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Pulmonary Immunotoxicity of Inhaled Ammonium Metavanadate in Fisher 344 Rats

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Male Fisher 344 rats were exposed to 2 mg vanadium(V)/m^3 (as ammonium metavanadate NH_4VO_3, 0.32 μm MMD) atmospheres for 8 hr/day for 4 days in a nose-only exposure system. In exposed rats, lung V burdens increased in a time-dependent fashion. Analysis of lung cells and lavage fluid 24 hr after the final exposure suggested that tissue damage and a strong inflammatory response was elicited; numbers of neutrophil and small macrophages (Mø), as well as levels of lavageable protein and lactate dehydrogenase, were significantly elevated as compared with levels observed with air-exposed rats. Vanadium also affected pulmonary alveolar Mø (PAM) capacities to produce and respond to immunoregulating cytokines. Inducible PAM production of tumor necrosis factor-α was significantly inhibited, as was the ability to increase cell surface Class II/I-A molecule expression in response to interferon-γ (IFNγ). PAM from V-exposed hosts were also inhibited in their ability to be primed by IFNγ to produce superoxide anion and hydrogen peroxide in response to stimulation with opsonized zymosan. These studies indicate that short-term repeated exposure of rats to atmospheric V, at levels encountered in an occupational setting, can alter host pulmonary immunomocompetence, with one major effect occurring at the level of cytokine-related functions. These alterations may be underlying mechanisms for the well-documented increases in bronchopulmonary infections and cancers in workers chronically exposed to V-containing atmospheres.
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Vanadium (V), a Group Vb transition metal, is used widely in the steel and chemical industries, and is a constituent of many ores, coals, and oils (ATSDR, 1991). 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 (containing both insoluble vanadium pentoxide and soluble vanadates) in many work environs (Nriagu and 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).

Vanadium is known to modulate immune responses in humans and experimental animals (reviewed in Zelikoff and Cohen, 1995). Epidemiological studies have demonstrated that exposure of workers to moderate-to-high levels of V-bearing particles resulted in an increased incidence of pulmonary diseases, i.e., asthma, pneumonia, and bronchitis, and deaths from respiratory failure secondary to infections or from lung cancers initiated by other agents (Stocks, 1960; Hickey *et al.*, 1967).

Though initial clearance of inhaled V is fairly rapid (≈40% in 1 hr), total clearance is never achieved (Conklin *et al.*, 1982; Sharma *et al.*, 1987), and particles may persist in the lungs. Studies of V-exposed humans demonstrated disturbances in pulmonary neutrophil numbers and morphology, plasma cell numbers, immunoglobulin production, and lymphocyte mitogenic responsiveness (Kivuoloto *et al.*, 1979, 1980, 1981). Concurrent changes in pulmonary function (decreased forced vital capacity and forced expiratory volume) as well as increased nonspecific pulmonary reactivity to V have also been documented (Kivuoloto, 1980; Lees, 1980; Musk and Tees, 1982). In exposed primates, V inhalation resulted in similarly altered lung morphology, physiology, and biochemistry (Knecht *et al.*, 1985; Kirkpatrick *et al.*, 1993). In exposed rodents, V was directly cytotoxic to tracheal epithelium (Schiff *et al.*, 1981; Schiff and Graham, 1984), altered lung insoluble collagen levels (Kowalska, 1989), and increased lung tissue lipid peroxidation (Inouye *et al.*, 1980; Donaldson and LaBella, 1983).

While studies using animal models to assess general toxicologic endpoints and kinetics following V inhalation/instillation have been performed, information regarding immunotoxicologic effects overall, and particularly in the lungs, is sparse. However, a few studies have demonstrated that in primates, V inhalation resulted in changes in host pulmonary function/reactivity along with significant changes in lung immune cell profiles (Knecht *et al.*, 1985; Kirkpatrick *et al.*, 1993). Rodents exposed to V by intraperitoneal injection, intratracheal instillation, or via the diet showed alterations in immune cell phagocytic activity, lymphoproliferative re-

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sponsiveness, lysosomal enzyme activity/release, microtubule structural integrity, and intracellular organelle pH (Zelikoff and Cohen, 1995). Exposed hosts also had immune system organ pathologies and displayed alterations in resistance to endotoxin/intact microorganisms and in pulmonary immune cell populations (Sharma *et al.*, 1981; Wei *et al.*, 1982; Cohen *et al.*, 1986, 1989; Al-Bayati *et al.*, 1992).

This study is the first to examine the immunotoxic effects of short-term, repeated inhalation of occupationally relevant concentrations of pentavalent vanadium in the rat lung. The results of this investigation may provide some insight into the mechanisms underlying V-induced suppression of host pulmonary immunocompetence.

METHODS

Materials. Tissue culture reagents were obtained from Gibco (Grand Island, NY), ammonium metavanadate (NH_4VO_3, 99.8% purity) from J.T. Baker Chemicals (Phillipsburg, NJ), reactive oxygen intermediate (ROI) assay reagents and cell differential dyes from Sigma Chemicals (St. Louis, MO), and rat interferon-γ (IFNγ) from Genzyme Corporation (Cambridge, MA).

Experimental animals. Upon arrival, 10-week-old pathogen-free male Fisher 344 rats (200–250 g, Charles River, Boston, MA) were quarantined for 2 weeks 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.*

Exposure regimen. For each experimental protocol outlined herein, separate groups of rats were exposed, nose-only, to atmospheres containing either filtered air or NH_4VO_3 (0.32-μm diameter). Vanadate, rather than the other commonly encountered form of V found in the workplace (i.e., V_2O_5), was used because of its high solubility at neutral pH, its prevalence in respirable dusts/fumes, and the lengthy period of time required (i.e., days) before polymerization to less soluble forms occurs. Specifically, ammonium metavanadate was selected for use in these studies because a substantial database for its immunotoxicity already exists (Cohen *et al.*, 1986, 1989; Vaddi and Wei, 1991a,b, 1996), and the ammonium ion has been shown not to impart immunotoxicologic effects at the concentrations employed.

