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Effect of Trace Metals on Phagocytosis by Alveolar Macrophages

JUDITH A. GRAHAM,* DONALD E. GARDNER, MICHAEL D. WATERS, AND DAVID L. COFFIN

Experimental Biology Laboratory, Pathobiology Research Branch, Environmental Protection Agency, Research Triangle Park, North Carolina 27711

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Experiments were performed to measure the effect of trace metals on a vital function of the alveolar macrophage (AM), phagocytosis. Since certain trace metals were found to reduce the viability of AMs, a technique was developed to permit examination of live cells only for phagocytosis. Evidence is presented that Ni^{2+} selectively altered the phagocytic activity of AMs at concentrations lower than those which caused cell death. It is further shown that a level of VO_3^- that caused extensive lysis and death did not reduce phagocytosis in surviving cells. The effects of Cd^{2+}, Cr^{3+}, and Mn^{2+} on AMs were also examined.

The polluted air of many industrial cities contains varying concentrations of potentially hazardous respirable metals, including nickel, vanadium, cadmium, chromium, and manganese (19). When particles containing these metals are inhaled and deposited in pulmonary spaces, several different mechanisms may affect their fate. They may be taken up by pulmonary phagocytes, remain as independent particles within the lung, or be absorbed or dissolved in the lung, later to be stored in other tissues or excreted from the body. Regardless of their ultimate disposition, all of these metals have a residence time within the lung, and there is some evidence that V, Cd, and Cr may accumulate in the lung with age (19).

Data from industrial exposures and epidemiological and animal studies have shown a relationship between airborne concentrations of several trace metals and the incidence of infectious respiratory diseases (4, 6, 9, 21). The establishment of pulmonary infections suggests a breakdown of the lung's defensive mechanisms. Through its phagocytic and bacteriolytic activity, the alveolar macrophage (AM) plays a vitally important role in protection of the host against viable and nonliving particles that reach the nonciliate portions of the lung. As a result of this activity, the AMs are often exposed to higher concentrations of toxic material than are other lung cells.

Using an in vitro model system for measuring the cytotoxicity of metals, we have previously shown that exposure of AM to certain concentrations of VO_3^-, Ni^{2+}, Cd^{2+}, Cr^{3+}, and Mn^{2+} results in loss in viability, and, in most cases, cell lysis (24, 25). However, in pulmonary defense, functional defects in AMs may be as detrimental to the host as cell death. Therefore a functional assay, using the parameter of phagocytosis, was employed. Since the metals to be studied were known to reduce cellular viability, the conventional phagocytic technique was modified to increase the sensitivity and reliability of cytotoxicity evaluations.

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MATERIALS AND METHODS

Rabbits. New Zealand white rabbits (Pel-Freeze Inc., Rogers, Ark.) of both sexes weighing 1.5 to 2 kg were used as a source of AMs. Purina Rabbit Chow and water were available ad libitum. No antibiotics or other drugs were administered for at least 2 weeks prior to experimental use of the rabbits.

Isolation and collection of AMs. The procedure of Coffin et al. (5) was used in procuring AMs. After the animals were sacrificed by injection of 3 ml of sodium pentobarbital (Nembutal, 50 mg/ml; Abbott, Chicago, Ill.) in the marginal ear vein, they were positioned on their backs, a tracheostomy was performed, and a catheter was inserted. Thirty milliliters of 0.85% saline at room temperature was instilled into the lungs, allowed to remain 15 min, and withdrawn by means of a siliconized glass syringe. This procedure, without the time delay, was repeated five more times and resulted in a lavage fluid containing the macrophages. Macrophages in a high state of purity (>97%) were recovered after centrifugation at 365 × *g* for 15 min at 5 C.

