JOURNAL of TOXICOLOGY and ENVIRONMENTAL HEALTH

ISSN: 0098-4108 (Print) (Online) Journal homepage: http://www.tandfonline.com/loi/uteh19

EFFECTS OF VANADIUM UPON POLYI:C-INDUCED RESPONSES IN RAT LUNG AND ALVEOLAR MACROPHAGES

Mitchell D. Cohen , Susanne Becker , Robert Devlin , Richard B. Schlesinger & Judith T. Zelikoff

EFFECTS OF VANADIUM UPON POLYI:C-INDUCED RESPONSES IN RAT LUNG AND ALVEOLAR MACROPHAGES

Mitchell D. Cohen

Nelson Institute of Environmental Medicine, New York University Medical Center, New York, New York, USA

Susanne Becker, Robert Devlin

Human Studies Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Chapel Hill, North Carolina, USA

Richard B. Schlesinger, Judith T. Zelikoff

Nelson Institute of Environmental Medicine, New York University Medical Center, New York, New York, USA

Hosts exposed to vanadium (V) display a subsequent decrease in their resistance to infectious microorganisms. Our earlier studies with rats inhaling occupationally relevant levels of V (as ammonium metavanadate, NH_4VO_3) indicated that several nascent/inducible functions of pulmonary macrophages (PAM) were reduced. In the present study, V-exposed rats were examined to determine whether some of the same effects might also occur in situ. Rats were exposed nose-only to air or 2 mg V/m^3 (as NH_4VO_3) for 8 h/d for 4 d, followed, 24 h later, by intratracheal (it) instillation of polyinosinic:polycytidilic acid (polyI:C) or saline. Analysis of lavaged lung cells/fluids after polyI:C instillation indicated that total lavageable cell/neutrophil numbers and protein levels, while significantly elevated in both exposure groups (as well as in saline-treated V-exposed rats), were always greater in V-exposed hosts. Exposure to V also affected the inducible production of interleukin 6 (IL-6) and interferon γ (IFNγ), but apparently not that of tumor necrosis factor-α (TNFα) or IL-1. Although polyI:C induced significant increases in lavage fluid IL-6 and IFNγ levels in both exposure groups, levels were greater in V-exposed rats. If calculated with respect to total lavaged protein, however, V-exposed rats produced significantly less cytokine. Following polyI:C instillation, there were no marked exposure-related differences in basal or stimulated superoxide anion production by pooled lavaged cells or PAM specifically. With V-exposed rats, pooled cells recovered 24 h after saline instillation displayed reduced production (in both cases) compared to the air control cells; PAM-specific production was affected only after stimulation. In both exposure groups, polyI:C caused decreased superoxide production in recovered cells. Though less apparent with pooled

Received 30 August 1996; sent for revision 21 October 1996; accepted 6 January 1997.

This research was supported by the National Institute for Occupational Safety and Health (grant OH03064-01) and is part of a National Institute of Environmental Health Sciences Center Program supported by (ES00260). The authors thank Joleen Soukup of the Health Effects Research Laboratory for her assistance with the cytokine assays.

Address correspondence to Dr. M. D. Cohen, Nelson Institute of Environmental Medicine, New York University, Long Meadow Road, Tuxedo, New York, NY 10987, USA. E-mail: cohenm@charlotte.med.nyu.edu

Journal of Toxicology and Environmental Health, 51:591–608, 1997

0098-4108/97 $12.00 + .00

cells, there was a time post polyI:C instillation-dependent decrease in stimulated PAM-specific superoxide production; this effect was greater in PAM from V-exposed rats than in PAM from air controls. Phagocytic activity of PAM from rats in both exposure groups was significantly increased by polyI:C instillation, although total activity in cells obtained from V-exposed rats was always significantly lower compared to air control cells. Our results indicate that short-term, repeated inhalation of occupationally relevant levels of V by rats modulates pulmonary immunocompetence. Modified cytokine production and PAM functionality in response to biological response modifiers (such as lipopolysaccharide, IFNγ, or polyI:C) may be, at least in part, responsible for the increases in bronchopulmonary disease in humans occupationally exposed to V.

The lungs are a primary target organ for environmental pollutants, and local pulmonary cell populations may be directly exposed to airborne toxicants. Since some of these cells, specifically the pulmonary macrophage (PAM), play a pivotal role in host pulmonary immunocompetence, their function in this regard may be compromised by pollutant exposure. This response, in turn, may be involved in the pathogenesis of environmentally induced pulmonary disease.

Within the lungs, effective removal of microbial pathogens relies primarily upon active PAM–lymphocyte intercellular signaling using interferons (IFNs), tumor necrosis factor-α (TNFα), and specific interleukins (i.e., IL-1α, -2, and -6). Lymphocyte-derived IFNγ, in conjunction with autocrine IL-1α and TNFα, prime PAM to heightened phagocytic/microbicidal states; to increased surface major histocompatibility complex (MHC) antigen expression; and to release of chemotactic/monocytopoietic products for activated PAM/monocyte influx into the lungs for eradication of infectious agents (Feldmann, 1996).

Vanadium (V) is widely used in the steel and chemical industries, and is a constituent of many ores, coals, and oils. Increased mining and milling of V-bearing ores and combustion of fossil fuels for heating/energy production result in high levels of respirable V particles/fumes in many work environs (Nriagu & Pacyna, 1988; ATSDR, 1991). Ambient V levels in these settings can reach >30 mg/m^3; the value for immediate danger to life or health is 70 mg/m^3 (NIOSH, 1985). Though initial clearance of inhaled V is fairly rapid (~40% in 1 h), animal studies have shown that total clearance is never achieved (Conklin et al., 1982; Sharma et al., 1987), and particles may persist in the lungs for long periods of time.

Vanadium is known to modulate immune responses in exposed humans and experimental animals (Zelikoff & Cohen, 1995). Epidemiological studies have demonstrated that acute and chronic exposure of workers to moderate to high levels of V-bearing particles resulted in an increased incidence of pulmonary diseases, such as asthma, pneumonia, and "Boilermaker's" bronchitis, as well as deaths from respiratory failure secondary to bacterial infections or from lung cancers initiated by other agents (Stocks, 1960; Hickey et al., 1967).

Studies of V-exposed humans demonstrated disturbed pulmonary neutrophil (PMN) numbers and morphology, plasma cell numbers and immunoglobulin production, and lymphocyte mitogenic responsiveness (Kivuoloto et al., 1979, 1980, 1981). Concurrent changes in pulmonary function, as well as increased nonspecific pulmonary reactivity to V, have also been documented (Kivuoloto, 1980; Lees, 1980; Musk & Tees, 1982). In studies using rodents exposed to V by intratracheal (it) instillation, intraperitoneal (ip) injection, or through the diet, hosts had altered lymphoproliferative responsiveness, as well as altered macrophage (Mϕ) phagocytic activity, lysosomal enzyme activity/release, microtubule structural integrity, and intracellular organelle pH (Zelikoff & Cohen, 1995). Exposed hosts also displayed altered resistance to endotoxin/intact microorganisms, modified pulmonary immune cell populations, and immune system organ pathologies (Sharma et al., 1981; Wei et al., 1982; Cohen et al., 1986, 1989; Al-Bayati et al., 1992).

