Differences in the biological activity of two PM_{3.3} components: carbonaceous and silica particles

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Summary. - In this study we compared the biological reactivity of $PM_{3,3}$ with those of carbon black (CB) and respirable silica particles, monitored by *in vitro* hemolytic potential and morphological alterations, in order to evaluate the correlations between the different physico-chemical characteristics of the three types of particulate and their biological effects. Carbon black and silica particles were used as reference environmental particles in order to limit the number of the urban PM variables, which is a mixture highly heterogeneous. Our data suggest that the urban $PM_{3,3}$ have a similar surface reactivity as CB. In fact, when the percent of hemolysis were plotted against particle surface per volume units, the $PM_{3,3}$ activity did not differ significantly from that of CB. This observation is in agreement with the SEM morphological evaluations of treated erythrocytes because the more abundant alteration in $PM_{3,3}$ -treated cells was the stomatocytic transformation (main feature of CB-treated red blood cells), followed by echinocytic transformation (observed in silica-treated cells).

Key words: hemolytic potential, urban PM_{3.3}, carbon black, silica, erythrocyte morphological alterations.

Riassunto (*Differenze nella reattività biologica di due componenti del* $PM_{3,3}$: particelle carboniose e di silice). - In questa indagine la reattività biologica del $PM_{3,3}$ è stata comparata con quella del carbon black e della silice respirabile, valutata in termini di potenziale emolitico e alterazioni morfologici indotte dai tre tipi di particolato sugli eritrociti, in modo da valutare le correzioni tra le loro differenti caratteristiche fisicochimiche ed i corrispondenti effetti biologici. Il carbon black e la silice sono stati usati come materiali di riferimento al fine di limitare il numero di variabili del PM urbano, estremamente complesso ed eterogeneo. I nostri risultati suggeriscono che il $PM_{3,3}$ ha una reattività superficiale simile a quella del CB; infatti quando la percentuale di emolisi viene graficata in funzione della superficie della particella per unità di volume della sospensione, l'attività del $PM_{3,3}$ non differisce in maniera significativa da quella del CB. Questa osservazione è in accordo con i risultati delle alterazioni morfologiche osservate al SEM sugli eritrociti trattati, dal momento che la più abbondante trasformazione negli eritrociti trattati con $PM_{3,3}$ è stata di tipo stomatocita (alterazione principale osservata negli eritrociti trattati con CB), seguita da quella di tipo echinocita (osservata negli eritrociti trattati con silice).

Parole chiave: potenziale emolitico, PM_{3,3} urbano, carbon black, silice, alterazioni morfologiche degli eritrociti.

Introduction

Exposure to airborne particulate matter in occupational and environmental air has been associated with a variety of diseases, including lung, immunological and cardiovascular diseases, in humans and experimental animals. Although a cause to effect relationship is proposed, the mechanisms of action remain elusive and, consequently, there is a need to assess in more details the biological reactivity of particles in terms of toxicity determinants, mechanisms of effects and potency. The biological effects of particles are determined by their physical and chemical nature, the physics of deposition and distribution in the respiratory tract, and the physiologic events occurring in response to the particle presence.

Urban PM is a highly heterogeneous, physicalchemical mixture which varies within and among cities, with season, weather condition and time of the day.

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Major questions remain as the physico-chemical parameters involved in particle toxicity, size, shape, surface, mass, chemical composition and reactivity. Experimental studies have shown a better correlation between particle surface area and effects, rather than particle mass relative to an equal chemical composition.

In order to limit the number of variables, in this study we elected to compare the biological reactivity of $PM_{3,3}$ with those of synthetic carbon black (CB) and respirable size crystalline silica particles as reference environmental particles.

Hemolysis by mineral particles has been used as a marker of cellular membrane damage and the role of membranolysis in the induction of particles-related pathogenic effects has been widely investigated [1, 2]. The biological activity of particles has been attributed to the surface functionalities and in particular to the generation of free radicals and the induction of oxidative stress which seem to be related to surface area. Razzaboni and Bolsaitis [3] have shown evidence of the correlation between oxidative mechanism and the hemolytic activity of silica particles.

In this study we investigated the *in vitro* hemolytic potential and morphological alterations of human erythrocytes exposed to three types of particles: carbon black (CB) (Fig. 1a), silica (Fig. 1b) and the fine fraction of urban particulate matter (Fig. 1c).

Materials and methods

Particulate suspension preparation

CB samples were kindly supplied by K. Donaldson. Silica particles were produced by grinding for 30 min Min-U-Sil 5-quartz by means of a micronizing mill (McCrone, London,UK).

Stock suspensions of silica and CB were prepared in isotonic buffer solution. To eliminate unbreatheable silica particles, the suspension was kept in the upright position for 10 min to allow for separation by sedimentation. 35 ml of the upper part of suspension, after the sedimentation, were used for experiments.