Preparation of tissue culture media with trace metals. The following metallic salts were used without further purification for this study: ammonium

vanadate (Pfaltz and Bauer, Inc., Flushing, N.Y.); cadmium chloride (Matheson, Coleman and Bell, Norwood, Ohio); nickelous chloride (International Nickel Company, Inc., Suffern, N.Y.); manganous chloride (Fisher Scientific Co., Fair Lawn, N.J.); and chromic chloride (J. T. Baker Chemical Co., Phillipsburg, N.J.). These metals were separately dissolved in Medium 199, with Hanks salts, supplemented with 20% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and kanamycin (100 μg/ml) (Gibco, Grand Island, N.Y.). The supplemented medium with the added metallic salts was filter sterilized through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.) and then added to the cytotoxicity test system as described below. Metal concentrations were tested for accuracy using a Perkin-Elmer model 306 atomic absorption spectrophotometer.

Test for cytotoxicity to AMs. The AMs were exposed to the metals as previously described by Waters et al. (25). Briefly, the AMs were resuspended in the supplemented medium without metals to a concentration of approximately 2×10^6 AMs/ml. Aliquots (12 ml) of this suspension were transferred to glass-roller culture vessels having a surface area of 240 cm^2 and placed in a 37 C incubator under a humidified atmosphere of 4% CO_2. After rotating for 3 h at 0.5 rpm, the unattached cells were poured off and discarded. Fresh medium (12 ml) containing the metallic salts was then added. Control vessels received 12 ml of the supplemented media without the dissolved metals. These vessels were returned to the incubator and rotated for an additional 20 h.

This time period was chosen so that the present phagocytosis measurements could be made after exposures under conditions similar to those employed in our previous investigations (24, 25). The 20-h incubation period was originally selected to maximize the opportunity for demonstration of cytotoxic effects in experimental cultures while minimizing attrition of cells in control cultures. After the exposure period, the medium containing unattached cells was decanted and saved. The attached cells were removed by adding 5 ml of 0.25% trypsin to the flask. The trypsin containing the suspended cells was effectively inactivated by combining the trypsinized cell suspension with the corresponding detached cells in medium that had been poured off earlier. In this manner, unattached as well as attached cells were recovered for analysis of residual phagocytic activity of the entire culture population. It was also found that after a 20-h exposure both attached and unattached cells exhibited similar viability. Furthermore, pooling the AMs increased the numbers of cells available for phagocytic studies. The cells were counted and viability was determined using the trypan blue exclusion technique (14). The AMs were then washed twice ($413 \times g$, room temperature, 10 min) in Medium 199 with Hanks salts, containing 10% heat-inactivated fetal calf serum, and the cells were resuspended in this medium at a concentration of 2.5×10^6 AMs/ml. This cell suspension was then used for the measurement of phagocytic activity.

Measurement of phagocytic activity. Phagocytosis was measured using a modification of the technique of Gardner et al. (10). Three milliliters of the prepared macrophage suspension was placed in a siliconized 25-ml Erlenmeyer flask, and cellular viability was again determined. A stock suspension of polystyrene latex spheres (1.1-μm diameter; Dow Diagnostics, Indianapolis, Ind.) was added to each flask to give a ratio of one AM to 120 latex spheres. The flasks were placed in a reciprocating shaker bath (60 cycles/min) for 1 h at 37 C. At least two flasks were employed for each measurement of phagocytic activity, and each metal was tested in three to six separate experiments.

After incubation and phagocytosis, the double-staining technique of deRenzis et al. (7) was used to differentiate viable from nonviable cells. This involved transferring the AM-latex sphere suspension to a tube and incubating for 10 min with 3 ml of 0.04% neutral red. This incubation was followed by the addition of 3 ml of 0.4% trypan blue. The suspension was then centrifuged ($413 \times g$, room temperature, 10 min), the supernatant was decanted, and the cell button was resuspended in 1 ml of the medium previously described for use in phagocytosis measurements. A drop was transferred to slides, and smears were made with a Pasteur pipette. Viability was determined on a duplicate slide. The slides were allowed to air dry, held in formalin vapor overnight, and then placed in xylene (10) to remove extracellular latex spheres. When examined under a light microscope, viable cells were stained reddish-orange by the neutral red, and the dead cells were stained blue by trypan blue. To obtain an accurate phagocytic index (PI), 200 live AMs were counted, and the percentage of these AMs containing intracellular spheres was determined (percentage of phagocytosis). This percentage was then divided by the percentage of live control cells containing intracellular spheres as follows: PI = (% live metal exposed AMs with spheres/% live control AMs with spheres) (100).