Our recent studies have shown that nose-only inhalation of V (as NH_4VO_3) by rats resulted in significant inflammation and immunomodulation within the lungs (Cohen et al., 1996b). Effects on host immunity were primarily at the level of ex vivo PAM cytokine-related biochemistry, that is, monokine formation and responsiveness to endotoxin (lipopolysaccharide, LPS) and IFNγ. These results were similar to those observed previously following in vitro V exposures of a mouse macrophage cell line (Cohen et al., 1993a, 1996a). The study presented here examined the effects of acute inhalation of V (at occupationally relevant levels) upon in situ activation of PAM within the rat lung. Using the biological response modifier polyinosinic-polycytidilic acid (polyI:C), formation of IFNγ, TNFα, IL-1, and IL-6 in the lungs, as well as changes in polyI:C-derived IFNγ-inducible PAM functions (i.e., superoxide anion formation and phagocytic activity), were examined. The results of this study provide further clarity as to the mechanisms by which inhalation exposure to V could give rise to compromised pulmonary immunocompetence.

MATERIALS AND METHODS

Reagents

Tissue culture reagents were from Gibco (Grand Island, NY); ammonium metavanadate (99.6% purity; NH_4VO_3) from J. T. Baker Chemicals (Phillipsburg, NJ); and superoxide anion assay reagents and polyI:C from Sigma Chemicals (St. Louis, MO).

Experimental Animals

Upon arrival, 10-wk-old pathogen-free male Fischer 344 rats (200–250 g, Charles River, Boston) were quarantined for 2 wk prior to

any exposures, then housed individually in stainless steel cages in temperature (20°C)/humidity (50% RH)-controlled rooms, and provided food (Purina rodent chow) and water ad libitum. Rats were routinely checked for respiratory infections by performing nasal swab checks.

Exposures

In each of 2 separate experiments, groups of 14 rats were placed in restrainers connected to a custom-designed 18-port nose-only exposure apparatus (CH Technologies, Westwood, NJ) and exposed to atmospheres containing either filtered air or NH_4VO_3 (0.32 μm MMD). During exposures, the restrainers permitted free movement of each rat's head; no sedation was required. Aerosols delivering 2 mg V/m^3 (V metal as NH_4VO_3) were generated from a suspension of 40 n*M* NH_4VO_3 in ultrapure water using a Laskin nebulizer. Exposure duration was 8 h/d for 4 d, approximating (in each 8-h period) the levels of V that could be encountered by workers exposed during an 8 to 10-h workday to the threshold limit value of 0.05 mg V_2O_5/m^3. Samples of the generated atmosphere were collected hourly on filters for gravimetric analysis of the actual V concentrations. Twenty-four hours following the last exposure, rats were anesthesized with halothane and then intratracheally instilled with 100 μl 1.5 mg/ml polyI:C (n = 8 rats/exposure groups) or with saline vehicle (n = 6 rats/exposure group); pilot studies indicated that halothane had no effect upon the polyI:C-inducible endpoints studied herein.

Isolation of Pulmonary Alveolar Macrophages

To provide PAM for all of the ex vivo experiments, at designated time points after polyI:C or saline instillation, rats were sacrificed by an overdose of Nembutal (80 mg/kg, ip). The trachea was exposed, a cannula was inserted and tied into place, and the lungs were lavaged 8–10 times with Ca^{2+},Mg^{2+}-free Hanks balanced salt solution (HBSS, pH 7.4, 37°C) (Cohen et al., 1996b). Recovered cells were then washed twice by centrifugation (1500 × g, 15 min, 4°C) with HBSS and resuspended in RPMI 1640. Cell differentials were performed on cytocentrifuged preparations stained with Diff-Quik to identify PAM and other immune cell types. Aliquots of the acellular lavage fluid were either assayed for total protein using a commercially available Bio-Rad assay, or quick-frozen and placed at –70°C for later analysis of either IFNγ, IL-1, IL-6, or TNFα.

Cytokine Quantitations

Levels of IFNγ in the first 10–15 ml of recovered lavage fluid were determined using a commercially available rat IFNγ enzyme-linked immunosorbent assay (ELISA) kit (limit of detection 250 pg/ml; Gibco). IL-1α levels were assessed indirectly by measuring activity

using an IL-1-dependent D10.G4.1 mouse T-helper cell line proliferation assay (Becker et al., 1989). TNFα activity was measured using a standard cytotoxicity assay against TNFα-sensitive WEHI-164 cells (Becker et al., 1989). IL-6 activity was quantitated using an IL-6-dependent 7TD1 hybridoma cell line proliferation assay (VanSnick et al., 1986; Becker et al., 1991). Production/biological activity of each cytokine was quantitated against curves generated using commercially available cytokine standards (Biosource International, Camarillo, CA, and R&D Systems, Minneapolis, MN).

Superoxide Anion Production

To evaluate changes in O_2^- production by PMN/PAM mixtures (as well as by the PAM specifically) following polyI:C/saline instillation, recovered cells (in RPMI/5% fetal bovine serum) were plated in 96-well microtiter plates at 10^5 lavaged cells/well (12 wells/rat). After a 2-h incubation at 37°C to permit cell attachment, nonadherent cells from each well were recovered (for later analysis of cell attachment). Each well was rinsed once with 200 μl ice-cold serum-free RPMI and O_2^- formation was measured using a previously described assay protocol (Zelikoff et al., 1993). Briefly, 200 m*M* ferricytochrome *c* in phenol red-free HBSS [with or without rat serum-opsonized zymosan (OZ; 50 μg/ml)] was added to half the wells; remaining wells received these same solutions supplemented with 600 U/ml superoxide dismutase (SOD). Plates were then incubated at 37°C for 60 min and well absorbances were measured at 550 nm on a microtiter plate reader. Superoxide levels (nmol, $O_2^-/10^5$ recovered cells) were calculated by subtracting the absorbances measured in SOD-bearing wells and correcting for any cell loss during well rinsing steps. PAM-specific O_2^- production was calculated as that amount remaining after accounting for the estimated contribution by residing PMN (determined from the differential counts).

Phagocytic Activity

Phagocytic activity of recovered PAM was measured using a suspension assay (Cohen et al., 1996b). Cell aliquots (5×10^5 lavaged cells/900 μl phenol-red free RPMI) were placed into polypropylene tubes along with 100 μl sterile, rat serum-opsonized polystyrene latex microspheres (3 μm, Duke Scientific, Palo Alto, CA) at a particle:PAM ratio of 100:1 (determined to yield a 50% phagocytic index in preliminary studies). Samples were then incubated at 37°C in a shaking waterbath for 90 min before aliquots were placed onto glass slides. Smears were air-dried, the cells fixed in 2% glutaraldehyde (in phosphate-buffered saline, PBS), and uningested particles removed by immersion of the slides into xylene for 24 h .Cells were then stained with Diff-Quik and phagocytic activity was determined by examination

of 200 PAM/slide; phagocytic activity was quantitated in terms of phagocytic index (PI, percentage of viable cells that ingested ≥1 latex particle) and capacity (PC, percentage of phagocytically active PAM that ingested ≥3 particles).

