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Decreased Sialidase Activity in Alveolar Macrophages of Guinea Pigs Exposed to Coal Mine Dust

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The origin of immune dysfunctions that are observed in pneumoconiotic miners still remains unknown. There is evidence that the carbohydrate moiety of membrane glycoconjugates is of primary importance in many functions of immunocompetent cells. The glycosylation, and especially the sialylation level of membrane components of various lymphocyte and macrophage subsets, vary depending on the state of cellular differentiation and activation. Sialidases, which may regulate the amount of sialic acids exposed on the cell membrane, can thus be considered as immunoregulatory enzymes. In this report, the sialidase activity has been measured in alveolar macrophages (AM) and in cell-free bronchoalveolar lavage fluid (BALF) from guinea pigs exposed for 4 months to coal mine dust at a concentration of 300 mg/m^3. The samples were collected by bronchoalveolar lavage 2 months after cessation of exposure. The sialidase activity in the cell-free fluid and in the purified alveolar macrophages showed a 10-fold decrease ($p < 0.001$). Kinetic parameters of the enzyme such as K_m and optimum pH did not change. This changed activity was specific for sialidase, as two other lysosomal glycosidases, β-galactosidase and *N*-acetylglucosaminidase, showed unchanged activities. These results suggest the possibility that, by inducing a decreased sialidase activity, exposure to coal mine dust may lead to a modified expression of AM membrane-associated sialic acids giving rise to altered immune functions (i. e., phagocytosis, antigen processing, response to cytokines, etc.).

Introduction

Pneumoconiosis, a chronic fibrotic reaction in the lung, is caused by the inhalation of dust particles containing silica (*1,2*). In experimental models of chronic exposure to mineral dust, it has been shown that alveolar macrophages (AM) play a critical role in the development and persistence of pulmonary inflammation (*3*). This inflammation is thought to be partly due to lysosomal enzymes released from lysed AM. Particulate silica ingested by AM has the potential to damage the integrity of cell membranes, and studies have suggested that lysosomal enzymes are discharged into the cytoplasm after phagolysosomal membrane disruption (*4-6*). The mechanisms by which particles are recognized and ingested by AM remain unclear. Evidence suggests that particles bind to cells through electrostatic forces (*7*). In that context, due to their exposed position on the cell membrane and their negative charge, sialic acid (SA) residues have been considered important. For instance, sialic acid has the capacity to mediate the initial binding of positively charged particles *in vitro* (*8*); also, it is implicated in the interactions of coal fly ash with mammalian cells (*9*). Furthermore, sialic acid residures are important in modulating the biochemical properties of many membrane glycoconjugates (*10*), expressed by immunocompetent cells (*11*). The sialylation of membrane sialoglycoconjugates is regulated by two kinds of enzymes, namely, sialyltransferases and sialidases. Factors that modify or alter sialidase activity can influence a diversity of physiological phenomena within cells, on cell surfaces, and in circulating body fluids (*12*). Sialidases (E.C.3.2.1.18; acylneuraminyl hydrolases) catalyze the hydrolysis of sialic acid residues α-ketosidically bound to oligosaccharides, glycoproteins, and glycolipids. These enzymes are widely distributed in eukaryotic and prokaryotic cells (*13*). A large body of evidence suggests that, in eukaryote, the total sialidase activity is actually a composite of at least three types of activities differing from each other both in their substrate specificity and in their localization within the cell. After tissue homogenization and differential centrifugation, sialidase activities have been demonstrated in the lysosomal, cytosolic, and membrane subcellular fractions; the lysosome-associated form being the predominant one (*13*).

In a rabbit model, lysosomal enzymes such as β-glucuronidase or acid phosphatases are released in the bronchoalveolar lavage fluid (BALF) after intratracheal injection of quartz (*14*). Because AM possess lysosomal sialidase activity (*15*) and because this enzyme may participate in the control of the degree of sialylation of cell surface glycoconjugates resulting in changed biological

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properties of the cells (*16,17*), we investigated the effect of coal mine dust exposure on the sialidase activity in AM and in BALF of guinea pigs.