Aerosols delivering approximately 2 mg V/m^3 [1.94 ± 0.04 mg/m^3 (±SD)] were generated from a suspension of 40 nM NH_4VO_3 in ultrapure water using a Laskin nebulizer connected in series with a nose-only exposure unit (Fig. 1). This concentration is based upon minute ventilations of ≈0.2 and 7.0 liters for the rat and human, respectively, and equivalent deposition efficiencies (i.e., 20%) for particles/droplets of this aerodynamic diameter. Exposure duration was 8 hr/day for 4 days approximating (in each 8-hr period) the levels of V that could be encountered by workers exposed to V-bearing dusts/fumes at 0.05 mg V_2O_5/m^3 per 8- to 10-hr workday. Samples of the generated atmosphere were collected hourly on filters placed in parallel lines for estimation of actual V concentrations. During all exposures, rats were placed in restrainers which allowed free movement of the head; no sedation was required.

Vanadium analysis in lung tissue. Immediately after each daily exposure, as well as 24 hr after the final exposure, rats (n = 2/timepoint) were euthanized by an overdose of Nembutal (80 mg/kg, ip) and their lungs removed, blot-dried, and trimmed of extraneous tissue. The left lungs were weighed and acid-digested by heating the tissues in 10 ml concentrated HNO_3 (Zelikoff *et al.*, 1993); 30% (v/v) hydrogen peroxide (H_2O_2) was added during digestion to aid in tissue matrix breakup. At near-dryness, the samples were removed from the heat and cooled; 5 ml concentrated HCl was then added and the samples were reheated. Samples were reconstituted in 2 ml ultrapure water (18 Mohm) for processing by graphite atomic absorption analysis (AAS; Thermal-Jarrel Ash Model 12 Spectorphotometer). Vanadium concentration was determined using a Fisher certified standard diluted to appropriate concentrations in 2% HNO_3. A calibration curve was generated by AAS and results were expressed in μgV/g lung tissue.

Isolation of rat pulmonary alveolar macrophages (PAM). To provide PAM for each *ex vivo* assay described, rats were sacrificed 24 hr after their last daily exposure. The trachea was exposed, a cannula inserted and tied into place, and PAM recovered by repeated (8–10) washings with warm (37°C) Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (HBSS, pH 7.4) (Schlesinger *et al.*, 1992). Cells in this bronchoalveolar lavage fluid (BAL) were then centrifuged at 250g for 15 min at 4°C and washed twice with RPMI 1640, and the total cell numbers and viabilities determined by hemacytometer counting and trypan blue exclusion, respectively. To monitor for influx of immune cells other than PAM resulting from V exposures, cell differentials were performed on cytocentrifuge preparations stained with Diff-Quik. Acellular lavage fluid was assayed for total protein and lactate dehydrogenase (LDH) activity, using commercially available Bio-Rad and Sigma LDH assay kits, respectively; results were expressed in mg protein in total BAL and B-B units/ml BAL, respectively.

TNFα production. To assess effects of V on TNFα production, lavaged cells (10^6 cells/1 ml serum-free RPMI) recovered from rats (n = 4/treatment group) were added into 35-mm dishes. After 2 hr incubation at 37°C to permit cell attachment, one-half of the plates received 1 μg *Escherichia coli* endotoxin (LPS, Type 0111:B4); the remaining plates received saline vehicle only. Following a 24-hr incubation at 37°C, the culture medium from each dish was collected and filtered through a 0.22-μm filter. The recovered volume was recorded and the TNFα-bearing samples were then analyzed for activity using a standard LM target cell cytotoxicity assay (Cohen *et al.*, 1994).

ROI production. To measure superoxide anion ($\cdot O_2^-$) and H_2O_2 production following host exposure to V or air (n = 10 rats/treatment group), recovered cells (in RPMI/5% FBS) were plated in 96-well microtiter dishes at 2.25 × 10^5 cells/well. After a 2-hr incubation at 37°C to permit PAM attachment, 10 μl rat IFNγ (1000 U/ml) or RPMI was added to the wells and the plates were reincubated for 24 hr at 37°C. Following incubation, wells received rat serum-opsonized zymosan (OZ) (50 μg/ml) or RPMI only (to assess spontaneous formation), and ROI formation was assessed using protocols previously described by Zelikoff *et al.* (1993).

Briefly, to quantitate $\cdot O_2^-$ production, 200 mM ferricytochrome c in phenol red-free Hanks balanced salt solution (HBSS) was added to half the wells; remaining wells received the solution supplemented with 600 U/ml superoxide dismutase (SOD). Plates were incubated at 37°C and well absorbances measured (at 550 nm) by an automated microtitre plate reader (Anthos 2001; Anthos Lab Instruments, Durham, NC). SOD-inhibitable $\cdot O_2^-$ levels were calculated using an extinction coefficient value of 15.8, and results expressed in nanomoles $\cdot O_2^-$/2.25 × 10^5 cells.