Data Analysis

All data were analyzed using a one-way analysis of variance (ANOVA) followed, when appropriate, by a Student–Newman–Keuls test. Results were considered significant at $p < .05$.

RESULTS

Instillation of polyI:C into both air- and V-exposed rats caused significant time-dependent increases in the total numbers of lavageable cells (Table 1). However, total cell recoveries (all cell types) from V-exposed rats were always significantly greater than those from their air-exposed counterparts. By the end of the study period (48 h post-instillation), the numbers of cells recovered from V-exposed rats were more than twice those of the air controls. No significant differences in cell recoveries were observed between the two exposure groups prior to or following saline instillation.

Although total cell recoveries were clearly modified by both host exposure and the type of instillate, no significant differences were observed between the air- and V-exposed rats in either the total numbers or viabilities of recovered PAM after saline or polyI:C instillation. Conversely, polyI:C instillation caused a significant time-dependent increase in lavage levels of PMN in both V-exposed (from 2.15 to 5.57 to 19.68 × 10^6/rat) and air-exposed rats (from 0.17 to 2.42 to 7.33 × 10^6/rat). In addition, V-exposed rats consistently displayed significantly greater level of PMN in their bronchoalveolar lavage (BAL) fluid than air-exposed counterparts. As a result, the relative change in lung PMN numbers in the air-exposed rats in the first 24 h after instillation was far greater than that in V-exposed rats (13.3- vs. 1.6-fold, respectively); however, from 24 to 48 h postinstillation, differences between the exposure groups in relative increases in PMN (2.0- vs. 2.5-fold, respectively) were not significant.

Because of this PMN influx, polyI:C-instilled air control rats had a significantly reduced percentage of PAM in their BAL fluid 24 h after instillation (as compared to their saline-instilled counterparts), while the percentage of PMN increased 11-fold. By 48 h postinstillation, the percentage of BAL-associated PAM in polyI:C-treated air control rats was decreased by an additional 50%, while that of PMN increased by an additional 53% (compared to the 24-h values).

Unlike the air controls, the V-exposed rats displayed a significant reduction in the percentage of lavageable PAM even in the absence

TABLE 1. Pulmonary immune cells recovered in rat BAL fluid

Exposure	PolyI:C treatment	Time post it instillation (h)	PAM		PMN		Total cells recovered[a]
			Total Numbers recovered[a]	Relative % of all cells recovered	Total numbers recovered[a]	Relative % of all cells recovered	
Air	–[b]	0	2.66 ± 0.66	98.00 ± 0.50	0.05 ± 0.01	1.50 ± 0.22	2.71 ± 0.67
	–	24	3.85 ± 0.13	94.83 ± 0.95	0.17 ± 0.01	4.17 ± 0.70	4.06 ± 0.13
	+	24	2.60 ± 0.42	49.62 ± 4.42[d]	2.42 ± 0.39[d]	46.25 ± 5.13[d]	5.23 ± 0.84
	+	48	2.58 ± 0.23	24.87 ± 3.42[e]	7.33 ± 0.66[e]	70.62 ± 2.83[e]	10.38 ± 0.90[e]
Vanadate	–	0	2.21 ± 0.40	78.08 ± 4.08[c]	0.62 ± 0.11	21.83 ± 4.07[c]	2.83 ± 0.51
	–	24	3.08 ± 0.49	57.83 ± 4.45[c]	2.15 ± 0.34[c]	40.50 ± 4.41[c]	5.32 ± 0.84
	+	24	2.79 ± 0.22	32.75 ± 5.96[c,d]	5.57 ± 0.44[c,d]	65.38 ± 5.89[c,d]	8.52 ± 0.68[c,e]
	+	48	2.16 ± 0.09	9.50 ± 1.34[c]	19.68 ± 0.78[c,e]	86.75 ± 1.39[c,e]	22.69 ± 0.90[c,e]

[a]Mean ($\times 10^6$) ± SE.

[b]Rats intratracheally instilled with 150 μg polyI:C (+) or saline vehicle (–).

[c]Significant ($p < .05$) differences between the V and air exposure groups at a single time/treatment point.

[d]Within each treatment, significant ($p < .05$) differences between polyI:C- and saline-instilled groups at a single postinstillation time point.

[e]Within each treatment, significant ($p < .05$) differences between polyI:C-instilled rats at 24 and 48 h postinstillation.

of polyI:C. When compared to saline-instilled V-exposed rats, polyI:C-instilled rats had a significantly lower percentage of PAM after 24 h; by 48 h, the percentage of recovered PAM from these rats decreased an additional 71%. While there were no significant differences between the exposure groups regarding the numbers of lavageable PAM, the percentage of PAM from V-exposed rats was always significantly lower than that in air controls. In addition, V-exposed rats consistently displayed significantly greater percentages of lavageable PMN than their air-exposed counterparts.

Analysis of the rat lung cytokine profile for lavage fluid IFNγ, IL-6, IL-1, and TNFα following polyI:C instillation demonstrated that inhalation of V altered host lung responsivity to this biological response modifier. In rats that received either polyI:C or saline, no obvious differences could be observed between V- and air-exposed groups in recoverable IL-1 and TNFα levels/activities at any postinstillation time-point examined, as all values were below the assay limit of detection (i.e., 8 and 1 U/ml, respectively). In contrast, the levels of biologically active IL-6 recovered in lavage fluid were consistently greater in all V-exposed rats compared to similarly-instilled air controls (Table 2). Within 24 h after polyI:C instillation, air control rats displayed an ~30-fold increase in IL-6, while the increase was only 11-fold in V-exposed rats. Although no significant increase in lung IL-6 in polyI:C-treated air controls over the period from 24 to 48 h postinstillation

TABLE 2. Total protein and total and relative levels of IL-6 recovered in rat BAL fluid

Exposure	PolyI:C treatment	Time post it instillation (h)	Total protein (mg)[a]	IL-6 (pg)[a]	IL-6 (pg/mg protein)[a]
Air	–[b]	0	77.94 ± 7.86	415 ± 184	5.32 ± 2.36
	–	24	84.93 ± 6.29	102 ± 21	1.19 ± 0.03
	+	24	172.63 ± 13.64	11,859 ± 1854[d]	68.70 ± 10.74[d]
	+	48	156.00 ± 9.80	14,156 ± 2376	90.74 ± 15.23
Vanadate	–	0	182.30 ± 45.02	4668 ± 2234[c]	25.61 ± 12.75
	–	24	162.78 ± 5.54	2096 ± 358[c]	12.87 ± 2.20
	+	24	6995.58 ± 324.77[c,d]	51,418 ± 15,606[c,d]	7.35 ± 2.23[c]
	+	48	8145.23 ± 501.92[c,e]	113,084 ± 17,955[c,e]	13.88 ± 2.20[c]

[a]Mean (±SE) of levels recovered in first 10–15 ml of BAL.