Materials and Methods

Animals and Dust Exposure

Two-month-old, noninbred, male Hartley guinea pigs (300 g) were divided into a control nontreated group ($n = 6$) and a coal mine dust-exposed group ($n = 13$). The coal mine dust was from Ostricourt (France). The size distribution of the particles as determined by electron microscopy was 3 μm<2.21% <4.5μm, 2 μm<6.94% <3 μm, 1 μm <25.87% <2 μm, and 64.98% <1 μm. Guinea pigs were housed in a stainless-steel exposure chamber (*18*). The controlled dust generators operated 6 hr/day, 5 days/week, for 4 months. The dust concentration was adjusted to 300 mg/m^3 of respirable dust. The animals were sacrificed 2 months after the end of exposure.

Bronchoalveolar Lavage

Guinea pigs were sacrificed by IP injection of a lethal dose of sodium pentobarbital. The chest was opened and the blood was collected by cardiac puncture. The trachea was intubated by tracheotomy, and bronchoalveolar lavage (BAL) was then performed with a total lavage volume of 100 mL warm phosphate-buffered saline Dulbecco buffer pH 7.2 (without Ca^{2+} or Mg^{2+}). Total cell count was performed with a Malassez hemocytometer, and differential cell counts were established on smears stained with May-Grunwald Giemsa (at least 200 cells were counted.)

The BAL was centrifuged at 300*g* for 10 min at 4°C. The BALF supernatant was concentrated 50-fold by filtration on a Diaflo PM 10 membrane (Amicon) at 4°C and then frozen and stored in 0.4-mL aliquots at −80°C. The cell pellet was resuspended in Hanks' saline solution at a concentration of 0.5 × 10^6 cells/mL, and 20 mL of the suspension was transferred into 110-mm diameter plastic petri dishes. After a 2-hr incubation at 37°C in a 5% CO_2 humid atmosphere, nonadherent cells were removed and the petri dishes were washed twice with Hanks' saline solution. Adherent cells were harvested with a Teflon scraper, resuspended at a concentration of 10^7 cells/mL in 3 mM imidazol buffer, pH 7.2, containing 250 mM sucrose and 1 mM ethylenediamine tetraacetate (EDTA). The cells were pelleted by centrifugation at 10,000*g* for 5 min at 4°C and immediately frozen at −80°C.

Enzymatic Assays

Alveolar macrophages were homogenized using a glass Teflon homogenizer with 20 strokes and assays were carried out immediately. The sialidase activity was assayed by using a fluorogenic substrate: sodium 4-methylumbelliferyl-α-D-neuraminate (Mu-Neu Ac) (*19*): 20 μL of cell homogenate or BALF was mixed with 2.5 μL of 2.5 × 10^{-3} M Mu-Neu Ac and 2.5 μL of 1 M acetic acid/sodium acetate buffer ranging in pH from 4 to 6 and incubated for 2 hr at 37°C. The reaction was stopped with 2.1 mL of 0.1 M NaOH. Fluorescence was measured on a Kontron SFM 25 fluorometer (excitation at 365 nm, emission at 450 nm). Pure methylumbelliferone was used as a standard. All assays were done in duplicate. One enzymatic unit was defined as the amount of enzyme that hydrolyzes 1 nmole of Mu-Neu Ac per hour at 37°C.

N-acetylglucosaminidase and β-galactosidase activities were assayed at their respective optimum pH using appropriate substrate (*20*). Protein concentration was determined using Bradford's method (*21*) with bovine serum albumin as a standard.

Statistical Evaluation

All the results are expressed as the mean ± SD. Statistical significance was determined by the Student's *t*-test.

Results

The number of bronchoalveolar cells obtained by lavage from the dust-exposed animals was significantly higher than from the controls (Fig. 1), 40.07 ± 13.6 and 13.10 ± 6.3 × 10^6 cells, respectively. Differential cell counts showed that the increased total cell number in dust-exposed animals was not attributable to a single cell type. In normal control guinea pigs, 77.6 ± 5.7% (Fig. 2) of the lavaged cells were alveolar macrophages, and 16.2 ± 5.0% were eosinophils; polymorphonuclear neutrophils (PMN) and lymphocytes each represented less than 3%. In dust-exposed

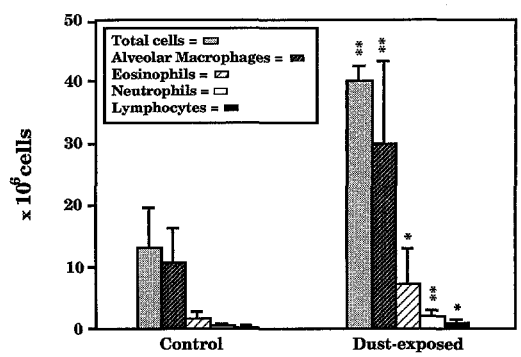

FIGURE 1. Total cell count in bronchoalveolar lavage fluid from control ($n = 6$) and dust-exposed ($n = 13$) guinea pigs. The results are expressed as means ± SD. (*) Difference between dust-exposed and control animals is significant, $p<0.05$; (**) difference between dust-exposed and control animals is highly significant, $p<0.005$.

