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Toxicity of Metal Ions to Alveolar Macrophages

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Significant concentrations of metals are found in the respirable particulate effluents associated with metallurgical smelters. In this investigation the effects of the metallic ions lead, cadmium, iron, mercury, aluminum, chromium, and nickel on various aspects of alveolar macrophage function were studied. The production of antibacterial substances (ie, reactive forms of oxygen) by these cells and oxygen consumption are very sensitive to the metals. Particle uptake displays moderate sensitivity, while lysosomal enzyme activity and membrane integrity are fairly resistant to metals. In addition, the effects of the organic solvents carbon tetrachloride, toluene, and xylene on alveolar macrophage function were tested. These solvents were found to inhibit oxygen consumption and the release of antibacterial substances while not greatly affecting membrane integrity. The results of these experiments indicate that some metals and some organic substances are toxic to alveolar macrophage function.

Key words: toxicity, metals, alveolar macrophages, oxygen consumption, chemiluminescence

INTRODUCTION

Environmental exposure to airborne contaminants in the metal industry has been studied extensively by those concerned with occupational health [Wagner, 1975; NIOSH Staff, 1978; Donaldson, 1978]. Some of these airborne particles have been shown to contain significant concentrations of metals in both soluble and insoluble forms [Wagner, 1975; Lee et al, 1972, 1973; Natusch et al, 1973]. Many of the insoluble metal particles are small enough to be inhaled into the lung and deposited in the alveoli [Lee et al, 1972; Natusch et al, 1973]. Once in the lung, these metal particles may affect the normal functions of the cells lining the alveoli.

Alveolar macrophages are free lung cells located on the surface of the small airways and the alveoli [Weibel, 1973]. These cells play an important role in the protection of the lung against airborne bacteria and particles. The response of the alveolar macrophages

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to inspired bacteria and particles is complex. Upon contact with the particles, alveolar macrophages release superoxide anion (O_2^-) [Drath et al, 1976, 1978; Sweeney et al, 1978] that may be involved in the detoxification of these foreign substances [Sagone et al, 1976]. The release of superoxide anion by the alveolar macrophages after exposure to foreign particles can be monitored by the measurement of chemiluminescence [Miles et al, 1978; Castranova et al, 1979]. Exposure to foreign particles also causes large increases in the consumption of oxygen [Drath et al, 1976, 1978; Hoidal et al, 1978; Castranova et al, 1979]. Finally, the alveolar macrophages engulf these foreign particles and digest them by releasing lysosomal enzymes into phagocytic vacuoles [Myrvik et al, 1961, 1967]. In this manner, these phagocytes cleanse the lung of bacteria and of foreign particles [Green, 1970].

Metals have been shown to affect a variety of properties of alveolar macrophages [Waters et al, 1974, 1975; Aranyi et al, 1977; Camner et al, 1978; Jarstrand et al, 1978; Loose et al, 1978]. Thus, airborne particles in the metal industry may be toxic to alveolar macrophages, and the normal defense mechanisms of the lung may be severely compromised. Therefore, an investigation of the effects of metallic ions on the function of alveolar macrophages is of great interest.

In this investigation, we measured the effects of in vitro exposure to several metallic ions on a variety of cellular and subcellular properties of rat alveolar macrophages. The objectives of this study were 1) to determine which metallic ions are most toxic to alveolar macrophages, and 2) to determine which cellular and subcellular responses are most susceptible to these toxic effects and, therefore, represent the most sensitive assays for metal toxicity. The parameters measured in this study are oxygen consumption, chemiluminescence, superoxide anion release, phagocytotic rate, lysosomal enzyme activity, and membrane integrity. In addition to metals, the effects of three organic solvents on alveolar macrophages are also reported.

METHODS

Male Long Evans hooded rats (250–300 gm) were anesthetized with sodium pentobarbital (0.2 gm/kg body weight) and exsanguinated by cutting the abdominal aorta. Alveolar macrophages were obtained by pulmonary lavage using an ice-cold, Ca^{++}-free solution (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, 5.5 mM glucose (pH = 7.4)) [Myrvik et al, 1961]. The cells were separated from the lavage fluid by centrifugation at 500g for five minutes and then washed twice with HEPES-buffered medium (140 mM NaCl, 5 mM KCl, 10 mM Na HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid), and 5.5 mM glucose (pH = 7.4)). HEPES buffer was used because it does not bind to metallic ions in solution [Good et al, 1966]. The metals used in these studies were added as the chloride or acetate salts.

