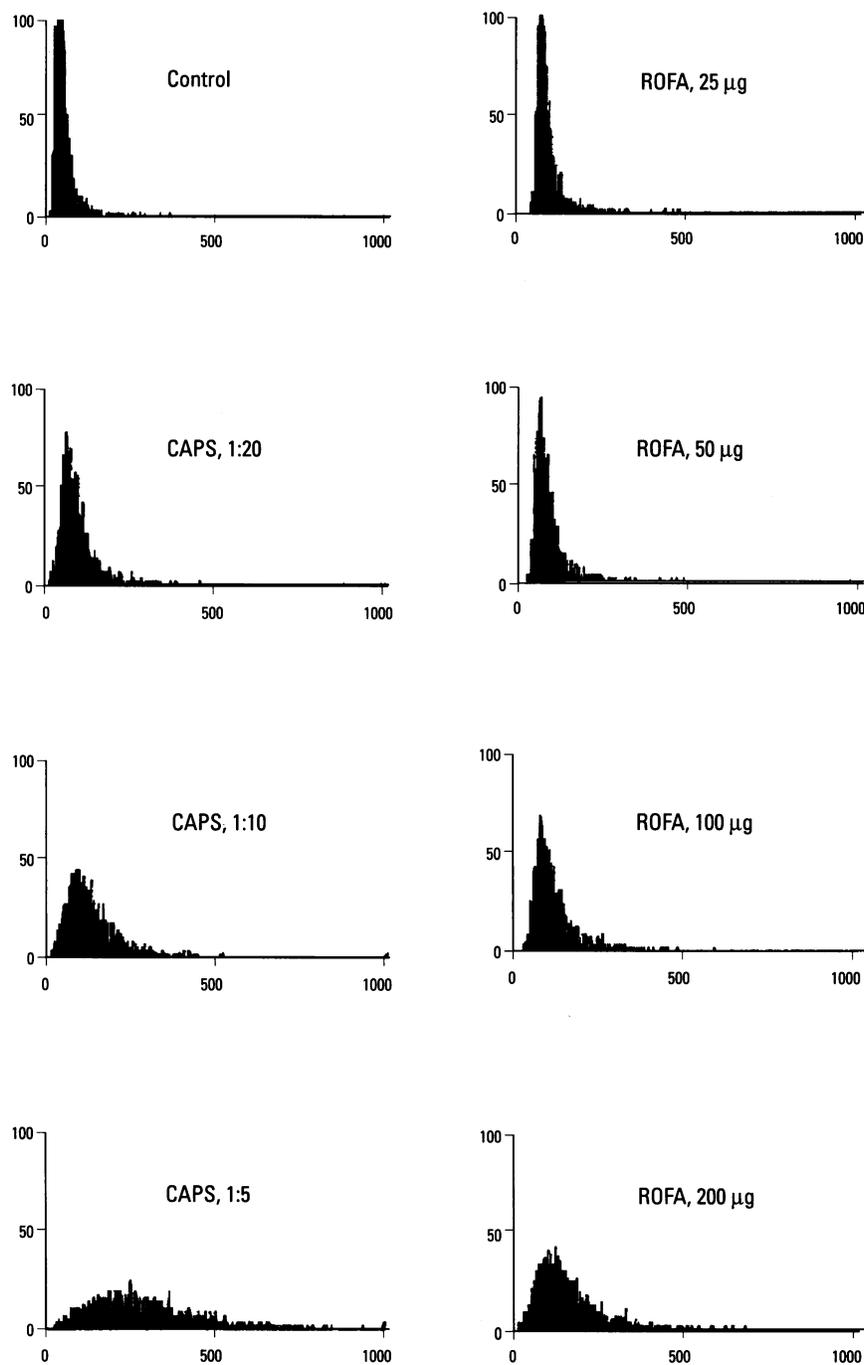


Figure 1. AM uptake of CAP and ROFA causes a dose-dependent increase in RAS, a flow cytometric parameter reflecting cell granularity. Histograms show RAS (x-axis) vs number of cells (y-axis) of AM incubated with either control buffer, CAP, or ROFA at the indicated concentrations per milliliter. CAP preparations were expressed as dilutions of particulates (see "Materials and Methods" and "Discussion"). These representative results illustrate data from 5000 cells per sample.

particulates as described above in the phagocytosis protocol. Cells were analyzed for both particle uptake by RAS measurements and for intracellular oxidative metabolism by green fluorescence.