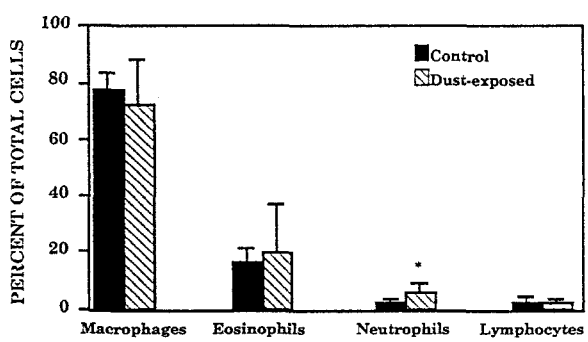

FIGURE 2. Differential cell count in bronchoalveolar lavage fluid of dust-exposed ($n = 13$) and control ($n = 6$) guinea pigs, means ± SD. (*) The fraction of neutrophils was significantly higher ($p < 0.05$) in dust-exposed than in control animals.

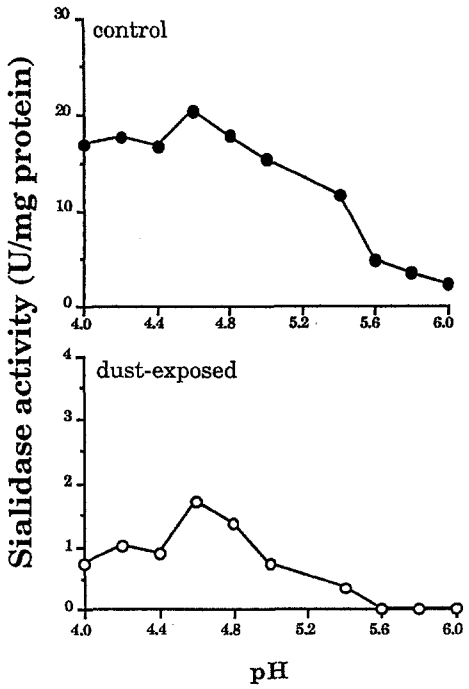

FIGURE 3. Effect of pH on alveolar macrophage sialidase activities prepared from control (●) and from dust-exposed guinea-pigs (○).

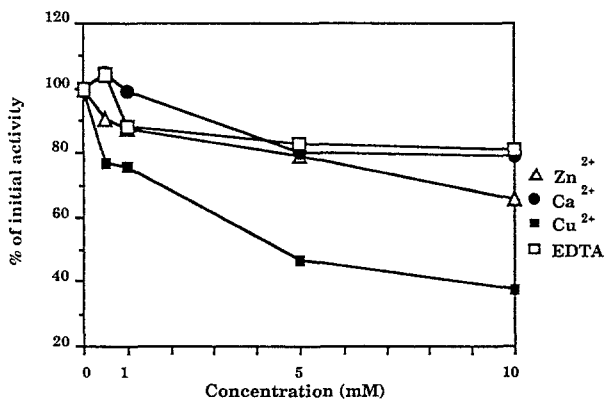

FIGURE 4. Effect of various divalent cations and EDTA on control alveolar macrophage sialidase activity. The results are expressed as percentage of the initial activity.

animals, the relative proportions of PMN increased (5.6 ± 3.6%), whereas those of the other cell types were unchanged. On smears stained with May-Grunwald Giemsa, more multinucleated giant cells were observed in dust-exposed animals (2%) than in controls (< 0.5%).

Six months after the beginning of exposure, the protein concentration in BALF was significantly ($p < 0.005$) increased: 1.65 ± 0.32 and 2.71 ± 1.03 mg/mL BALF from control and exposed animals, respectively.