To quantitate H_2O_2 formation, 0.25 mg/ml horseradish peroxidase:0.5 mg/ml phenol red (in HBSS) solution was added to each well. At hourly timepoints, designated wells were made alkaline by addition of 10 μl 1 N NaOH. Levels of H_2O_2 (expressed in nanomoles H_2O_2/2.25 × 10^5 cells) were determined spectrophotometrically (at 600 nm) after correcting for background absorbance (using cell-free wells containing all reagents) and using an extinction coefficient value of 16.2. For both ROI species, data were corrected for cell detachment.

IFNγ-induced I-A expression. To assess any modification of IFNγ-inducible surface I-A antigen expression in PAM from hosts exposed to V or air (n = 5 rats/treatment group), lavaged cells were placed in 24-well plates (10^6 cells/well in 0.9 ml RPMI/5% FBS) and incubated for 2 hr at 37°C. Interferon-γ (50 ng/0.1 ml RPMI) or RPMI alone was then added, and the cells were incubated 48 hr to induce maximal surface I-A expres-

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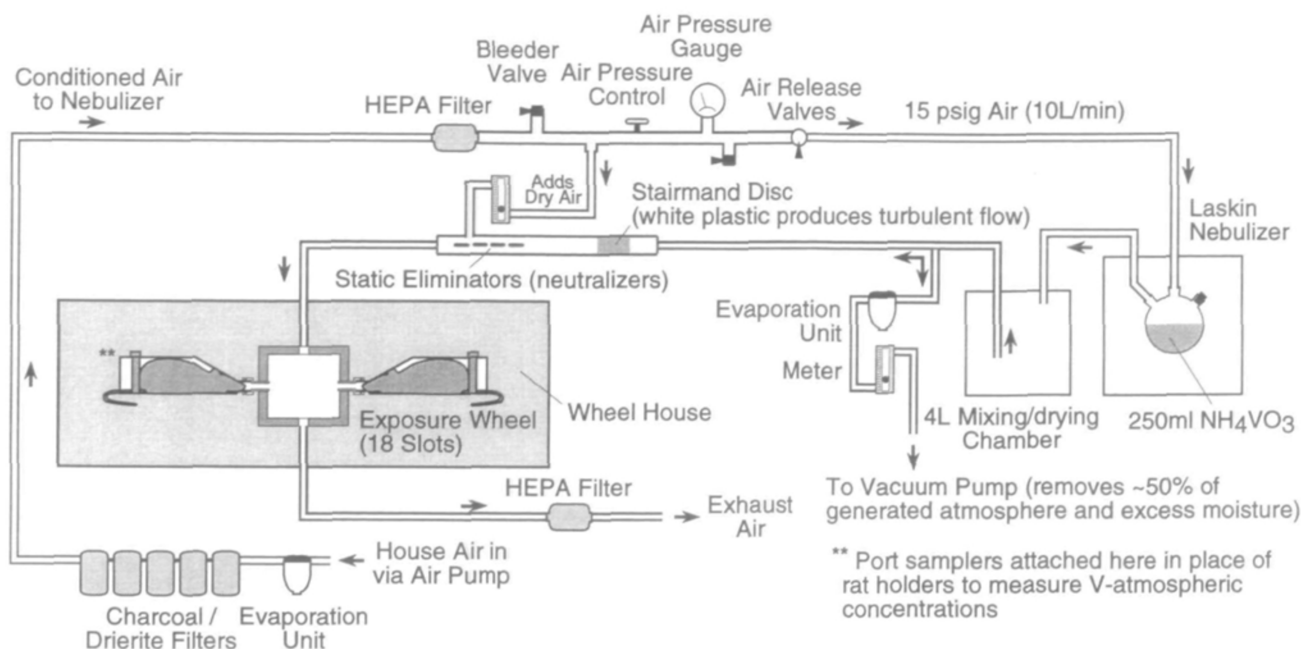

FIG. 1. The generation/exposure system for nose-only delivery of the NH_4VO_3-containing atmosphere to rats.

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sion. PAM were then harvested by gentle trypsinization and analyzed for I-A expression. To block nonspecific binding of anti-I-A antibody by F_c receptors, PAM were incubated with 10% rat serum–PBS solution for 20 min at 4°C, and then washed twice with ice-cold PBS. These cells were then incubated on ice in the dark for 1 hr in 40 μl antibody solution containing 10 μg Fisher 344-specific FITC-mouse anti-I-A monoclonal IgG_{1a} (MAb, OX3, Harlan Bioproducts, Indianapolis, IN) and 20 μg unlabeled nonspecific mouse IgG (to minimize nonspecific MAb binding). Free MAb was removed by washing with PBS containing 0.2% bovine albumin and 0.1% sodium azide. Cells resuspended to 10^6 cells/ml in 1% paraformaldehyde were evaluated using FACS analysis (Coulter EPICS II multiparameter cell sorter) and the percentage of $I\text{-}A^+$ cells was calculated by histogram subtraction; mean fluorescence intensity of $I\text{-}A^+$ cells was used to measure I-A density.

Phagocytic activity. Phagocytic activity of recovered cells ($n = 5$ rats/treatment group) was measured using a suspension assay (Schlesinger, 1987). Cell aliquots (5×10^5 lavaged cells/900 μl; 10 rats/treatment group) 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:cell ratio of 100:1 (determined to be optimal in preliminary studies). After microscopically analyzing a total of 200 PAM/slide, phagocytic activity was quantitated in terms of phagocytic index (PI; percentage of viable cells found to have ingested ≥1 latex particle) and phagocytic capacity (PC; percentage of phagocytically active PAM that ingested ≥3 particles).

Data analysis. All data were analyzed using a one-way ANOVA followed, when appropriate, by a Student–Newman–Keuls test. Results were considered significant at $p < 0.05$. Correlation of lung V burdens with length of exposure was performed using standard linear regression analysis.