Oxygen consumption was measured using an oxygraph equipped with a Clark electrode. Alveolar macrophages (3×10^6 cells) were preincubated at 37°C for 15 minutes in HEPES-buffered medium (1.65 ml) in either the presence or absence of metallic ions or organic solvents. The cell suspension was then transferred to a temperature controlled chamber (37°C) and oxygen consumption measured for ten minutes. Cells were exposed to particles by adding zymosan (20 mg/10^7 cells) to the suspension in the oxygraph chamber at zero time.

Chemiluminescence (CL) was measured as counts per minute in the tritium channel of a liquid scintillation counter operated in the out-of-coincidence mode [Miles et al, 1978]. Alveolar macrophages (5×10^6 cells) were preincubated in 5 ml of HEPES-buffered medium at 37°C for 15 minutes in either the presence or absence of metallic ions before CL was measured and the maximum counts per minute determined. In these experiments, zymosan was added immediately before the CL measurement (zero time).

Phagocytotic rate was quantified using a suspension of diisodecyl phthalate particles containing oil-red 0 and coated with opsonized E coli lipopolysaccharide [Cox and Stossel, 1976]. Alveolar macrophages (2×10^7 cells) were added to 2 ml of HEPES-buffered medium containing 1.26 mM $CaCl_2$ and 1 mM $MgCl_2$. This suspension was preincubated at 37°C for ten minutes in either the presence or absence of metallic ions. Uptake was then initiated by the addition of 0.5 ml of oil suspension. At 0 and 15 minutes, 0.5 ml samples were taken and uptake terminated by dilution with 4.5 ml of a saline solution (154 mM NaCl and 1 mM N-ethyl maleimide). These samples were then centrifuged and the cells washed three times with saline solution to remove extracellular oil-red 0. The ingested oil-red 0 was then extracted from the cells with 1.2 ml of dioxane and the optical density determined at 525 nm.

Alveolar macrophages (10^5–10^6 cells) were suspended in 200 μl of 0.9% NaCl and were ruptured by the addition of 50 μl of 1% Triton X -100 to release the lysosomal enzymes. These samples were then preincubated at 37°C in either the presence or absence of metallic ions for ten minutes prior to the initiation of the enzyme reaction by the substrate. Acid phosphatase was measured at pH = 5.0, using p-nitrophenyl phosphate as substrate [Turnbull and Neil, 1969], and β-glucuronidase was measured at pH = 5.0, using p-nitrophenyl-β-D-glucuronidate as substrate [Lockard and Kennedy, 1976].

Cell viability was determined by the trypan-blue exclusion method [Phillips, 1973]. Alveolar macrophages (4×10^6 cells) were preincubated at 37°C for 30 minutes in 1.48 ml of HEPES-buffered medium in either the presence or absence of metallic ions or organic solvents. The suspensions were then centrifuged and the cells resuspended in fresh medium containing trypan-blue dye (0.4%). After four minutes, the cells were observed under a light microscope and cell viability determined.

Superoxide anion release was measured directly by determining the reduction of cytochrome c [Babior et al, 1973]. Alveolar macrophages (4×10^6 cells) were added to 5 ml of HEPES-buffered medium containing 0.12 mM cytochrome c. This suspension was preincubated at 37°C for ten minutes in either the presence or absence of organic solvents. Then a zero-time sample was taken, centrifuged at 6000g for 30 seconds at 2°C, and the optical density of the supernatant measured at 500 nm with a spectrophotometer. Later, a 30 minute time sample was taken and the optical density measured. The amount of superoxide-dependent cytochrome c reduction is proportional to the difference in optical densities measured over this 30 minute period. Zymosan (0.8 mg/ml) was added to the suspension at zero time to study the effects of particle exposure.