Method for testing effects of viability on PI. To determine the effect of reduced cell viability on the PI, the following experiment was performed. Freshly harvested AMs were divided into two aliquots in two tubes. One tube was placed in a boiling water bath for 10 min, which killed all of the cells without lysing them. The second tube contained 97% viable cells. The heat-killed and viable cells were each washed once in the medium used for phagocytosis and mixed in proportions that resulted in 11 different viabilities ranging from 48 to 98%. Measurement of phagocytic activity was performed as reported above and the PI was determined.

Method for testing the efficacy of the double-staining technique. The slides prepared to examine the effect of viability on the PI (see above) were also used to examine the effectiveness of the double-staining technique for determination of percentage of phagocytosis by viable cells. Each slide was examined in two ways. For each field, the viable cells were scored for the percentage of AM with spheres. The same field was then reexamined, and all cells were

counted for percentage of phagocytosis in the total population. This latter count is intended to represent the use of a single nonvital stain technique such as Giemsa. Enough fields were examined so that 200 cells/slide were counted.

RESULTS

Effect of viability on PI. The PI of AMs exhibiting 11 different viabilities (48 to 98%) was a mean of 100.4 ± 1.2 standard error. This indicated that under these extremely varying experimental conditions the proportion of viable cells had no effect on the PI when measured by our system.

Efficacy of double-staining technique on PI. In evaluating the effectiveness of the double-staining technique, the PI was measured using three different suspensions of AMs having viabilities ranging from 63 to 94%. Table 1 illustrates the results when cell viability (column 1) was determined by trypan blue, and the percentage of phagocytosis (column 2) was determined using a single stain (Giemsa), or the percentage of phagocytosis (column 3) was determined using the double-staining technique. Interestingly, when all intact cells were counted, the percentage of phagocytosis decreased with viability. This was due to the fact that dead cells were unknowingly counted as non-phagocytizing live cells. When the double-staining technique was used and only live cells were scored, no difference was seen in phagocytosis by viable cells.

Effect of trace metals on phagocytosis. The effects of the metals studied on the PI of AMs are shown in Fig. 1. The bars represent means ± standard error. Metal concentrations were originally chosen to achieve a viability of approximately 80% (25). The actual viabilities determined are reported within each bar. Statistical analysis using Dunnett's test (a multiple comparison test for testing several treatments jointly against a control) revealed that the PI for cells exposed to all metals except VO_3^- was significantly lower than controls at $P < 0.01$. A one-way analysis of variance indicated that the PIs for AMs incubated with VO_3^- and all concentrations of Ni^{2+} and all other metals were different from each other at $P < 0.05$. However, the PIs determined for Cd^{2+}- , Cr^{3+}- , and Mn^{2+}-exposed cells were not different from one another, even though each was different from VO_3^- and Ni^{2+} AMs.

Figure 2 compares the action of VO_3^- and

TABLE 1. *Effect of viability and different staining techniques on phagocytosis by AMs*

Flask no.	Viability (1)	% Phagocytosis[a]	
		Single stain (2)	Double-viability stain (3)
1	93	43.3	43.7
2	72.5	31.0	46.0
3	64.3	28.1	46.1

[a] Percentage of phagocytosis is percentage of cells with intracellular spheres. Column 1, Viability was determined with trypan blue. Column 2, Percentage of phagocytosis when a single stain was used and all whole cells were counted. Column 3, Percentage of phagocytosis when a double-viability stain was used and only whole live cells were counted. The same microscopic fields were examined for cell counts in columns 2 and 3.