RESULTS

The analysis of lung V burdens indicate that inhalation of NH_4VO_3 at 2 mg V/m^3 for 8 hr/day resulted in a significant ($r^2 = 0.94$) time-dependent increase in lung V content (Fig. 2). Lung V burdens were increased by 44% between Days 1 and 2; thereafter, the daily increases approximated 10%. Twenty-four hours after the fourth and final exposure, lung V levels decreased ≈39%, from 26.9 to 16.5 μg/g lung; there were no significant changes in lung weight over the exposure period (data not shown).

In V-exposed rats, the absolute numbers of neutrophils and small macrophages (Mø; <15 μm diameter) were increased compared to levels observed in the air-exposed control rats (PMN, 1.72×10^6/rat vs 0.03×10^6/control rat; small Mø, 0.64×10^6/rat vs 0.06×10^6/control rat, respectively); in addition, the relative percentages of these cell types were also significantly increased by 38- and 7-fold, respectively (Fig. 3). Although the percentage of total cells which were PAM was reduced in V-exposed rats by 24% compared to in control rats, because total cell counts in the BAL were significantly ($p < 0.05$) increased by V exposure (7.10×10^6/V-rat vs 4.96×10^6/control rat), the total numbers of PAM recovered did not significantly differ from control animals (5.38×10^6/V-rat vs 4.93×10^6/control rat). Lung cell damage due to V exposure was evidenced by significant increases in LDH activity and protein levels measured in the BAL fluid (Fig. 4A and 4B).

Studies evaluating V-induced effects upon PAM functionality indicated that the capacity of PAM to generate TNFα and ROIs, to actively phagocytize inert opsonized particles, and to increase the surface expression of Class II I-A antigen in response to IFNγ were altered. LPS-induced production/

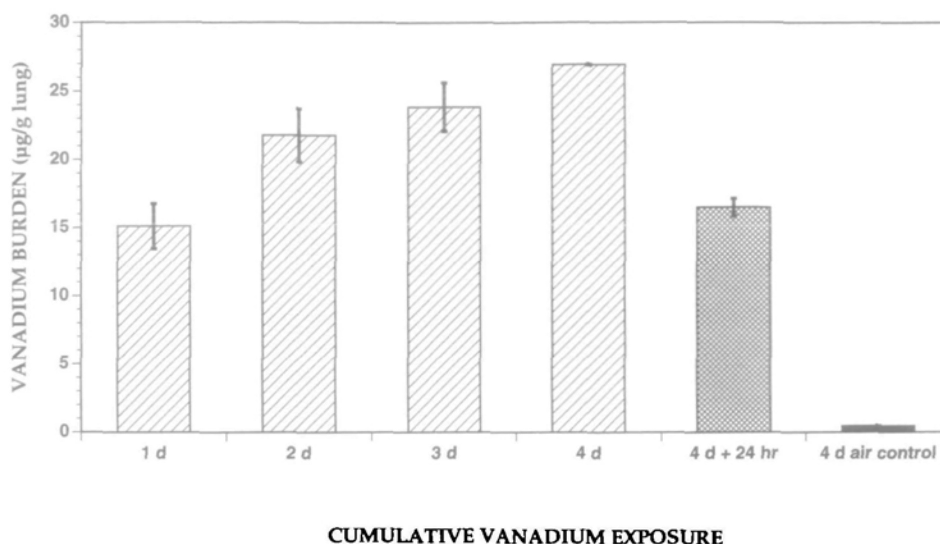

FIG. 2. Vanadium levels in rat lungs. Levels indicated (µg/g lung wet wt) are the average V burdens in two lungs for each exposure duration indicated.

release of functionally active TNFα from PAM recovered from V-exposed hosts was significantly reduced (by 39%) as compared to that released by PAM from air-treated control rats (Fig. 5); spontaneous TNFα formation in both exposure groups was minimal (<8 units; data not shown). The phagocytic activity of PAM from V-exposed hosts was also significantly reduced; the PI was decreased by 29% compared to that of control PAM, and the PC of these phagocytically active PAM was reduced by 34% (Fig. 6).

The ability of PAM to produce ROI (i.e., $\cdot O_2^-$ and H_2O_2) following host exposures to V appeared to be most severely affected when cells were first primed for 24 hr with IFNγ prior to OZ stimulation. Although spontaneous $\cdot O_2^-$ production (IFNγ-primed, but without OZ) was slightly increased in PAM from V-exposed hosts, this change in production was not statistically or biologically significant. In PAM incubated with OZ but without IFNγ priming, $\cdot O_2^-$ production was equivalent in both exposure groups (Fig. 7). When V-exposed PAM were primed with IFNγ prior to the addition of OZ, there was a significant (49%) reduction in $\cdot O_2^-$ generation as compared with that observed in their unprimed treatment counterparts; control rat cells displayed no significant differences due to IFNγ priming before OZ addition.

As observed with $\cdot O_2^-$, V-exposed PAM did not spontaneously produce greater amounts of H_2O_2 than that from control rats (Fig. 8); however, stimulation with OZ induced significantly greater production of H_2O_2 by these cells (2.41 vs 1.62 nmol $H_2O_2/2.25 \times 10^5$ PAM in control rat PAM). Again, while priming of control PAM with IFNγ did not produce a significant increase in H_2O_2 production above that due to OZ alone, H_2O_2 formation in V-exposed cells was found to be reduced (by 63%) compared with OZ-treated unprimed V-exposed PAM. In addition, increased length of OZ incubation reduced H_2O_2 production even further (by 63% after 1 hr and by >76% after 2 hr).