[b]Rats intratracheally instilled with 150 μg polyI:C (+) or saline vehicle (–).

[c]Significant ($p < .05$) differences between the V and air exposure groups at a single time/treatment point.

[d]Within each treatment, significant ($p < .05$) differences between polyI:C- and saline-instilled groups at a single postinstillation time point.

[e]Within each treatment, significant ($p < .05$) differences between polyI:C instilled rats at 24 and 48 h postinstillation.

TABLE 3. Total protein and total and relative levels of interferon-γ recovered in rat BAL fluid

Exposure	PolyI:C treatment	Time post it instillation (h)	Total protein (mg)[a]	IFNγ (ng)[a]	IFNγ (pg/mg protein)[a]
Air	–[b]	0	77.94 ± 7.86	0.10 ± 0.04	1.09 ± 0.26
	–	24	84.93 ± 6.29	0.10 ± 0.01	1.20 ± 0.09
	+	24	172.63 ± 13.64	0.75 ± 0.32	4.35 ± 1.81[d]
	+	48	156.00 ± 9.80	0.72 ± 0.17	4.44 ± 0.80
Vanadate	–	0	182.30 ± 45.02	0.11 ± 0.02	0.72 ± 0.19
	–	24	162.78 ± 5.54	0.08 ± 0.01	0.48 ± 0.07
	+	24	6995.58 ± 324.77[c,d]	2.02 ± 0.64[c,d]	0.29 ± 0.09[c]
	+	48	8145.23 ± 501.92[c,e]	2.93 ± 0.20[c,e]	0.36 ± 0.01[c]

[a]Mean (±SE) of levels recovered in first 10–15 ml of BAL.

[b]Rats intratracheally instilled with 150 μg polyI:C (+) or saline vehicle (–).

[c]Significant ($p < .05$) differences between the V and air exposure groups at a single time/treatment point.

[d]Within each treatment, significant ($p < .05$) differences between polyI:C- and saline-instilled groups at a single postinstillation time point.

[e]Within each treatment, significant ($p < .05$) differences between polyI:C-instilled rats at 24 and 48 h postinstillation.

was observed, levels in similarly instilled V-treated rats doubled. By 48 h, IL-6 levels were 8-fold higher in lavage fluid from V-exposed rats compared to the air-exposed controls; however, the total increase above background IL-6 levels (i.e., at 0 h) in these rats was only 24-fold, compared to 34-fold in the controls.

Instillation of polyI:C also significantly increased total levels of IFNγ in the lung lavage fluids of rats from both exposure groups (Table 3). While background and saline-instilled rats in both exposure groups had equivalent lung IFNγ levels, control and V-exposed rats demonstrated 7- and 20-fold increases, respectively, by 24 h post-polyI:C instillation. In addition, in the subsequent 24 h, IFNγ levels increased by an additional 45% in V-exposed rats while remaining unchanged in their air-exposed counterparts.

Another effect of polyI:C instillation was an increase in the total amount of protein found in the BAL (Tables 2 and 3). However, while BAL protein levels doubled in air-exposed rats over the entire 48-h period after instillation, levels in the V-exposed rats increased by 38- to 45-fold. As a result, when IFNγ and IL-6 levels at both post-polyI:C instillation timepoints were evaluated in relation to the total protein in the BAL, V-exposed rats had significantly lower levels of cytokine than those measured in the air-exposed controls.

No differences in either PMN/PAM mixture or PAM-specific O_2^- production were observed between the exposure groups prior to instil-

lation of either polyI:C or saline (Figure 1 and Table 4). At 24 h after saline instillation, both spontaneous and OZ-stimulated O_2^- production by all cells recovered from V-exposed rats were significantly reduced compared to that by cells from V-exposed rats sacrificed immediately after saline instillation or from air control 24-h counterparts; PAM-specific O_2^- production was significantly reduced only after stimulation. Saline instillation had no effect on O_2^- production in air-exposed rats.

At 24 h following polyI:C instillation, there were no significant host exposure-dependent differences in either spontaneous or OZ-stimulated O_2^- formation by the PAM/PMN mixtures or by PAM specifically. However, while production from cell mixtures obtained from air-exposed rats was uniformly reduced compared to that of cells from their saline-instilled counterparts, only stimulated production was significantly reduced in the cells of V-exposed hosts. When analyzed at the level of PAM-specific production, this polyI:C effect was only apparent after zymosan stimulation, regardless of host preinstillate exposure.

By 48 h post-polyI:C instillation, spontaneous O_2^- formation by the PAM/PMN mixtures of both exposure groups was reduced further; however, no further reduced production was observed in OZ-stimulated mixtures. Conversely, spontaneous PAM-specific production appeared to be unaffected while that following stimulation continued to decrease in cells from both exposure groups. The data herein also indicated that a time post polyI:C-dependent effect upon OZ-stimulated PAM O_2^- production was evident; this effect was greater in PAM from rats exposed to V prior to polyI:C instillation than in similarly instilled air control animals.

PolyI:C instillation produced significant increases in the phagocytic activity of PAM recovered from rats in both the air and V exposure groups. At 24 h postinstillation, the phagocytic index (PI) was increased by 46% and 40% with PAM recovered from air- and V-exposed rats, respectively (Figure 2A). Saline-induced increases in PI were not significant in cells obtained from either exposure group. Although increases in PI after polyI:C-instillation were similar for both the air and V exposure groups, PI values in PAM from V-exposed rats were always significantly lower than those in cells recovered from the air controls. A similar pattern was observed with the phagocytic capacity (PC) (Figure 2B); polyI:C significantly elevated the PC of PAM from both exposure groups (52% and 85% above preinstillation values for air- and V-exposed rats, respectively). However, in V-exposed rats that received saline there was also a significant increase in the PC of recovered PAM to a level approximating that of their polyI:C-treated counterparts (28% vs. 30%, respectively); no saline-induced increase in PC was observed in air-exposed rat cells.