Results

Alveolar Macrophage Uptake of Particulates

We found that normal hamster AM showed avid uptake of particulates in both ROFA and CAP samples. This uptake could be seen easily by microscope (not shown) and resulted in quantifiable increases in RAS, a parameter relating to cell granularity that was measured by flow cytometry. Figure 1 shows histogram data from a representative experiment that illustrate the increases in RAS found after AM interaction with increasing concentrations of ROFA or CAP. Figure 2 summarizes the dose-response data obtained in several experiments with ROFA. Similar dose-response data were obtained with CAP (Table 1). In addition, CAP samples collected on several different days gave similar results (not shown). For both particle types, histograms of RAS show increases of the whole population rather than preferential particle uptake by a discrete subpopulation (Figure 1).

Role of Scavenger-type Receptors

Scavenger-type receptors (SR) mediate AM binding of certain inert and environmental particulates (latex beads, titanium dioxide, quartz, iron oxide) but not others (diesel engine exhaust dust) (9). The macrophage-type SR found on AM binds several, but not all, polyanions. Hence, we measured the effect of a specific polyanionic inhibitor of the macrophage-type scavenger receptor (polyinosinic acid [PI]) on AM binding of

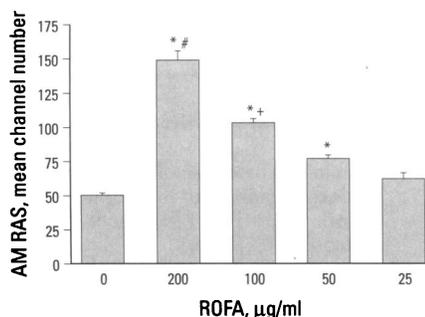


Figure 2. RAS increase after AM uptake of ROFA. A dose-dependent increase in AM RAS (expressed as mean channel number) is reproducibly seen. ($n > 4$). * $p < 0.05$ vs negative control; # $p < 0.05$ vs 100 µg, 50 µg, and 25 µg; + $p < 0.05$ vs 50 µg.

ROFA and CAP. The polyanions chondroitin sulfate and heparin, which do not inhibit SR binding, were used as control reagents. We found substantial inhibition of ROFA and CAP binding as measured by increases in AM RAS when AM were pre-treated with PI (Figures 3 and 4). Chondroitin sulfate and heparin had no effect. While inhibition was 80 to 90% for ROFA binding, Figure 3 shows that a smaller portion of CAP particulates are bound by SR pathways (see "Discussion").

Alveolar Macrophage Intracellular Oxidant Stress

Intracellular oxidant stress within AM may contribute to signaling, activation and subsequent production of proinflammatory mediators (12-14), which, in turn, could mediate health effects associated with particulates. To measure AM intracellular

oxidant stress, we used the well-characterized reporter DCFH (6,7), which is loaded into cells as a nonfluorescent precursor but becomes fluorescent upon oxidation. We measured the intracellular oxidation of DCFH in AM incubated with buffer only, phorbol myristate acetate (PMA), a known AM activator, and ROFA or CAP particulates. We found substantial increases in intracellular fluorescence, indicating particle-associated oxidant stress. Figure 5 shows typical histograms of AM fluorescence for control cells, PMA-stimulated, and particle-stimulated cells. We observed similar DCFH oxidation in AM with comparable uptake of ROFA and CAP, as measured by increases in RAS over control (Figure 5; see "Discussion"). A dose-response relationship in DCFH oxidation was observed for ROFA particles (Figure 6) and for CAP samples (Table 1).

Table 1. AM interaction with CAP results in increased RAS and intracellular oxidant stress (DCFH oxidation).