When PAM were analyzed for basal and IFNγ-inducible expression of Class II/I-A surface antigen, PAM from V-exposed rats again showed modified IFNγ responsivity. Although background I-A levels on the V-exposed PAM were not significantly different from those measured on control rat cells (31.7 vs 25.5%, respectively), after a 48-hr incubation with IFNγ, I-A expression was significantly lower (30%) in PAM from V-exposed hosts (60 vs 85% $I\text{-}A^+$ in air controls) (Fig. 9). These increases in I-A expression after IFNγ treatment were >225% above background for control rat PAM, but only 86% in PAM from V-exposed rats.

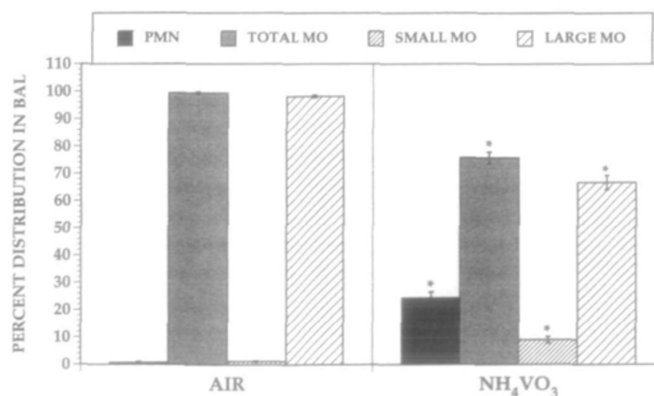

FIG. 3. Profile of cells recovered from 4-day V- or air-exposed rats. Mean percentage (±SE) of each cell type recovered in the lavages of 10 rats per treatment group. Values significantly different from the air-exposed controls (*$p < 0.01$) are indicated.

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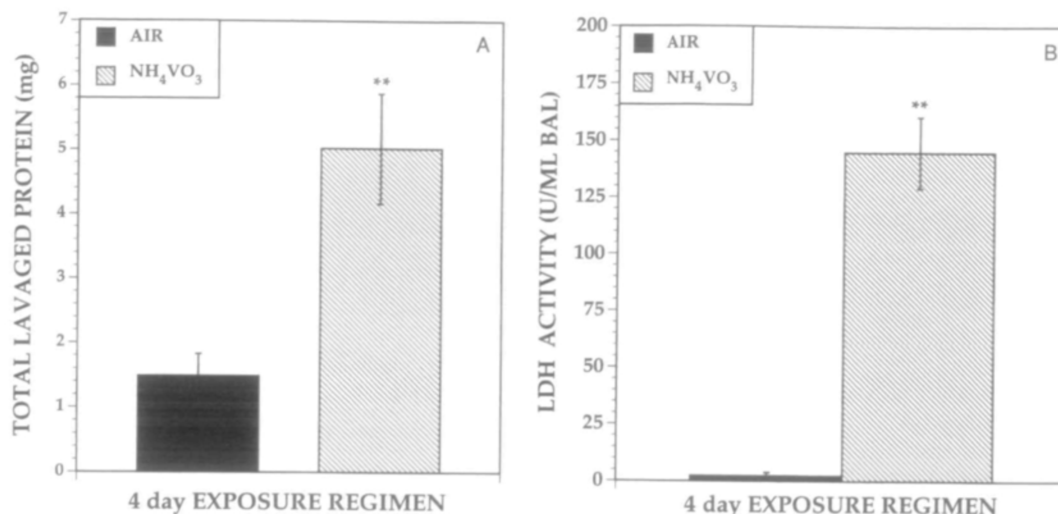

FIG. 4. Analysis of lung lavage from 4-day V- or air-exposed rats. Mean (±SE) (A) lactate dehydrogenase activity, and (B) total protein, measured in acellular BAL fluid obtained from 10 rats per treatment group. Value significantly different from the air-exposed controls ($^{**}p < 0.01$) is indicated.

DISCUSSION

In earlier studies (Cohen *et al.*, 1986, 1988, 1989; Cohen and Wei, 1988; Vaddi and Wei, 1991a, b, 1996), intraperitoneal exposure of mice to soluble pentavalent V (as NH_4VO_3) modulated immune responses both systematically and at the site of injection. The present study indicates that short-term, repeated inhalation of NH_4VO_3, at a concentration encountered by humans over a typical workweek (and adhering to government standards of acceptable airborne soluble V levels), also altered pulmonary immune cell functions, and produced significant changes in the lungs themselves.

Of the few studies dealing with V compounds and their effects upon rodent lungs, most have utilized the more frequently encountered, poorly soluble vanadium pentoxide (V_2O_5). In addition, these studies have, for the most part, used a single intratracheal instillation, rather than inhalation as the route of exposure (Conklin *et al.*, 1982; Sharma *et al.*, 1987; Zychlinski *et al.*, 1991). In these studies, 70 and 90% of an instilled dose of V_2O_5 and sodium vanadate ($NaVO_3$), respectively, was cleared from the lungs within 24 hr. In the present study, NH_4VO_3 clearance was 40% within 24 hr of the final exposure. It is not clear whether the route of V administration or V toxicity itself was responsible for the differences between previously reported vanadate clearance results and those observed in this study. It has been shown previously that normal clearance of inhaled particles can be overwhelmed if particle exposure is continuous or if the particles themselves cause a progressive toxicity that alters the cell (i.e.,