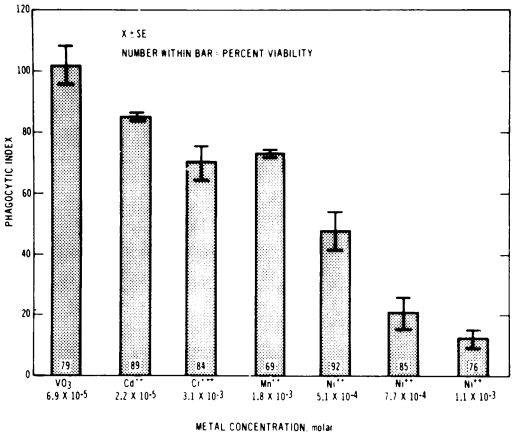

FIG. 1. *Effect of various metal concentrations on phagocytosis by live AMs.*

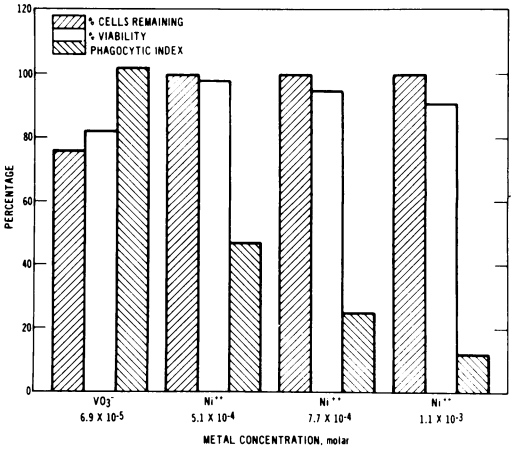

FIG. 2. *Effect of various metal concentrations on phagocytosis, viability, and percentage of cells remaining of AMs. Viability and percentage of cells remaining is according to Waters et al. (25).*

Ni^{2+} on AM viability and percentage of cells remaining as reported by Waters et al. (25) and the effects of these metals on phagocytic activity. At the concentrations employed, VO_3^- caused noticeable cellular destruction, whereas Ni^{2+} produced very little loss in AM viability or cellular lysis. Vanadate lysed and killed AMs at relatively low concentrations (6.9×10^{-5} M), but the remaining whole live cells were completely capable of functioning in phagocytosis. Conversely, Ni^{2+} at higher concentrations (1.1×10^{-3} M) produced only minimal physical damage to macrophages, but many cells had a severe functional defect as measured by their lack of ability to phagocytize polystyrene latex spheres.

DISCUSSION

The role of the AM in defense against infectious disease is well known. The macrophage is a critical cell in determining pulmonary responses to a wide range of external agents. In general, these responses depend upon phagocytosis or plasma membrane-particle interactions. Any material that depresses the phagocytic action of these cells can adversely affect the host's susceptibility to infection (12, 13). A variety of gaseous environmental pollutants have been shown to affect phagocytosis by AMs and to lead to a higher incidence of respiratory infections (4, 5, 11). However, measurements on the effect of environmental particulates on AM phagocytic ability have been limited.

To study phagocytosis in the presence of cytotoxic substances, techniques had to be developed to eliminate the inherent problem of lowered cellular viabilities. Firstly, it was decided to investigate the effect of viability alone on phagocytosis to ensure that the results reflected actual toxicant damage rather than simply the effect of lower viability. Brandt (3) had reported that phagocytosis of staphylococci and yeast cells by leukocytes was reduced as the concentration of erythrocytes in the suspension increased. It was hypothesized that, since phagocytosis depends on chance contact between phagocyte and particle, the increased number of erythrocytes reduced chance of leukocyte-bacteria or yeast contact, thereby lowering cellular ingestion. In view of this hypothesis, it seemed possible that a dead AM in our system could play the same role as an erythrocyte in Brandt's. However, in the phagocytic system reported here, excess dead cells had no effect, possibly due to the high ratio of AMs-latex spheres.