Treatment	Experiment 1		Experiment 2	
	Particle uptake, RAS	AM DCFH oxidation	Particle uptake, RAS	AM DCFH oxidation
Buffer	56	76	47	95
Buffer 1:2	56	74	46	99
Buffer 1:5	58	73		
CAP	110	186	279	240
CAP 1:2	87	132	138	266
CAP 1:5	71	105	89	186

AM loaded with DCFH were incubated with serial dilutions of CAP prepared as described in "Materials and Methods." Actual mass for the CAP solutions used in these two representative experiments was approximately 20, 10, and 4 µg/ml, respectively. Both particle uptake (mean RAS) and AM DCFH oxidation (mean green fluorescence channel number) were measured by flow cytometry for 5000 cells per sample. Controls (buffer) were matched volumes of diluent (water) used for the CAP samples.

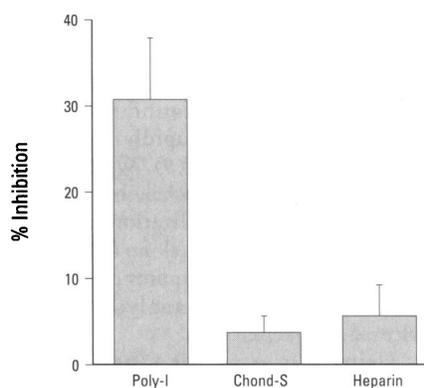


Figure 3. Inhibition of AM binding of CAP by the scavenger-receptor ligand PI (10 µg/ml). PI causes a $31.0 \pm 7.0\%$ inhibition but control polyanions (Poly-I), chondroitin sulfate (Chond-S) and heparin (10 mg/ml), caused no significant change. The heterogeneous particles within CAP include those that interact with AM SR as well as a substantial proportion that bind via other receptor(s).

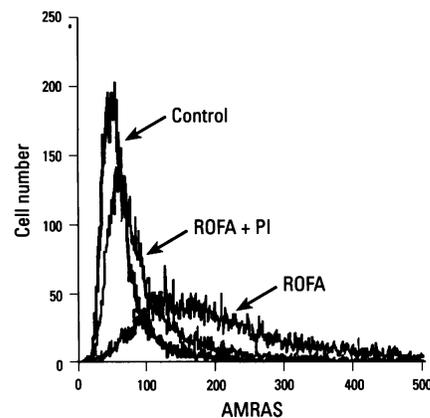


Figure 4. Inhibition of AM binding of ROFA by the scavenger-receptor ligand PI (10 mg/ml). This representative histogram illustrates findings that PI causes an almost complete inhibition in AM RAS increase (particle binding) caused by ROFA.

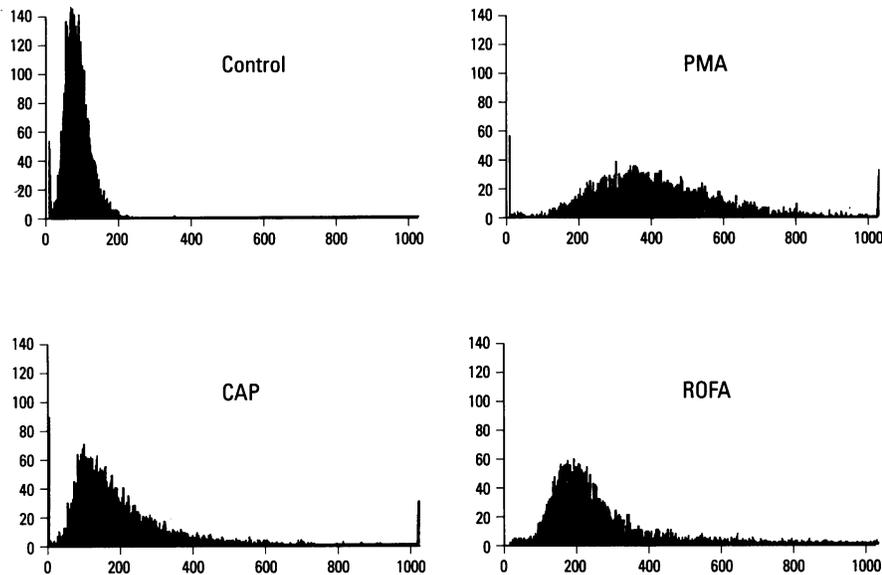


Figure 5. AM uptake of CAP and ROFA causes a dose-dependent increase in intracellular oxidation of the reporter molecule DCFH. Histograms show green fluorescence channel number (x-axis) vs cell number (y-axis) of AM incubated with either control buffer, positive control PMA (10^{-7} M), CAP (1:5 dilution), or ROFA (100 μ g/ml). In this experiment these concentrations of particulates resulted in similar particle uptake as reflected in identical increases in AM RAS (RAS mean channel number: 56, 110, and 110 for control, CAP, and ROFA, respectively). These results illustrate data from 5000 cells per sample.