The airborne particulate was sampled in Rome, in an area with moderate vehicular traffic, by an eight-stage cascade impactor (Andersen particle fractionating sampler) with a preseparator stage able to eliminate particles with aerodynamic diameter above 10 μ m. The flow rate of the sampler was 28,317 l/min. The sampling was conducted in April and lasted 15 days. In this work we studied the "fine" particulate fraction with aerodynamic diameter ranging from 3.3 to 0.4 μ m (PM_{3,3}). The PM_{3,3} concentration was 9.2 ± 0.1 μ g/m³.

After sampling the stainless disks of the cascade impactor were put in a sonication bath with ethyl alcohol to remove the particulate matter collected.



Fig. 1. - a) Carbon black (CB). SE image (6000x); b) Silica particles. SE image (2000x); c) Fine fraction of urban particulate ranging from 3.3 to 0.4 μ m aerodynamic diameter (PM_{3.3}). SE image (2000x).

Particulate matters analysis by automated scanning electron microscopy (SEM)

Suspensions of CB, silica and $PM_{3.3}$, were filtered on polycarbonate membranes and portions of filters were mounted on SEM stubs, coated with a thin carbon film.

The analysis of the particle samples were performed by a SEM Philips XL30 equipped with a thin-window EDAX DX4 system for X-ray microanalysis by energy dispersion spectrometry [4]. For each sample at least 3000 particles were randomly selected and automatically detected by an increase in the secondary electron (SE) and in the back-scattered electron video signal above a preset video threshold. Twenty X-ray regions of interest (ROI) were used to detect the presence of C, O, Na, Mg, Al, Si, S, Cl, Cd, K, Ca, Ti, Cr, Fe, Ni, Cu, Zn, Pb and Br in the fine fraction (PM_{3,3}) of urban particulate matter and an EDX spectrum was acquired for 40 s at the particles centre. The intensities of the characteristic X-ray lines were converted in the corresponding atomic concentration by a standardless ZAF correction method [5].

The data set obtained by X-ray microanalysis of $PM_{3,3}$ were analysed using the hierarchical cluster analysis to classify the particles into groups with similar chemical composition [6].

We adopted the "squared Euclidean distance" as the distance measure between particles and the Ward's error sum method in order to agglomerate the clusters.

Hemolysis experiments

The in vitro cell cytotoxicity assay we used in this comparative study was the red blood cells lysis assay as a membrane integrity endpoint. The assay is based on short term (1 h) exposure of human red blood cells obtained by healthy volunteers to particulate matter concentrations ranging from 60 to 600 µg/ml. Blood samples were washed in isotonic salt solutions at 2500 rpm for 10 min until clarification of supernatants. Finally, the red blood cells were suspended at a concentration of about 4% in isotonic saline solution. These red blood cells suspensions were added with particles suspensions in the same medium at the appropriate concentrations. Incubation was carried out at 20 °C for 60 min with gentle shaking. The degree of hemolysis was quantified by spectrophotometric measurement of the absorbance at 541 nm to determine hemoglobin release [7]. Results were expressed as percentage hemolysis relative to the total lysis control.

Erythrocyte morphology by electron microscopy

Following 1 h exposure to particles, the erythrocytes were centrifuged and then fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer. Fixed erythrocyte were let adhere to polylysine-coated coverslips and, after rinsing in 0.1 M phosphate buffer, postfixed in 1% OsO₄ in 0.1 M cacodylate buffer for 1 h. After thorough rinsing in 0.1 M phosphate buffer, specimens were dehydrated in a graded series of ethanol and transferred into liquid CO₂ in a critical point dryer. The dried specimens were mounted, sputter-coated with gold and examined in a SEM at 25 kV.

Results

Particles analysis results

The mean equivalent diameter of the three particulate samples were determined as being 280 nm for the CB, 820 nm and 920 nm for silica and PM_{3,3}, respectively.

The hierarchical cluster analysis allowed us to identify seven principal particle types in the fine particulate matter: C-rich particles, Ca-carbonates, Casulphates, silica, silicates, Fe-rich particles and metals compounds (Fig. 2).

The C-rich particles prevail in the PM_{3,3} and the most significant source is motor vehicle exhausts. These particles show a surface coating containing S and N, or sometimes S, K, Na, Ca. The abundance of C-rich particles with the "sulfuric" coating is 39%. Ca-carbonates, silica and silicates derive by the soil erosion and building structure erosion and on the whole in the PM_{3,3} their abundance amount to about 18%. Ca- sulphates abundance is 8%; on the whole the abundance of Ferich particles (Fe > 50%) and metals compounds (Al, Cr, Ni, Ti, Zn, Cu) amounts about to 4%.