Sialidase Activity in Guinea Pig Alveolar Macrophages

Sialidase activity, with an optimum pH at 4.6, was detected in the crude homogenate of control guinea pig alveolar macrophages (Fig. 3). The mean specific activity was 21.60 ± 13.49 U/mg protein. The apparent K_m value for Mu-Neu Ac at pH 4.6 was $1.08 \times 10^{-4} \pm 0.17 \times 10^{-5}$ M. The effect of EDTA and of several divalent cations (Zn^{2+}, Ca^{2+}, Cu^{2+}) at concentrations ranging from 0.5 to 10×10^{-3} M using their respective chlorides (Fig. 4). Cu^{2+} and Zn^{2+} caused a marked inhibition of the sialidase activity. At a 10 mM concentration, Cu^{2+} was able to inhibit about 60% of the initial activity. On the contrary, at low concentration (0.5 mM), Ca^{2+} and EDTA slightly activated the sialidase activity.

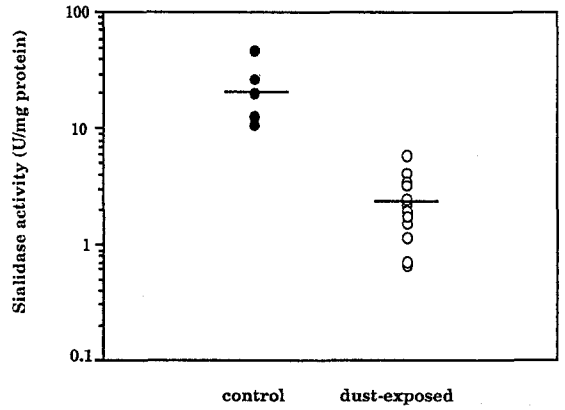

FIGURE 5. Sialidase activity in alveolar macrophages from control (●) and dust-exposed animals (○). The activity is expressed as enymatic units per milligram of protein.

Sialidase Activity in Alveolar Macrophages from Dust-Exposed Animals

To determine if any quantitative and/or qualitative changes in sialidase content occurred after coal mine dust exposure, the sialidase activity was assayed in AM from 13 dust-exposed guinea pigs. The results obtained are summarized in Figure 5. Control and exposed animals formed two distinct groups, the specific AM sialidase activity being significantly ($p < 0.025$) decreased in all dust-exposed animals. This drop in sialidase activity reached up to 90% of the initial activity: 2.35 ± 1.47 U/mg protein and 21.6 ± 13.49 U/mg protein in the exposed and control group, respectively. Although the sialidase activity decreased in the dust-exposed group, kinetic parameters such as K_m ($0.51 \pm 0.18 \times 10^{-4}$ M) or the optimum pH at 4.6 of the enzyme did not change significantly.

The major sialidase activity is known to be associated with lysosomes. One can assess that the decreased sialidase activity was specific for this enzyme because the two other lysosomal glycosidase activities that were assayed, namely, β-galactosidase and *N*-acetylglucosaminidase, were not significantly modified.

Sialidase Activity in BALF

In BALF from normal animals, a sialidase activity with an optimum pH at 4.6 was detected. A BALF sialidase activity with the same optimum pH was detected in 12 out of the 13 exposed animals. The major change in dust-exposed guinea pigs was a decrease in the total BALF sialidase activity (Fig. 6): 1.83 ± 1.46

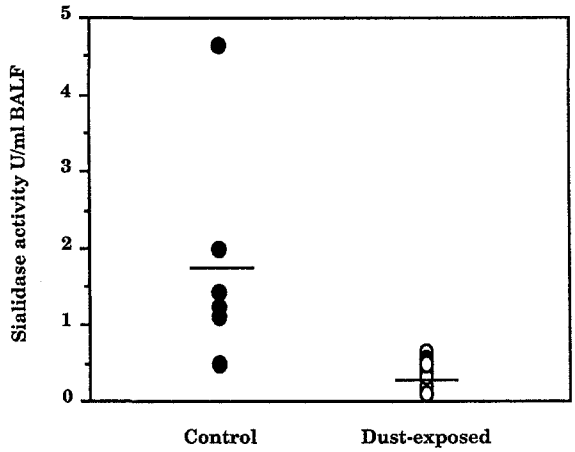

FIGURE 6. Sialidase activity in the cell-free bronchoalveolar lavage fluid (BALF) from control (●) and dust-exposed animals (○). The activity is expressed as enzymatic units per milliliter of concentrated BALF.

and 0.319 ± 0.169 U/mL BALF in the control and the exposed group, respectively.