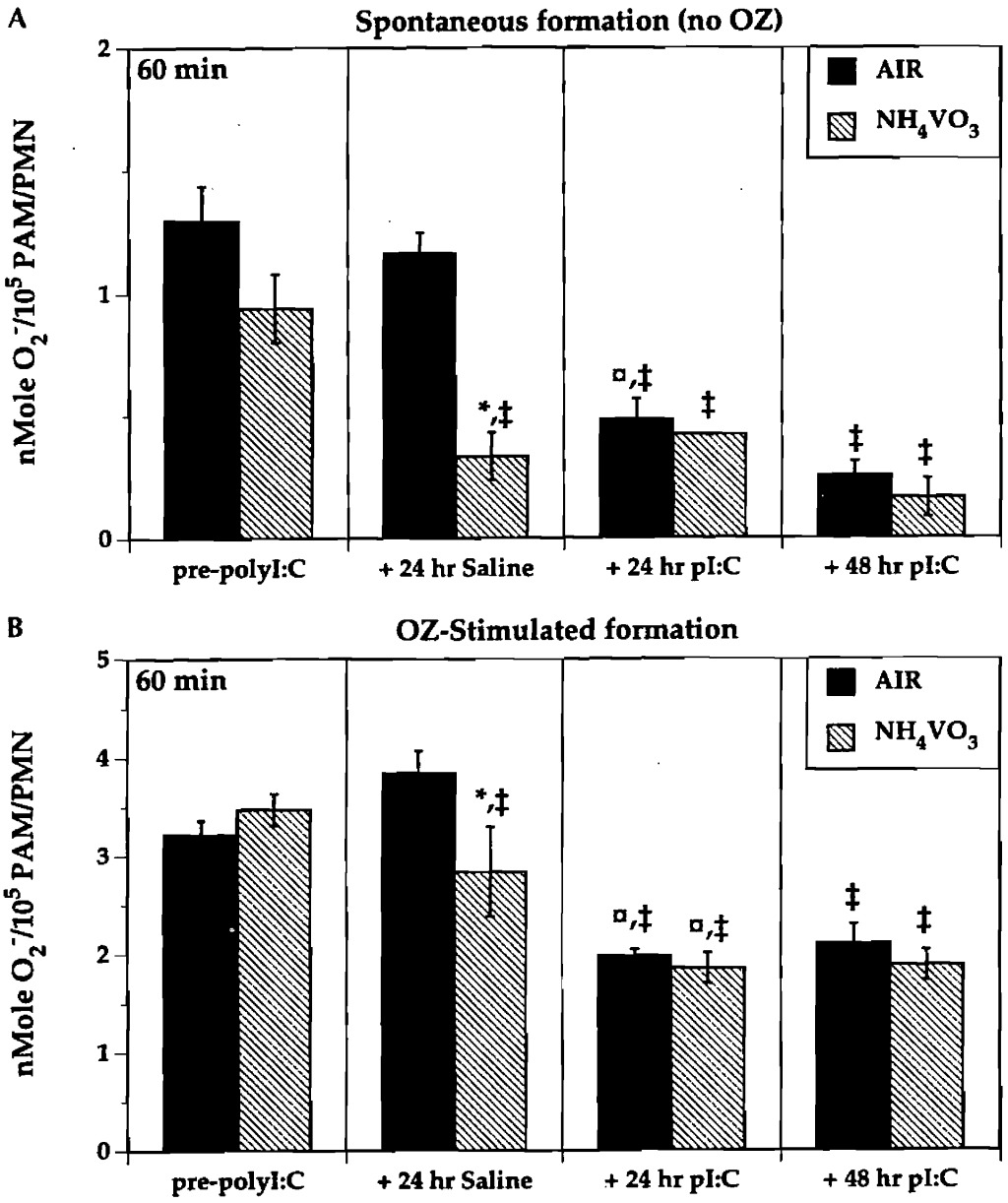

FIGURE 1. (A) Spontaneous and (B) opsonized zymosan (OZ) stimulated superoxide anion (O_2^-) production in pooled lavaged cells recovered from rats exposed for 4 d to NH_4VO_3 or air and then instilled with polyI:C or saline. Values shown are mean ± SE (number of rats per group described in Materials and Methods) PAM production levels after normalizing for numbers of adherent cells. Asterisk indicates values significantly different between the exposure groups ($p < .05$) at the same time/treatment point. Within each exposure group, values significantly different due to the type of instillate ($^{\square}p < .05$) or significantly different from the pre-polyI:C value ($^{\ddagger}p < .05$) are also shown.

TABLE 4. Superoxide formation by Rat PAM after accounting for PMN presence

Exposure	PolyI:C treatment	Time post it instillation (h)	Contribution to O_2^- production from PMN present (%)[a]	nmol O_2^-/ 10^5 PAM/ 60 min[b]
		Spontaneous production		
Air	–[c]	0	0	1.321 ± 0.146
	–	24	0	1.229 ± 0.088
	+	24	0	0.974 ± 0.170
	+	48	0	1.017 ± 0.237
Vanadate	–	0	0	1.204 ± 0.180
	–	24	0	0.577 ± 0.169
	+	24	0	1.288 ± 0.013
	+	48	0	1.716 ± 0.835
		OZ-stimulated production		
Air	–	0	0.79	3.188 ± 0.145
	–	24	1.44	3.786 ± 0.222
	+	24	30.86	1.368 ± 0.045[d,e]
	+	48	48.92	1.072 ± 0.104[e]
Vanadate	–	0	11.82	3.062 ± 0.144
	–	24	23.33	2.177 ± 0.351[e,f]
	+	24	40.66	1.100 ± 0.093[d,e]
	+	48	61.85	0.720 ± 0.059[e]

[a]Assumes no O_2^- production by PMN in unstimulated samples and a rate of 0.045 nmol $O_2^-/10^5$ PMN/min in stimulated samples (R. Thrall, personal communication). Value calculated as 5 min PMN production/estimated 5 min production in PMN + PAM (based on mixture production rate).

[b]Mean (±SE) after accounting for PMN contribution (assumes that percentage calculated for 5 min PMN remains constant over 60-min incubation period).

[c]Rats intratracheally instilled with 150 μg polyI:C (+) or saline vehicle (–).

[d]Within each treatment, significant ($p < .05$) differences between polyI:C- and saline-instilled groups at a single postinstillation time point.

[e]Within each treatment, significant ($p < .05$) difference from the 0 h saline-instilled rat value.

[f]Significant ($p < .05$) differences between the V and air exposure groups at a single time/treatment point.

DISCUSSION

The results from the studies presented here indicate that short-term, repeated inhalation of NH_4VO_3 by rats can alter local pulmonary immune responses to the biological response modifier polyI:C. This is supported by findings from previous studies that demonstrated that V exposure can alter ex vivo and in vitro macrophage responses to LPS and IFNγ (Cohen et al., 1993a, 1993b, 1996a, 1996b).