Hemolysis results

Hemolysis test results for the three types of particulate are reported in Fig. 3 for weight per volume unit (Fig. 3a) and particle surface per volume unit of the suspension under investigation (Fig. 3b).

When the weight per volume unit (μ g/ml) was used for particle dosing, our results showed: a) a greater activity of silica compared with the carbonaceous particles; b) a dose-dependent percent hemolysis for all kinds of particulate matters; c) a hemolytic potential of PM_{3.3} higher than that of CB.

When the surface per volume unit (μ m²/ml) was used the hemolysis curves of the PM_{3.3} and CB did not show significant differences whereas for a given surface per unit volume value, silica had a hemolytic activity much greater than that of fine particulate and CB.



Fig. 2. - Principal particle types (clusters) identified in the $PM_{3,3}$ by hierarchical cluster analysis.

Our results suggest that the hemolytic activity of $PM_{3,3}$ could be dependent on the chemical composition of the $PM_{3,3}$ which is composed by 69% of carbonaceous particles.

Morphological evaluation results

In order to express semiquantitatively the extent of the types of shape transformations [8] we used the morphological index (MI) defined as below:

MI: = \sum (morphological score) x transformed cell number / total cell number.

Morphological scores from 1 to 4 were assigned according to various stages of the shape change.

The larger the MI figure, the more severe the shape change. Erythrocytes morphology showed alterations in all specimens: echinocytic transformation was observed in silica-treated red blood cells (MI = 0.75) (Fig. 4c); stomatocytic transformation was the main feature of CB (MI = 1.30) (Fig. 4b) treated red blood cells while both shapes were present in PM_{3.3} treated cells. Erythrocytes exposed to PM_{3.3} showed either echinocytic (up to 27% of total cell number) with MI = 0.60 or stomatocytic alterations (50% of total cell number) with MI = 1.48 (Fig. 4d).

Discussion

In this study we compared the hemolytic potential of different particles as a screening test for cytotoxic potential of particulate matter and in order to address the correlations between their physico-chemical characteristics and the biological effects.

Indepentently from the x-axis choice (Fig. 3) the hemolitic activity of fractured silica particles resulted to be higher than that exibited by both CB and $PM_{3.3}$ particles.

When the percent of hemolysis is plotted against particle weight, the $PM_{3,3}$ activity seems to be stronger than that of CB. However, when the results were presented in terms of surface per volume unit the hemolysis curves of the $PM_{3,3}$ and CB did not show significant differences.

A large body of experimental studies suggest that the biological activity of mineral particles depend on their surface chemistry. Our results suggest that ambient $PM_{3,3}$ particles have a similar surface reactivity as CB particles. This could be explained by the chemical composition of $PM_{3,3}$, which is formed for up to 69% of carbonaceous particles similar to CB.

Highly hemolytic silica particles are also present in $PM_{3,3}$ sample, but their activity is not detectable. This could be explained either by its small percentage (2%) in the ambient PM, or by the surface modification of silica in the ambient samples and/or by a different activity due to the influence of the carbonaceous particles in the mixture. These observations are consistent with previous findings from other authors as well as with ours. In fact, in a previous study [4] on the PM_{10} composition we observed a "sulfuric" coating on 30% of the total number of Sirich particles (silica and silicates) in the sample which could influence the surface activity of silica particles. Moreover Liu *et al.* [9] showed that coal fly ash (Si-content 53%) hemolytic activity was about on fifth that of pure silica at equal particle size and the hemolysis did not correlate with Si-content. The authors concluded that coal fly ash had an attenuating effect on the quartz induced effects.

SEM morphological evaluations of treated erythrocytes, performed to evaluate the induction of shape transformations and, consequently, the membraneperturbing effects of particles, are in agreement with



Fig. 3. - a) Hemolysis percentage *vs* particulate concentration; b) Hemolysis percentage *vs* particle surface per volume unit.



Fig. 4. - a) Control red blood cells showing normal discocytic shape. SE image (3000x); b) treatment with CB particles (600 μ g/ml) induces the discocyte-stomatocyte transition. SE image (3200x); c) silica-treated red blood cells (600 μ g/ml) showing significant echinocytic alterations. SE image (2000x); d) shape alterations induced by exposure to PM_{3.3} particles (600 μ g/ml). SE image (1641x).

this observation. Silica, CB and $PM_{3.3}$ used in this study induced different patterns of shape transformation. Echinocytic transformation was observed in silica-treated red blood cells, stomatocytic transformation was the main feature of CB treated red blood cells, whereas stomatocytic transformation represent the more abundant morphological alteration (50% of total cell number) in $PM_{3.3}$ treated cells followed by echinocytic transformation (27% of total cell number). It is possible that these different patterns reflect a different surface chemistry of particles when interacting with cell membranes. Experiments are in progress to evaluate the involvement of free radicals and metals in the induction of effects.

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