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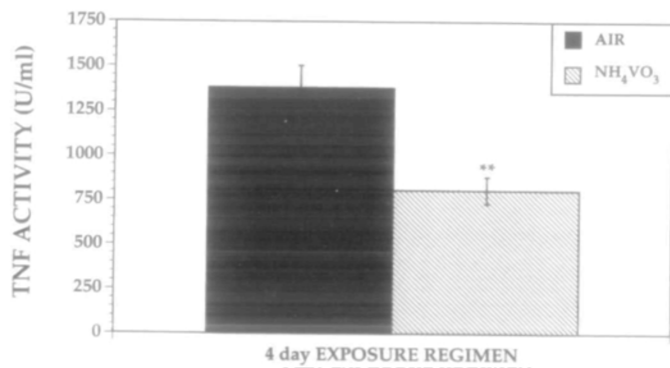

FIG. 5. LPS-induced TNFα production by lavaged cells from 4-day V- or air-exposed rats. Values are mean (±SE) TNFα levels recovered in medium from duplicate plates (from each of four rats per treatment group) containing 10^6 cells stimulated 24 hr with 1 μg/ml LPS. Value significantly different between the exposure groups ($^{**}p < 0.01$) is indicated.

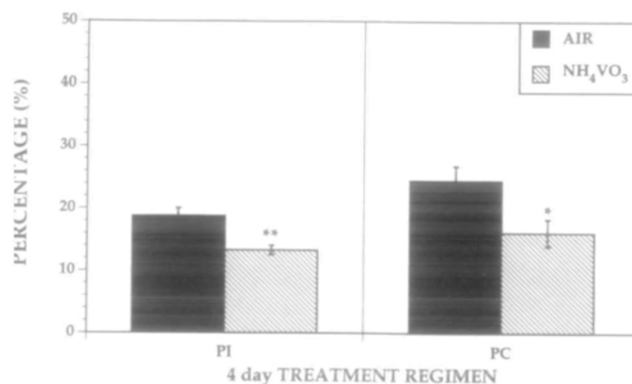

FIG. 6. Phagocytic index/capacity of PAM from 4-day air- or V-exposed rats. Percentages (±SE) of cells ingesting particles (PI), and those phagocytically active cells bearing ≥3 particles per cell (PC) are indicated. Each bar represents examination of 200 cells/slide, with five slides/rat (10 rats per treatment group). Values significantly different between the exposure groups ($^{*}p < 0.05$; $^{**}p < 0.01$) are indicated.

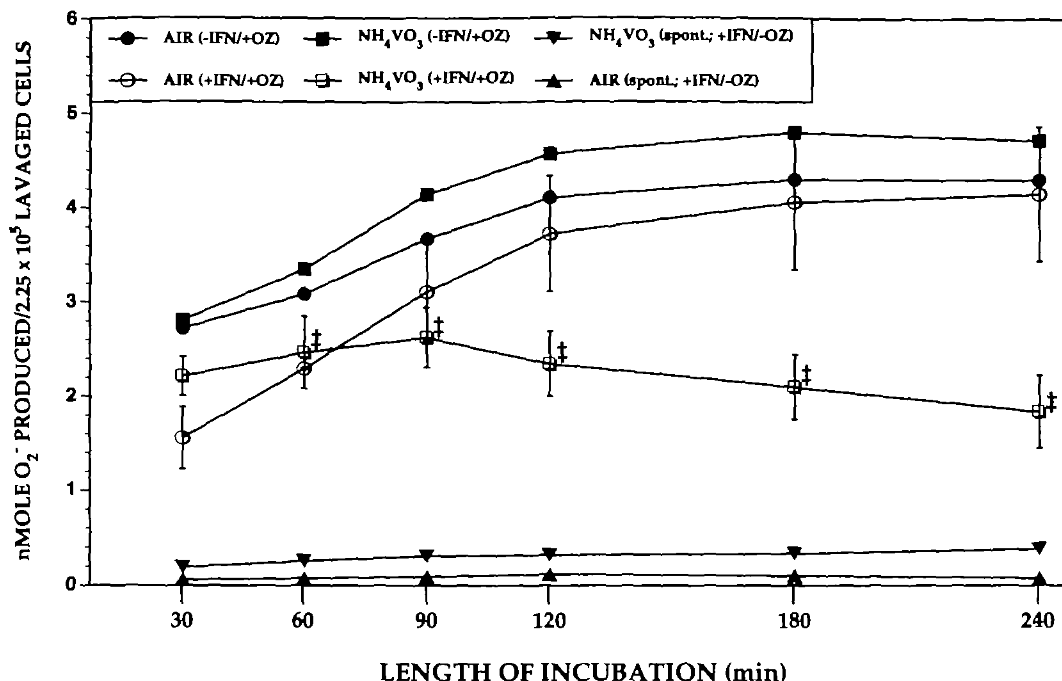

FIG. 7. Superoxide anion (O_2-) production in IFNγ-primed/unprimed opsonized zymosan (OZ)-stimulated lavaged cells recovered from rats exposed for 4 days to V or air. Values shown are mean (±SE; n = 10 rats/group) production levels after normalizing for numbers of adherent cells. Within an exposure group, values significantly (‡$p < 0.01$) different due to IFNγ priming are indicated.

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Mø) capacity to ingest/process particles (Glaser *et al.*, 1985, 1986); a similar mechanism may underlie the reduced vanadate clearance observed in this study.