Discussion

As assessed by a 3-fold increase in the number of cells harvested by bronchoalveolar lavage, the initial response of the lung in guinea pigs that had been exposed to coal mine dust was characterized by an acute inflammatory reaction. As a single intratracheal injection of pure silica led to the same observation in guinea pigs (*22*), silica in coal mine dust is likely to be responsible for this reaction. The local cellular recruitment could be due to the secretion of chemoattractants by dust-loaded AM (*23,24*). The relative proportion of AM in the bronchoalveolar cell population of dust-exposed animals was similar to that seen in controls. As compared to controls, more PMN were present in BAL from dust-exposed animals. In addition, the fraction of PMN was slightly but significantly increased. PMN seem to play a critical role in early cellular events occurring in several types of experimental models of pneumoconiosis (*25,26*). A controversy still exists concerning the precise role of PMN during pulmonary diseases. Depending on the experimental model, both protective and causal (*27*) roles have been attributed to PMN in the development of pulmonary interstitial diseases.

The cellular inflammation was associated with a rise in the BALF protein concentration. In another experimental model where hamsters were exposed to pure silica, a 7-fold increase in the BALF protein concentration was demonstrated (*28*). Proteins appearing in the airways may originate from various sources including serum exudation, cell lysis (*29*), local protein synthesis (*14*), and enzyme secretion. For example, increased lysosomal enzyme activities were found in BAL from mice injected intratracheally with silica (*25*) and in BAL from silica-exposed workers (*30*).

In AM-crude homogenate from control guinea pigs, the sialidase activity was optimum at pH 4.6. Similar observations were reported for rabbit AM (*15,31*). The limited number of cells recovered by bronchoalveolar lavage did not allow us to study the subcellular distribution of this activity. Previous reports have provided evidence that the bulk of sialidase activity displays acidic optimum pH and is associated with the fraction enriched in lysosomes (*15,32–34*). A cytosolic sialidase with an optimum activity at pH 5.4 (*15*) and a sialidase associated with the plasma membrane, active at pH 4.5 (*35*), have also been described. As already shown (*36*), we observed that divalent cations such as Ca^{2+} on the one hand, and Cu^{2+} and Zn^{2+} on the other hand, have opposite effects on the sialidase activity.

Sialidase activity is present in normal guinea pig BALF (*37*), whereas in humans this enzyme is only present in significant amounts in BALF from patients with interstitial lung diseases such as idiopathic pulmonary fibrosis or sarcoidosis (*38*). Nevertheless, to date, the cellular origin of this enzyme has not been definitively demonstrated. However, because AM are numerous in bronchoalveolar spaces and because sialidase activities of AM and BALF have the same optimum pH, one can speculate that BALF sialidase represents an activity released from AM.

In dust-exposed animals, sialidase activities decreased both in AM and in BALF; however, kinetic parameters such as K_m and optimum pH were not modified. Because particulate silica has been shown to alter the cellular metabolism, the decreased BALF sialidase activity may result from a decreased synthesis and/or liberation of cellular sialidase into the respiratory secretions. In the absence of cell lysis, the decreased BALF sialidase activity can also be the consequence of an enzymatic inhibition or inactivation. Numerous mechanisms including inactivation by proteases (*39*) and inhibition by cations (*36*) may result in a decreased catalytic function of sialidase in BALF from dust-exposed animals.

Macrophage sialidase activity can be up- or downregulated. Treatment of rat peritoneal macrophages with inactivated *Streptococcus pyogenes* fragments resulted in a 9-fold increase in sialidase activity (*35*). *In vivo* bacille Calmette-Guérin (BCG) stimulation of rabbits induced an upregulation of AM lysosomal sialidase (*15*), and a decreased sialidase activity was found in AM from guinea pigs treated with a glycoproteic extract from *Klebsiella pneumoniae* (*37*). So far, the precise mechanism of sialidase regulation and its direct effectiveness on the degree of sialylation of sialoglycoconjugates is not fully understood. Modifications of sialidase content with a concurrent decline in membrane-bound sialic acid have been reported. For instance, T-cell activation is accompanied by an increase in the endogenous level of sialidase activity and an hyposialylation of class I major histocompatibility complex antigen molecules (*16,17*). Previous desialylation of the target cells improves recognition and ingestion by macrophages (*40*). These results point out the importance of membrane sialic acids in cellular differentiation, activation, and biological properties including phagocytosis. It is therefore conceivable that a sialidase-mediated differential expression of sialic acids on the AM cell membrane may be a mechanism through which impaired cellular functions may participate in the development of pneumoconiosis. A study of the relative sialylation of AM membrane sialoglycoconjugates from either normal control or dust-exposed guinea pigs should help to answer this question.

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