RESULTS AND DISCUSSION

Alveolar macrophages play an important role in the protection of the organism against bacteria and foreign substances that may be inhaled into the lungs. The responses of these cells to particle exposure are complex and include increases in the utilization of

oxygen, the release of superoxide anion, the release of lysosomal enzymes, and the phagocytosis of the foreign substances. In this study the effects of various metallic ions on alveolar macrophages were determined. In addition, an effort was made to determine which of the cellular responses to particle exposure mentioned above are the most sensitive to metal ions.

In order to determine the responses most sensitive to metals, various parameters were screened, using exposures to Cd^{++} and Ni^{++}. The ED_{50} values for each response—ie, the concentrations of metals that produce 50% of the maximal inhibition—were determined from double reciprocal plots. It was on the basis of ED_{50} values that the most suitable assays for metallic toxicity were selected; these data are shown in Table I. The generation of chemiluminescence by alveolar macrophages exposed to particles is very sensitive to the toxic effects of both Cd^{++} and Ni^{++}; ie, ED_{50} values are in the order of 10^{-5} M. Oxygen consumption is inhibited by the metals with ED_{50} values in the range of 10^{-4} M. Lysosomal enzyme activities (acid phosphatase and β-glucuronidase activity) are very insensitive to treatment with metals; ie, ED_{50} values are greater than 10^{-3} M. The phagocytotic rate of alveolar macrophages is inhibited by 10^{-4} M concentrations of Cd^{++} but much higher levels of Ni^{++} are required for inhibition. The results of these experiments indicate that the cellular responses most sensitive to metals are chemiluminescence and oxygen consumption. Therefore, these two responses were used to assess the effects of metals on alveolar macrophages in the remainder of this study.

The consumption of oxygen by the alveolar macrophages was measured with the cells at rest and after exposure to particles. A tracing from the Gilson Oxygraph is shown in Figure 1. The resting level of oxygen consumption for rat alveolar macrophages is 7.7 ± 0.6 nmoles/10^7 cells/min. After the exposure of the macrophages to zymosan particles, oxygen consumption increases by about 170% to 20.4 ± 2.4 nmoles/10^7 cells/min. The oxygen consumption of these cells is markedly affected by treatment with metal ions (Table II). Metallic ions inhibit oxygen consumption both at rest and after exposure to zymosan by about 70–90%. The order of effectiveness of the metals in inhibiting oxygen consumption in resting cells, based on ED_{50} values, is $Hg^{++} > Al^{+++} \approx Fe^{+++} \approx Cd^{++} \approx Pb^{++} \approx Cr^{+++} > Ni^{++}$. The order of potency in the cells exposed to particles is $Hg^{++} > Fe^{+++} \approx Al^{+++} \approx Ni^{++} \approx Cd^{++} \approx Cr^{+++} > Pb^{++}$.

The exposure of alveolar macrophages to zymosan particles stimulates the release of superoxide anion from the cells. This release of superoxide anion is commonly monitored by measuring the reduction of cytochrome c [Babior et al, 1973]. However, we have shown that the metallic ions bind to cytochrome c [Castranova et al, 1979]; therefore, this assay for superoxide release cannot be used in this study. Fortunately, superoxide anion release from alveolar macrophages can be measured in another way. We have shown previously that the exposure of alveolar macrophages to zymosan produces chemiluminescence which occurs as a result of the reaction between the superoxide and zymosan [Miles et al, 1978]. Therefore, the effect of the metallic ions on the release of superoxide from the cells can be measured by monitoring chemiluminescence.

The time course of the chemiluminescence generated when rat alveolar macrophages were exposed to zymosan is shown in Figure 2. The peak response occurs approximately 5 minutes after exposure of the cells to zymosan, and then the chemiluminescence gradually diminishes over the next 90 minutes. All of the results are summarized in Table III. Maximal inhibition of chemiluminescence is approximately 70–85% with the metals tested. The order of the effectiveness of the metallic ions in inhibiting chemiluminescence,