Cytologic analyses of the lungs and recovered BAL fluid demonstrated that V inhalation caused increases in the levels of PMN and small PAM. At this point, it is not clear whether

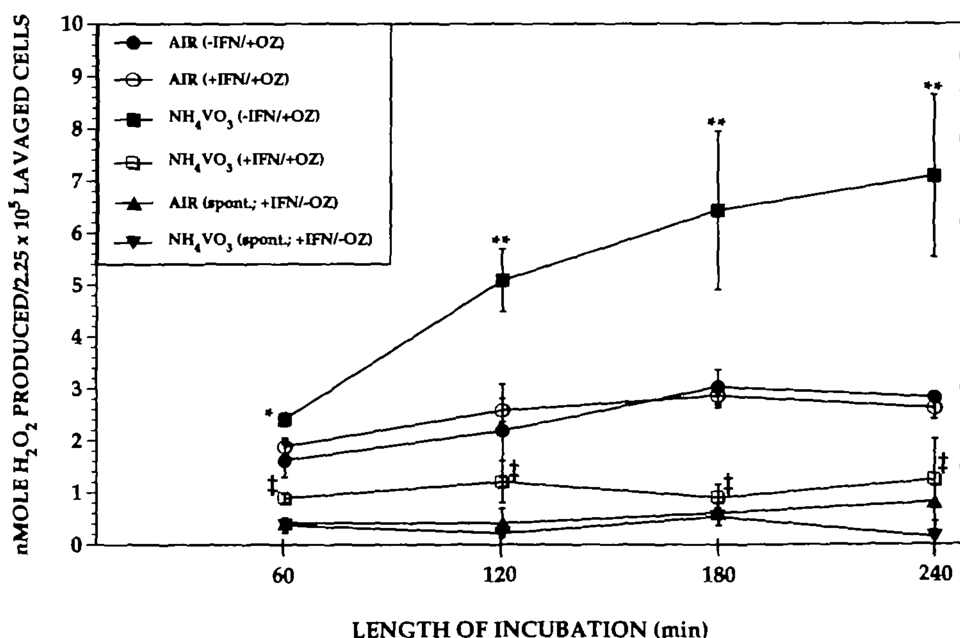

FIG. 8. Hydrogen peroxide (H_2O_2) production in IFNγ-primed/unprimed opsonized zymosan (OZ)-stimulated lavaged cells from rats exposed for 4 days to V or air. Values shown are mean (±SE; n = 10 rats/group) production levels after normalizing for numbers of adherent cells. Within an exposure group, values significantly (‡$p < 0.01$) different due to IFNγ priming are indicated. Values significantly (*$p < 0.05$; **$p < 0.01$) different between the exposure groups, irrespective of priming, are also shown.

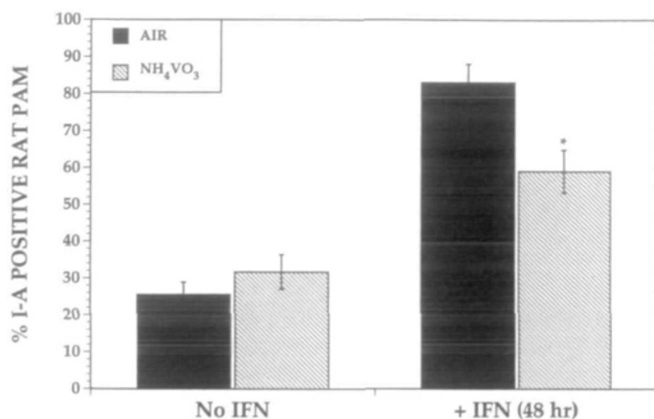

FIG. 9. Interferon-γ-induced I-A expression on PAM from rats exposed for 4 days to V or air. Values shown represent I-A expression on PAM incubated 48 hr without (left set) or with 250 U IFNγ (right set) prior to FACS analysis. Each bar represents the mean I-A expression (±SE) measured in PAM populations from five rats per treatment group (analyzed in duplicate). Values significantly ($*p < 0.01$) different between the exposure groups are indicated.

the small PAM are, in fact, immature or resident Mø. These observed effects are similar to those noted in cynomolgus monkeys exposed in whole-body chambers, either acutely or subchronically, to 5 or 0.5 mg V (as V_2O_5)/m^3, respectively (Knecht *et al.,* 1985; Kirkpatrick *et al.,* 1993). However, in contrast to the primate studies, significant increases in total BAL protein and LDH activity were observed in the exposed rats. Apart from obvious species differences, solubility differences between V_2O_5 and NH_4VO_3 might have contributed to these discrepancies. Variable effects upon biological responses as a function of metal solubility (and subsequent variations in bioavailability) are well known for several transition metals (Waters *et al.,* 1974; Labedzka *et al.,* 1989; Zelikoff and Cohen, 1995; Cohen *et al.,* 1996a).

Ex vivo studies utilizing adherent PAM indicated that several Mø functions critical for host immunocompetence were compromised following NH_4VO_3 inhalation. The reduced formation/release of functional TNFα by LPS-stimulated PAM from V-exposed rats parallels results observed *in vitro,* wherein mouse WEHI-3 Mø-like cells displayed concentration-dependent decreases in TNFα production following V exposure (Cohen *et al.,* 1993). We hypothesized then, and here as well, that effects upon TNFα arose from alterations in cellular levels of TNFα-regulating agents (i.e., cAMP, PGE_2) or from effects upon post-translational processing of TNFα precursor molecules rather than from inhibitory interactions between V and fully-processed TNFα trimers.

Altered cell surface receptor expression/binding activity represents another mechanism by which PAM functionality may be modified by inhaled V. Our earlier *in vitro* studies demonstrated that WEHI-3 cell responsivity to IFNγ was reduced by V, in part, by this mechanism (Cohen *et al.,* 1996b). Although not directly assessed here, reduced expression/functionality of LPS-binding and IFNγ receptors on PAM could have led to the observed reductions in TNFα production and IFNγ-inducible I-A expression, respectively, following inhalation of V.