For a high degree of accuracy in estimating phagocytic activity, viability must always be considered and accounted for in some manner. In the present study, a double-viability stain was used to eliminate nonfunctioning dead cells from the phagocytic measurement. Other methods are available, i.e., differential centrifugation on albumin to separate living and dead macrophages (18) or mathematical manipulations; but the staining technique used in these experiments appeared to combine ease and accuracy.

All of the metals tested in this experiment, V, Cd, Mn, Cr, and Ni, are usually present in community air pollution and are associated with the smallest particles collected, that is, within the respirable range of 5 μm (15). Table 2 compares the metal concentrations used in these experiments with those found in the human lung, industrial air, and urban air and the industrial threshold limit values.

Data from human studies show a close correlation between inhalation of some of the metals studied and increases in respiratory infections (1, 9, 12). The recent studies of C. Port et al. (Abstr. Environ. Health Perspectives, **10**:268, 1975, Conference on Heavy Metals in the Environment, Duke University, Durham, N.C., 1974) and R. Maigetter et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, M84, p. 80), in which mice

TABLE 2. *Comparisons of metal concentrations used in these experiments with those found in the environment*

Metal	In phagocytosis (μg/ml)	Maximum in human lung (μg/ml)[a]	Avg in community air ($\mu g/m^3$)[b]	Maximum in community air ($\mu g/m^3$)[b]	Threshold limit values ($\mu g/m^3$)[c]
Cd	2.5	930	0.002	0.350	200
Cr	160	2,000	0.015	0.350	500
Mn	100	1,700	0.10	10	5,000
Ni	30, 45, 65	8,000	0.032	0.690	1,000
V	3.5	680	<0.003-0.90[d]	1.4[d]	500

[a] From ref. 19.
[b] From ref. 23.
[c] From ref. 8.
[d] Also see ref. 17.

were exposed to metal aerosols (NiO, MnO_2), have demonstrated an increased susceptibility to induced pulmonary infections mimicking the increase in human infectious diseases resulting from occupational exposures to these pollutants. These responses could have been related to the effect of these metals on AMs.

The fact that nickel selectively inhibits phagocytosis at concentrations below those that caused lysis or cell death permits speculation upon its mechanism of action. The energy required for phagocytosis comes from the utilization of cellular adenosine 5'-triphosphate (ATP), and therefore any substance that reduces the availability of ATP would affect the cells' phagocytic activity. Since Ni binds with ATPase, thereby reducing ATP energy release (16) and with ATP itself to form a stable binary complex (20) that also inhibits ATP utilization, these actions could be responsible for the effect of nickel on phagocytosis. Currently, experiments are being conducted to determine the cellular levels of ATP in AMs exposed to metals using the techniques described in this paper. Preliminary data indicates that there is a concomitant decrease in ATP and phagocytic activity (M. Waters et al., In Vitro **10**:342, 1975).

Metals such as Ni, which reduce the phagocytic function of AMs, may tend to increase the pulmonary burden of other potentially harmful metals that coexist with Ni. Furthermore, additional Ni-containing particles in the deep lung may have a longer residence time, and this could be related to Ni's role as a possible carcinogen or cocarcinogen (22). In vitro studies have shown that microphages phagocytize tumor cells (2), and Ni would presumably prevent or reduce this. It would be of toxicologal interest to examine other metals that have been implicated with pulmonary cancer to see if they too depress phagocytosis.

In summary, these studies illustrate the differing mechanisms of cellular toxicity produced by exposure to soluble trace metals. Hence, they emphasize the importance of measuring functional as well as cytological and biochemical parameters when attempting to estimate the toxicity of a substance. Additionally, the data suggest that certain metallic air pollutants can cause severe functional defects in AM phagocytic activity that may be linked ultimately to infectious pulmonary disease.

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