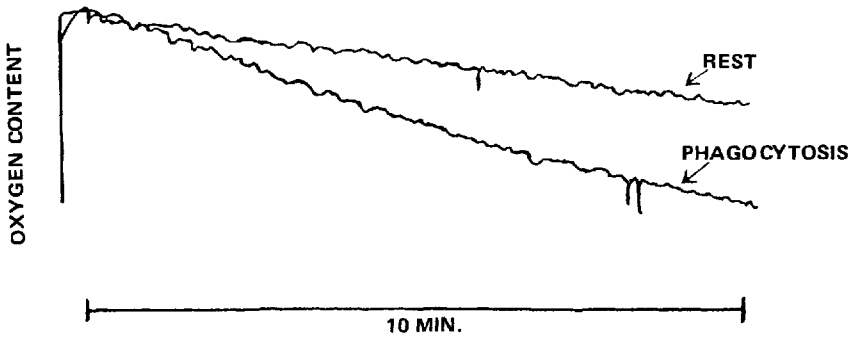

Fig. 1. A tracing from the Gilson Oxygraph showing oxygen consumption in rat alveolar macrophages at rest and during particle exposure. The resting value for oxygen consumption is 7.7 ± 0.6 nmoles/10^7 cells/min. When the cells are exposed to zymosan, oxygen consumption increases two- to threefold to 20.4 ± 2.4 nmoles/10^7 cells/min.

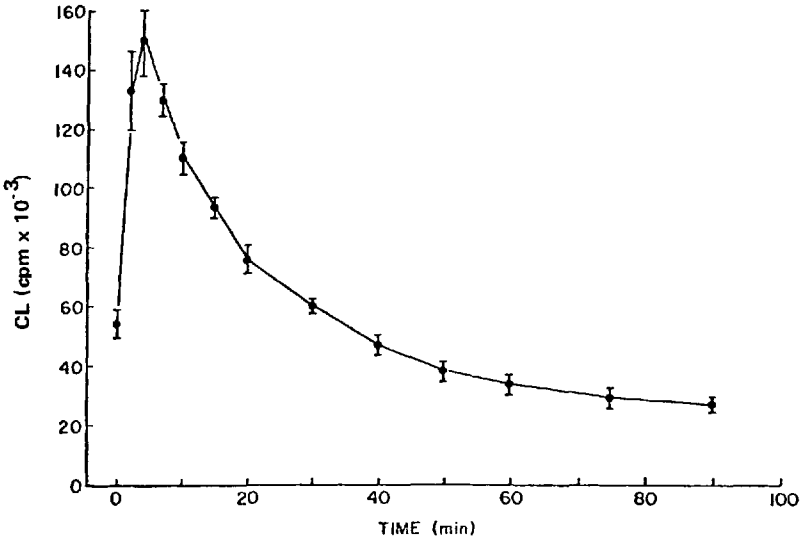

Fig. 2. Time course of the chemiluminescence produced when rat alveolar macrophages were exposed to zymosan. Zymosan was added to the cell suspension at zero time. The points are mean values for six experiments, and the bars are the standard errors of the means.

based on the ED_{50} values, is $Hg^{++} > Ni^{++} \approx Cd^{++} > Pb^{++} \approx Al^{+++} \approx Fe^{+++} \approx Cr^{+++}$. In general, these ED_{50} values are slightly lower than those obtained for oxygen consumption, suggesting that particle-induced superoxide release is very sensitive to metallic ions.

TABLE I. Effects of Cd^{++} and Ni^{++} on Various Parameters of Rat Alveolar Macrophages

Parameter	ED_{50} (M)[a]	
	Cd^{++}	Ni^{++}
Chemiluminescence	$7.6 \pm 2.8 \times 10^{-5}$	$6.6 \pm 3.1 \times 10^{-5}$
O_2 consumption	$4.1 \pm 0.8 \times 10^{-4}$	$3.8 \pm 2.0 \times 10^{-4}$
Phagocytotic rate	$8.4 \pm 4.8 \times 10^{-5}$	$> 10^{-3}$
Enzyme activity		
Acid phosphatase	$> 10^{-3}$	—
β-glucuronidase	$> 10^{-3}$	$> 10^{3}$

[a]ED_{50} values are the concentrations of metals that produce 50% of the maximal inhibition. The numbers shown are mean values of at least three experiments ± SEM.