Another example of V-induced modification in surface receptor expression has been shown with Mø opsonin receptors in V-exposed rodents (Vaddi and Wei, 1991b). This effect, in conjunction with V-induced effects upon microtubule integrity/function (Wang and Choppin, 1981; Bennet *et al.,* 1993; Correia *et al.,* 1994), and upon biochemical/enzymatic pathways critical for providing energy necessary for phagocytosis (Cohen *et al.,* 1986, 1988), may help to explain why PAM-associated phagocytic activity was reduced in this study. If this, in fact, is true, then the amount of time required for the processing of any one particle by PAM from a V-exposed host might be extended, and fewer particles would be ingested within a given time period. A similar mechanism likely underlies earlier reports of decreased bacterial uptake by peritoneal Mø (PEM) from V-exposed mice (Cohen *et al.,* 1989).

The effects of V upon select enzymes/pathways might also explain, at least in part, the alterations in ROI production observed in this study. NAD(P)H oxidase, critical for ROI formation, is dependent upon several enzymes previously shown to be inhibited in PEM by *in vivo* V exposures (Cohen and Wei, 1988). However, unlike the mouse PEM, non-IFNγ-primed rat PAM had no significant reductions in $\cdot O_2^-$ production after OZ stimulation. While this was unexpected in light of the observed reduction in PAM phagocytic activity and earlier observations that V exposure decreased Mø levels of reducing equivalents required for NAD(P)H oxidase $\cdot O_2^-$ generation, the apparent lack of effect on OZ-induced $\cdot O_2^-$ production may be due to: (1) ROI formation arising from intracellular V redox cycling and reactions between peroxides and vanadate/vanadyl ions (Ramasarma *et al.,* 1981; Stankiewicz *et al.,* 1991), and/or (2) greater reducing equivalents/NAD(P)H oxidase levels in PAM compared to those in PEM (Lasser, 1983; Bowden, 1984; Valberg and Blanchard, 1991). Thus, while expected reductions in PAM $\cdot O_2^-$ production may have been compensated for by these mechanisms, any V-induced increase in basal levels of H_2O_2 would then not be apparent.

While these mechanisms may explain the observed results for ROI production in unprimed V-exposed PAM, explanations for the sharp reductions in ROI formation following initial IFNγ priming are more complicated. It is interesting to note that in air-exposed control PAM, IFNγ priming did not elicit increased ROI formation after OZ stimulation. Normally, IFNγ primes cells for increased ROI formation after proper antigenic stimulation (Nathan and Tsunawaki, 1986). However, a recent study has indicated that treatment of rat PAM with IFNγ de-

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creased select IFNγ-inducible endpoints (Prokhorova *et al.*, 1994). The findings from this study indicate that IFNγ either had no effect overall (unlikely in light of I-A expression results), or had no impact upon ROI induction, specifically. As IFNγ-primed PAM from V-exposed hosts did not demonstrate the "no effect" response observed with control PAM, it could be concluded that exposure to V elicited a toxicity dependent upon the presence of IFNγ.

A mechanism by which IFNγ could specifically contribute to the observed deactivation/decreased ROI production in PAM from V-exposed hosts involves alterations in protein tyrosine phosphorylation and/or prostaglandin E_2 (PGE_2) production. Previous *in vitro* studies showed that PGE_2 production was increased in V-exposed WEHI cells (Cohen *et al.*, 1993). It is possible that a similar V-induced increase in *in situ* PGE_2 formation, in combination with PGE_2 formed in response to any IFNγ binding, might allow threshold levels of PGE_2 to be attained and, thus, result in reduced ROI formation (Parnham and Englberger, 1985; Becker *et al.*, 1991). Similarly, levels of tyrosine-phosphorylated proteins, beyond those arising from V-dependent inhibition of tyrosine phosphatases (Nechay, 1984; Imbert *et al.*, 1994; Conde *et al.*, 1995), may reach inhibitory levels once IFNγ is bound and *de novo* phosphorylation begins (Green *et al.*, 1992; Schindler *et al.*, 1992; Igarishi *et al.*, 1994).

Because the V-exposed rats yielded heterogenous lavaged cell populations, possible contributions by PMN to some of the selected endpoints (i.e., TNFα and ROI production) need to be considered. The recovery of a heterogenous population might imply that in both the ROI and TNFα studies, the reported results might overestimate actual production by PAM. However, this would suggest that TNFα production by PAM from V-exposed hosts was depressed even more so than indicated; similarly, the differences arising in ROI production between the air- and V-exposed rat cells that were primed with IFNγ would be even more dramatic than described. In addition, as PMN are not considered IFNγ-sensitive (Feldmann, 1996), any heterogeneity in the V-exposed rat cell populations would not impact upon the analysis of comparative effects from IFNγ priming. Because analysis of phagocytic activity was based upon the direct microscopic observation of cells, and because PMN are not generally considered Class II/I-A-expressive (and are gated out during the FACS analysis), the presence of PMN in these assays would not influence the reported outcomes.

In summary, these studies indicate that short-term, repeated inhalation of V (as NH_4VO_3) by rats, at levels encountered in the workplace, can modulate immune responses in the lungs. A major target of the toxicity of inhaled V is the PAM, with significant immunotoxicity occurring at the level of PAM cytokine-related function. As proper PAM production of, interaction with, and responsivity to cytokines are critical for maintaining *in situ* immunocompetence, alterations in these functions may contribute to the increased incidence of bronchopulmonary disease, such as infections and lung cancer, observed in workers exposed to V-containing workplace atmospheres.

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