In several species, double-stranded polyribonucleotides, such as polyI:C, act as potent immunomodulators for some immune functions,

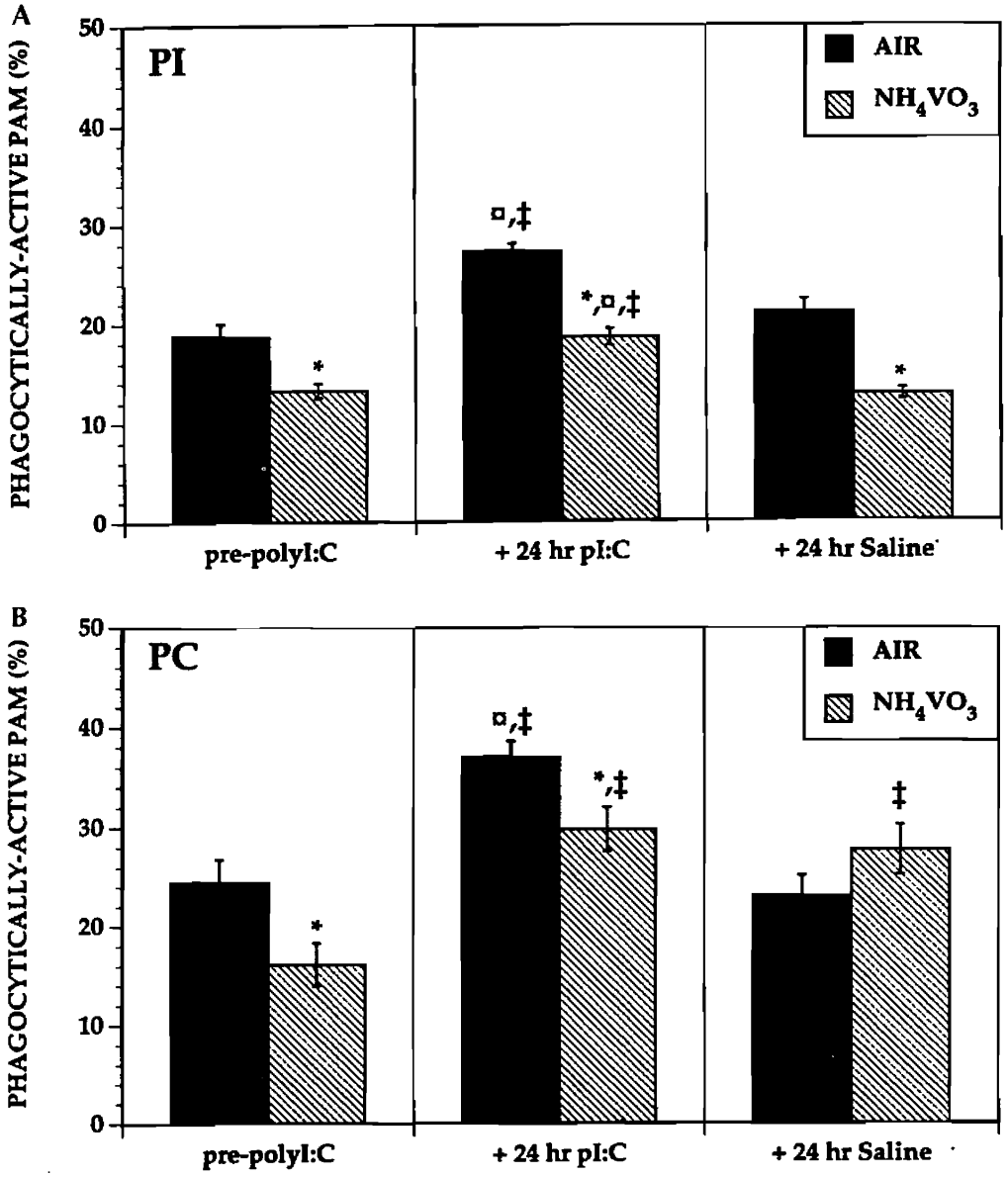

FIGURE 2. (A) Phagocytic index and (B) capacity of PAM recovered from rats exposed for 4 d to NH_4VO_3 or air and then instilled with polyI:C or saline. Percentages ± SE (number of rats per group described in Materials and Methods) of cells ingesting particles (PI) and those phagocytically active cells bearing ≥3 particles per cell (PC) are indicated. Each bar represents examination of 200 PAM/slide, with 5 slides/rat. Asterisk indicates values significantly different between the exposure groups ($p < .05$) at the same time/treatment point. Within each exposure group, values significantly different due to the type of instillate ($¤p < .05$) or significantly different from the pre-polyI:C value ($^{\ddagger}p < .05$) are also shown.

including IFN synthesis, antibody production, natural killer cell activity, and resistance against certain viral agents (Black et al., 1992) and tumors (Levine et al., 1982); as such, polyI:C is a useful model for demonstrating altered immune cell functions as a result of toxicant exposure. For example, polyI:C produces alterations in rat PAM respiratory burst activity, IL-1α synthesis, and tumoricidal activity following in vitro exposure (Meunier et al., 1986). When administered by intratracheal (it) instillation or ip injection, polyI:C modifies PAM ex vivo tumoricidal/candidacidal activity, Fc-mediated erythrophagocytosis, and O_2^- production, as well as increasing Mϕ IFNα/β production (Burleson et al., 1987; Weiss et al., 1990; Kaplan et al., 1995).

In studies with rats intratracheally instilled with polyI:C, there was an immediate and persistent (up to 7 d) PMN influx into the lungs (Weiss et al., 1990; Kaplan et al., 1995). In these aforementioned studies, PMN levels increased from 6 to 57% within the first 24 h and then declined slowly to baseline levels. In our study, air-exposed rats displayed these same trends. However, as rats exposed for 4 d to NH_4VO_3 already demonstrated significantly elevated levels of PMN in their lungs prior to polyI:C instillation, instillation with polyI:C did not cause similar large augmentations of PMN levels to occur as seen with the air control rats.

While there are no reports demonstrating effects of polyI:C on rat BAL fluid protein or cytokine levels, in a study using mice intratracheally instilled with polyI:C, no effect upon BAL fluid protein levels (compared to saline-instilled controls) was previously observed (Hyde & Giri, 1990). In our study, polyI:C instillation more than doubled the BAL protein levels in air-exposed rats. In comparison, V-exposed polyI:C-instilled rats demonstrated a 35- to 45-fold increase in protein levels. While increases in recoverable protein levels previously observed in rodents repeatedly exposed to NH_4VO_3 (Cohen & Wei, 1988; Cohen et al., 1996b) were initially hypothesized to be due to effects from V upon protein metabolizing enzymes (Nechay, 1984; Pillai & Zull, 1985), the extensive increase in V-exposed rat BAL protein levels following polyI:C instillation cannot be due to this modification alone. The observed increased levels of select cytokines in the BAL from V-exposed polyI:C-instilled rats suggested that altered cytokine formation may have impacted upon total protein levels; however, these increases in cytokine levels would only yield a small contribution to overall BAL protein levels. Therefore, based on our findings, as neither the contribution from lung cell damage due solely to V nor those from increased cytokine production alone could completely explain the increased BAL protein levels, we surmise that V and polyI:C acted together to bring about the observed effect.