TABLE II. Effects of Metallic Ions on Oxygen Consumption in Alveolar Macrophages*

Metal	Rest		Exposure to zymosan	
	Maximal inhibition (%)	ED_{50} (M)	Maximal inhibition (%)	ED_{50} (M)
Cd^{++}	69 (± 3)	$3.6 (\pm 0.9) \times 10^{-4}$	76 (± 1)	$4.1 (\pm 0.8) \times 10^{-4}$
Hg^{++}	76 (± 2)	$2.5 (\pm 1.1) \times 10^{-5}$	82 (± 4)	$1.4 (\pm 0.3) \times 10^{-5}$
Ni^{++}	73 (± 3)	$3.9 (\pm 2.0) \times 10^{-3}$	87 (± 2)	$3.8 (\pm 2.0) \times 10^{-4}$
Pb^{++}	79 (± 4)	$5.6 (\pm 1.3) \times 10^{-4}$	80 (± 3)	$2.6 (\pm 1.4) \times 10^{-3}$
Al^{+++}	88 (± 5)	$2.2 (\pm 0.2) \times 10^{-4}$	82 (± 2)	$2.5 (\pm 1.0) \times 10^{-4}$
Cr^{+++}	92 (± 4)	$6.6 (\pm 2.1) \times 10^{-4}$	90 (± 3)	$5.0 (\pm 0.9) \times 10^{-4}$
Fe^{+++}	80 (± 2)	$2.5 (\pm 0.4) \times 10^{-4}$	85 (± 5)	$2.2 (\pm 0.8) \times 10^{-4}$

*The cells were incubated in the presence of the metal cations for 15 minutes before the measurements were made. Oxygen consumption was measured over a ten-minute period. Zymosan was added to the cells just prior to the time during which oxygen consumption was measured. The ED_{50} is the concentration of metal that produces 50% of the maximal inhibition. Maximal inhibition was produced by the following metal concentrations: $Cd^{++} = 10^{-3}$ M, $Hg^{++} = 10^{-4}$ M, $Ni^{++} = 10^{-2}$ M, $Pb^{++} = 10^{-2}$ M, $Al^{+++} = 10^{-3}$ M, $Cr^{+++} = 5 \times 10^{-3}$ M, and $Fe^{+++} = 10^{-3}$ M. The numbers shown are mean values for six experiments ± SEM. Normal values for oxygen consumption of rat alveolar macrophages are 7.7 (± 0.6) nmoles/10^7 cells/min at rest and 20.4 (± 2.4) nmoles/min after exposure to zymosan.

TABLE III. Effects of Metallic Ions on Maximal Chemiluminescence*

Metal	Maximal inhibition (%)	ED_{50} (M)
Cd^{++}	73 (± 1)	$7.6 (\pm 2.8) \times 10^{-5}$
Hg^{++}	80 (± 2)	$2.3 (\pm 0.8) \times 10^{-6}$
Ni^{++}	84 (± 3)	$6.6 (\pm 3.1) \times 10^{-5}$
Pb^{++}	75 (± 1)	$1.2 (\pm 0.5) \times 10^{-4}$
Al^{+++}	71 (± 4)	$1.4 (\pm 0.3) \times 10^{-4}$
Cr^{+++}	82 (± 3)	$6.5 (\pm 1.6) \times 10^{-4}$
Fe^{+++}	80 (± 3)	$1.7 (\pm 0.4) \times 10^{-4}$

*The cells were incubated in the presence of the metals for 15 minutes before the measurements were made. The ED_{50} is the concentration of metal that produces 50% of the maximal inhibition. Maximal inhibition was produced by the following metal concentrations: $Cd^{++} = 10^{-3}$ M, $Hg^{++} = 10^{-4}$ M, $Ni^{++} = 10^{-2}$ M, $Pb^{++} = 10^{-2}$ M, $Al^{+++} = 10^{-3}$ M, $Cr^{++} = 5 \times 10^{-3}$ M, and $Fe^{+++} = 10^{-3}$ M. The numbers shown are mean values for six experiments ± SEM.

TABLE IV. Effects of Metallic Ions on Trypan-Blue Exclusion*

Metal (concentration)	% Cells that excluded dye
Control	92 (± 1)
Cd^{++} (10^{-3} M)	90 (± 1)
Hg^{++} (10^{-4} M)	93 (± 1)
Ni^{++} (10^{-2} M)	90 (± 2)
Pb^{++} (10^{-2} M)	89 (± 2)
Al^{+++} (10^{-3} M)	89 (± 3)
Fe^{+++} (10^{-3} M)	83 (± 4)
Cr^{+++} (5×10^{-3} M)	91 (± 3)

*The cells were incubated at 37°C for 30 minutes either in the presence or absence of heavy metals before the measurements were made. The percentages of the total number of cells that excluded trypan-blue dye were determined. The numbers shown are mean values for three experiments ± SEM.