Discussion

In vitro analysis of AM interaction with air pollution particulates may provide mechanistic insights into their biologic effects. Measurement of the amount of particle uptake (or dose) associated with subsequent AM responses (e.g., oxidant production, cytokine release, etc.) is important to establish dose-response relationships and to gauge biologic relevance. We have found that flow cytometry offers a useful, rapid method to measure the relative amount of air pollution particulate uptake and intracellular oxidant response by AM *in vitro*. We reported that AM uptake of inert particles such as latex beads or well-defined, homogeneous environmental particles (TiO_2 , α -quartz, iron oxide, diesel engine dust) results in increases in AM RAS easily measured by flow cytometry (8,9). We report here that this method can also provide quantitation of AM uptake of the heterogeneous particulates found in ROFA or CAP.

The advantages of this methodology include ease, rapidity, and the ability to combine particle quantitation with other fluorescent probes of cell responses (e.g., DCFH for oxidant stress). When using the same source of particulate (the ROFA sample) in several experiments using AM from different animals, the reproducibility of dose-related RAS increases was satisfactory

(Figure 2). Similar results were noted with repeated analyses of the same CAP sample (results not shown).

There are a number of limitations to this methodology. It does not provide an absolute number or mass for particle uptake, but only a relative measure compared to control cells. Because quantitation by counting the number of particles per cell is laborious, the rapidity, ease, and reproducibility of RAS as a relative measure of uptake makes this method a desirable alternative. The assay measures particle uptake—a combination of particle binding and internalization. Previous studies found that under these conditions, AM rapidly internalize most bound particles (8,9). When necessary experimentally, cytochalasin D can be used to prevent internalization, allowing measurement of binding alone (9). A final disadvantage is that it requires cells in suspension, precluding analysis of AM cultured as adherent cells.

Using this assay, we found a dose-dependent increase in AM oxidant stress upon uptake of either ROFA or CAP. In preliminary studies, we found oxidant components in both the particulate and soluble fraction of ROFA and CAP suspensions. Additional studies are under way to further characterize these components. We also obtained evidence that macrophage-type SR mediate a substantial proportion of AM

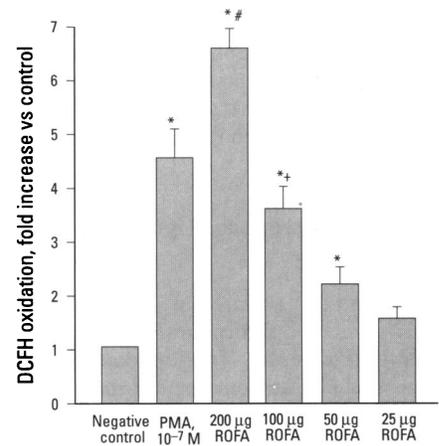


Figure 6. Increase in intracellular oxidant stress after AM uptake of ROFA. A dose-dependent increase in AM oxidation of intracellular DCFH reporter molecule (expressed as fold increase of mean green fluorescence channel number over control) is reproducibly seen; $n > 4$. * $p < 0.05$ vs negative control; # $p < 0.05$ vs PMA, 100 μ g, 50 μ g, and 25 μ g ROFA; + $p < 0.05$ vs 50 μ g ROFA.

binding of these particles. The ROFA we used has been extensively combusted and contains little carbonaceous particulate (J Godleski, personal communication). In contrast, the CAP samples contain substantial amounts of carbon-based particles, which we interpret as responsible for the non-SR component of particle uptake observed in Figure 4. We previously found no effect of SR ligand inhibitors on AM uptake of carbonaceous diesel dust (9). Since the SR mediate uptake of both inert (e.g., TiO_2) and proinflammatory (e.g., quartz) particles without evidence of receptor-mediated cell activation, our working hypothesis is that particle-associated components (e.g., transition metals) are likely to mediate intracellular oxidant stress and proinflammatory activation.

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