Observed increases in lung IFNγ and IL-6 levels/activity support this last notion. While levels of IL-6 and IFNγ formation did not

increase from 24 to 48 h after polyI:C treatment of the air-exposed control rats, levels in V-exposed rats continued to rise during this period. That an increase in levels of inflammatory cells alone could underlie these changes is not supported by data from this study. In air control rats, although PMN levels increased significantly during the 24- to 48-h period after polyI:C instillation, IL-6 and IFNγ levels were unchanged. In addition, if IL-6 levels from air- and V-exposed rats are compared when PMN levels were comparable (48 and 24 h postinstillation, respectively), levels remain ~4-fold greater in the V group. This suggests that short-term repeated V exposure might modulate mechanisms that regulate in situ polyI:C-induced cytokine production.

Stimulated O_2^- production by lavaged lung cells after polyI:C instillation was expected to be significantly reduced compared to that produced by cells from saline-treated rats (Weiss et al., 1990). While this effect was observed in both the air- and V-exposed groups, the most significant effect upon O_2^- production was not observed with PAM/PMN mixtures from V-exposed polyI:C-treated rats but rather from 24 h post-saline-instilled V-exposed rats. This decreased production could be attributed to the fact that the BAL from these latter rats was more heavily contaminated with PMN (which produce significantly less O_2^- after simulation than PAM; R. Thrall, personal communication) than that recovered from their air-exposed counterparts. However, if all exposure-related differences in O_2^- production were directly correlated with variations in recovered cell types, then pooled cells recovered from V-exposed rats sacrificed immediately after saline instillation should have had lower levels of O_2^- production than their air-exposed counterparts. In addition, air-exposed rats sacrificed 24 h after saline instillation had 4 times as many PMN as their counterparts sacrificed immediately after saline instillation, yet demonstrated no marked changes in O_2^- production; conversely, V-exposed rats analyzed 24 h after instillation with saline had a near-doubling of PMN levels, but displayed reduced O_2^- production compared to counterparts sacrificed immediately after saline instillation. While it is impossible to ignore the contribution from contaminating PMN upon measured O_2^- production, until more quantitative studies explaining the contribution of individual phagocytic cell types to oxyradical production are performed, to claim that the observed reduction in O_2^- production in saline-instilled V-exposed rats as a result of a "diluting-out" effect alone would be misleading.

As noted, cells recovered from both air- and V-exposed rats displayed reduced OZ-stimulated O_2^- production capacity following polyI:C instillation. While no marked differences between the total production by pooled cells at 24 and 48 h postinstillation were evident in either exposure group, when the contributions from contaminating PMN were accounted for (assuming that V had no effect upon PMN O_2^- produc-

tion), a time-postinstillation-dependent trend in the reduction in PAM-specific O_2^- production became apparent for both groups. In addition, within this trend, the reduction in stimulated O_2^- production (over the 24- to 48-h period post-polyI:C instillation) by PAM from V-exposed rats was ~60% greater than that with PAM from air control rats (34.5 vs. 21.6%, respectively). As this result is in agreement with our earlier observations in which PAM responsiveness to OZ after stimulation with IFNγ was decreased after V exposure (Cohen et al., 1993b, 1996a, 1996b), we hypothesize that PAM responsiveness to polyI:C, like that to IFNγ, was affected by V.

This hypothesis is further supported by results from the phagocytic activity studies in which PAM recovered from V-exposed hosts displayed: (1) significantly different activities compared to cells from polyI:C-treated controls, (2) a lack of an increase in phagocytic capacity (PC) above those levels obtained with PAM from saline-instilled V-exposed rats, and (3) smaller increments in phagocytic indices (PI) (compared to saline-instilled counterparts) than obtained with cells from air-exposed rats.

Effects upon Mϕ/PAM responsiveness to IFNγ and LPS following host/cell exposure to V have been previously demonstrated (Cohen et al., 1993, 1996a, 1996b). As it has been shown that polyI:C acts upon Mϕ using signaling processes similar to that of these agents (Torres & Johnson, 1985), it is possible that our earlier hypothesized mechanisms by which V might modulate cell responsivity to these biological response modifiers (i.e., via altered receptor distribution/functionality) may also underlie altered responses to polyI:C. Until exact mechanisms by which polyI:C acts upon immune cells are elucidated, a more precise explanation of how V might be affecting PAM ability to respond to this modulator remains uncertain. Still, the results here, in conjunction with our previous studies, clearly indicate that short-term, repeated inhalation of V in the form of NH_4VO_3 alters the capacity of pulmonary immune cells to respond to important biological response modifiers. As such, modification of the lungs' ability to properly respond to agents that serve, in part, to regulate production of critical cytokines now appears certain to be an underlying factor in the observed increased incidence of respiratory diseases in humans occupationally exposed to V.

REFERENCES

Agency for Toxic Substances and Disease Registry. 1991. *Toxicological profile for vanadium and compounds.* Atlanta, GA: U.S. Public Health Service.

Al-Bayati, M. A., Culbertson, M. R., Schreider, J. P., Rosenblatt, L. S., and Raabe, O. G. 1992. The lymphotoxic action of vanadate. *J. Environ. Pathol. Toxicol. Oncol.* 11:19–27.

Becker, S., Devlin, R. B., and Haskill, S. 1989. Differential production of tumor necrosis factor,

macrophage colony stimulating factor, and interleukin-1 by human alveolar macrophages. *J. Leukocyte Biol.* 45:353–361.

Becker, S. Madden, M. C., Newman, S. L., Devlin, R. B., and Koren, H. S. 1991. Modulation of human alveolar macrophage properties by ozone exposure in vitro. *Toxicol Appl. Pharmacol.* 110:403–415.

Black, P. L., Hartmann, D., Pennington, R., Phillips, H., Schneider, M., Tribble, H. R., and Talmadge, J. E. 1992. Effect of tumor burden and route of administration on the immunotherapeutic properties of polyinosinic-polycytidilic acid stabilized with poly-L-lysine in carboxymethyl cellulose [Poly(I,C)-LC]. *Int. J. Immunopharmacol.* 14:1341–1353.

Burleson, G. R., Fuller, L. B., Menache, M. G., and Graham, J. A. 1987. Poly(I):poly(C)-enhanced alveolar and peritoneal macrophage phagocytosis: Quantification by a new method utilizing fluorescent beads. *Proc. Soc. Exp. Biol. Med.* 184:468–476.

Cohen, M. D., and Wei, C. I. 1988. Effects of ammonium metavanadate treatment upon macrophage glutathione redox cycle activity, superoxide production, and intracellular glutathione status. *J. Leukocyte Biol.* 44:122–129.

Cohen, M. D, Wei, C. I., Tan, H., and Kao, K. J. 1986. Effect of ammonium metavanadate on the murine immune response. *J. Toxicol. Environ. Health* 19:279–298.

Cohen, M. D., Chen, C. M., and Wei, C. I. 1989. Decreased resistance to *Listeria monocytogenes* in mice following vanadate exposure: Effects upon the function of macrophages. *Int. J. Immunopharmacol.* 11:285–292.