TABLE V. Effects of Organic Solvents on Rat Alveolar Macrophages*

Superoxide release[a]		
Solvent	Maximal inhibition (%)	ED_{50} (M)
Carbon tetrachloride	95 ± 3	0.0112 ± 0.0017
Toluene	100 ± 0	0.0117 ± 0.0013
Xylene	100 ± 0	0.0062 ± 0.0004
Oxygen consumption[b]		
Solvent	Maximal inhibition (%)	ED_{50} (M)
Carbon tetrachloride	74 ± 5	0.0118 ± 0.0017
Toluene	67 ± 7	0.0120 ± 0.0018
Xylene	72 ± 6	0.0099 ± 0.0022
Trypan-blue exclusion[c]		
Solvent	% Cells that exclude dye	
Carbon tetrachloride	87 ± 2	
Toluene	89 ± 2	
Xylene	87 ± 2	

*Values shown are the means of at least five experiments ± SEM. Maximum concentrations of the solvents used were 0.0628 M carbon tetrachloride, 0.0570 M toluene, and 0.0504 M xylene.

[a]The normal value for superoxide release from rat alveolar macrophages exposed to zymosan is 135 ± 4 nmoles of cytochrome c reduced/4 × 10^6 cells/30 min.

[b]The normal value for oxygen consumption in rat alveolar macrophages exposed to zymosan is 20 ± 2 nmoles of oxygen consumed/10^7 cells/min.

[c]Normally, 95 ± 1% of untreated rat alveolar macrophages exclude trypan-blue.

The effect of metals on membrane integrity of alveolar macrophages was determined by measuring trypan-blue exclusion; these results are shown in Table IV. Note that the treatment of alveolar macrophages with relatively high doses of metals causes very little cell damage; ie, approximately 90% of the cells excluded the dye. Therefore, we concluded that the metallic ions show toxic effects on oxygen consumption and superoxide anion release at doses that have no effect on membrane integrity.

In recent years, there has been an increasing use of organic reagents for the extraction of metals from crude ore. In light of this fact, we studied the effect of several organic solvents on superoxide anion release, on oxygen consumption, and on trypan-blue exclusion by alveolar macrophages. The solvents used in this study were carbon tetrachloride, toluene, and xylene. These results are summarized in Table V. Organic solvents completely blocked the release of superoxide anion as measured by cytochrome c reduction; ED_{50} values for these solvents are on the order of 10^{-2} M. The organic solvents also caused about a 70% inhibition of the oxygen consumption of alveolar macrophages; again, the ED_{50} values are about 10^{-2} M. Although these solvents block superoxide release and oxygen consumption, there is little effect on membrane integrity as measured by trypan-blue exclusion; ie, at least 87% of the cells excluded the dye. Thus, these results indicate that organic solvents may be toxic to alveolar macrophage function. Although the solvents used in this study are not the organic reagents used in the metal industry, their toxicity may suggest a danger in the use of this class of compound. This possibility requires further study.

In summary, the toxicity of selected metallic ions to rat alveolar macrophages was investigated by monitoring several cellular and subcellular responses, such as superoxide anion release (measured as chemiluminescence), oxygen consumption, phagocytotic rate, enzyme activities, and membrane integrity (measured as trypan-blue exclusion). Superoxide anion release and oxygen consumption were found to be the cellular responses most sensitive to the metallic ions. The classical test for cellular viability; ie, trypan-blue exclusion, was found to be the least sensitive. All of the metallic ions tested were found to inhibit both superoxide anion release and oxygen consumption, with Hg^{++} being the most toxic of the metals. In addition, three organic solvents were also found to inhibit both superoxide anion release (measured as cytochrome c reduction) and oxygen consumption. The results of these experiments indicate that two substances found in the atmosphere in metallurgical smelters—ie, metals and organic solvents—are toxic to alveolar macrophages. In particular, the release of superoxide anion, an antibacterial substance, from the cells is affected by these substances.

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