Cohen, M. D., Parsons, E., Schlesinger, R. B., and Zelikoff, J. T. 1993a. Immunotoxicity of *in vitro* vanadium exposure: Effects on interleukin-1, tumor necrosis factor, and prostaglandin E_2 by WEHI-3 macrophages. *Int. J. Immunopharmacol.* 15:437–446.

Cohen, M. D., Schlesinger, R. B., and Zelikoff, J. T. 1993b. Vanadium-induced alterations in macrophage responses to biological response modifiers *in vitro*. *Toxicologist* 13:422.

Cohen, M. D., McManus, T. P., Yang, Z., Qu, Q., Schlesinger, R. B., and Zelikoff, J. T. 1996a. Vanadium affects macrophage interferon-γ binding and -inducible responses. *Toxicol. Appl. Pharmacol.* 138:110–120.

Cohen, M. D., Yang, Z., Zelikoff, J. T., and Schlesinger, R. B. 1996b. Pulmonary immunotoxicity of inhaled ammonium metavanadate in Fischer 344 rats. *Fundam. Appl. Toxicol.* 33:254–263.

Conklin, A. W., Skinner, C. S., Felten, T. L., and Sanders, C. L. 1982. Clearance and distribution of intratracheally instilled vanadium compounds in the rat. *Toxicol. Lett.* 11:199–203.

Feldmann, M. 1996. Cell cooperativity in the antibody response. In *Immunology,* eds. I. Roitt, J. Brostoff, and D. Male, pp. 8.1–8.16. London: Mosby.

Hickey, R. J., Schoff, E. P., and Clelland, R. C. 1967. Relationship between air pollution and certain chronic disease death rates. *Arch. Environ. Health* 15:728–739.

Hyde, D. M., and Giri, S. N. 1990. Polyinosinic-polycytidilic acid, an interferon inducer, ameliorates bleomycin-induced lung fibrosis in mice. *Exp. Lung Res.* 16:533–546.

Kaplan, J. M., Herzyk, D. J., Ruggieri, E. V., O'Leary Bartus, J., Esser, K. M., and Bugelski, P. J. 1995. Effect of TNFα production inhibitors BRL 61063 and pentoxifylline on the response of rats to polyI:C. *Toxicology* 95:187–196.

Kivuoloto, M. 1980. Observations on the lungs of vanadium workers. *Br. J. Ind. Med.* 37:363–366.

Kivuoloto, M., Rasanen, O., Rinne, A., and Rissanen, M. 1979. Effects of vanadium on the upper respiratory tract of workers in a vanadium factory. A macroscopic and microscopic study. *Scand. J. Work Environ. Health* 5:50–58.

Kivuoloto, M., Pakarinen, A., and Pyy, L. 1980. Clinical laboratory results of vanadium-exposed workers. *Arch. Environ. Health* 36:109–113.

Kivuoloto, M., Rasanen, O., Rinne, A., and Rissanen, A. 1981. Intracellular immunoglobulin in plasma cells of nasal biopsies taken from vanadium-exposed workers. *Annt. Anz. Jena* 149:446–450.

Lees, R. 1980. Changes in lung function after exposure to vanadium compounds in fuel oil ash. *Br. J. Ind. Med.* 37:253–256.

Levine, A. S., Durie, B., Lampkin, B., Leventhal, B. F., and Levy, H. B. 1982. Interferon induction, toxicity, and clinical efficacy of poly ICLC in hematologic malignancies and other tumors. In *Immunotherapy of human cancer,* eds. W. D. Terry and S. A. Rosenberg, pp. 411–418. Amsterdam: Elsevier-North Holland.

Meunier, P. C., Badger, A. M., Kellet, A., Weiss, R. A., and Bugelski, P. J. 1986. Biological response modifier (BRM) stimulation of rat alveolar macrophages: Dissociation of superoxide release and tumoricidal activity. *Fed. Proc.* 45:331.

Musk, A. W., and Tees, J. G. 1982. Asthma caused by occupational exposure to vanadium compounds. *Med. J. Aust.* 1:183–184.

National Institute for Occupational Safety and Health. 1985. *Pocket guide to chemical hazards,* 5th ed., pp. 234–235. Washington, DC: Department of Health and Human Services.

Nechay, B. R. 1984. Mechanisms of action of vanadium. *Annu. Rev. Pharmacol. Toxicol.* 24:501–524.

Nriagu, J. O., and Pacyna, J. M. 1988. Quantitative assessment of worldwide contamination of air, water, and soils by trace metals. *Nature* 333:134–139.

Pillai, S., and Zull, J. E. 1985. Effects of ATP, vanadate, and molybdate on cathepsin D-catalyzed proteolysis. *J. Biol. Chem.* 260:8384–8389.

Sharma, R. P., Bourcier, D. R., Brinkerhoff, C. R., and Christensen, S. A. 1981. Effects of vanadium on immunologic functions. *Am. J. Ind. Med.* 2:91–99.

Sharma, R. P., Flora, S. J., Brown, D. B., and Oberg, S. G. 1987. Persistence of vanadium compounds in lungs after intratracheal instillation in rats. *Toxicol. Ind. Health* 3:321–329.

Stocks, P. 1960. On the relations between atmospheric pollution in urban and rural localities and mortality from cancer, bronchitis, pneumonia, with particular reference to 3,4-benzopyrene, beryllium, molybdenum, vanadium, and arsenic. *Br. J. Cancer* 14:397–418.

Torres, B. A., and Johnson, H. M. 1985. Lipopolysaccharide and polyribonucleotide activation of macrophages: Implications for a natural triggering signal in tumor cell killing. *Biochem. Biophys. Res. Commun.* 131:395–401.

VanSnick, J. S., Cayphas, S., Vink, A., Uttenhove, C., Coulie, P., Rubira, M. R., and Simpson, R. J. 1986. Purification and NH_2-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas. *Proc. Natl. Acad. Sci. USA* 83:9679–9683.

Wei, C. I., Al-Bayati, M. A., Culbertson, M. R., Rosenblatt, L. S., and Hansen, L. D. 1982. Acute toxicity of ammonium metavanadate in mice. *J. Toxicol. Environ. Health* 10:673–687.

Weiss, R. A., Meunier, P. C., Kelley, A., Klinkner, A., Badger, A. M., and Bugelski, P. J. 1990. Activation of rat pulmonary lavage cells by intratracheal administration of polyinosinic-polycytidilic acid. *J. Biol. Respir. Modif.* 9:411–419.

Zelikoff, J. T., and Cohen, M. D. 1995. Immunotoxicity of inorganic metal compounds. In *Experimental immunotoxicology,* eds R. J. Smialowicz and M. P. Holsapple, pp. 189–228. Boca Raton, FL: CRC Press.

Zelikoff, J. T., Thomas, D. T., Parsons, E., and Schlesinger, R. B. 1993. Inhalation of particulate lead disrupts pulmonary macrophage-mediated functions important for host defense and tumor surveillance in the lung. *Environ. Res.* 67:207–222.