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Vanadium-Induced Apoptosis and Pulmonary Inflammation in Mice: Role of Reactive Oxygen Species

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Pulmonary exposure to metals and metal-containing compounds is associated with pulmonary inflammation, cell death, and tissue injury. The present study uses a mouse model to investigate vanadium-induced apoptosis and lung inflammation, and the role of reactive oxygen species (ROS) in this process. Aspiration of the pentavalent form of vanadium, V (V), caused a rapid influx of polymorphonuclear leukocytes into the pulmonary airspace with a peak inflammatory response at 6 h post-exposure and resolution by 72 h. During this period, the number of apoptotic lung cells which were predominantly neutrophils increased considerably with a peak response at 24 h accompanied by no or minimum necrosis. After 24 h when the V (V)-induced inflammation was in the resolution phase, an increased influx of macrophages and engulfment of apoptotic bodies by these phagocytes was observed, supporting the role of macrophages in apoptotic cell clearance and resolution of V (V)-induced lung inflammation. Electron spin resonance (ESR) studies using lavaged alveolar macrophages showed the formation of ROS, including $O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$ radicals which were confirmed by inhibition with free radical scavengers. The mechanism of ROS generation induced by V (V) involved the activation of an NADPH oxidase complex and the mitochondrial electron transport chain. The ROS scavenger, catalase (H_2O_2 scavenger), effectively inhibited both lung cell apoptosis and the inflammatory response, whereas superoxide dismutase (SOD) ($O_2^{\cdot -}$ scavenger) and the metal chelator, deferoxamine (inhibitor of $^{\cdot}OH$ generation by Fenton-like reactions) had lesser effects. These results indicate that multiple oxidative species are involved in V (V)-induced lung inflammation and apoptosis, and that H_2O_2 plays a major role in this process. J. Cell. Physiol. 195: 99–107, 2003. Published 2003 Wiley-Liss, Inc.†

Increasing evidence indicates that pulmonary exposure to environmental and occupational airborne particulate matter (PM) results in an increased incidence of cardiopulmonary diseases (Dockery and Pope, 1994). Chemical analysis of PM samples reveals that they can contain various trace metal ions, such as vanadium, chromium, and nickel (Kennedy et al., 1998; Kodavanti et al., 1998). While these metal ions have been postulated to contribute to the pathologic effects of inhaled PM, the biological mechanisms involved in the disease process remain unclear. The present study investigates the role of apoptosis in vanadium-induced lung inflammation and the involvement of reactive oxygen species (ROS) in this process. Vanadium is a transition metal that exists in various oxidative states ranging from −1 to +5. The pentavalent form, V (V), is one of the most abundant forms in nature and is also the most toxic (Barceloux, 1999). Previous studies have shown that V (V) can cause apoptosis in a variety of cells in vitro (Hehner et al., 1999; Huang et al., 2001). However, in vivo evidence of this process has not been

Abbreviations: TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end-labeling; ESR, electron spin resonance; ROS, reactive oxygen species; $O_2^{\cdot -}$, superoxide anion; H_2O_2, hydrogen peroxide; $^{\cdot}OH$, hydroxyl radical; SOD, superoxide dismutase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DPI, diphenylene iodonium; PBS, phosphate-buffered saline; V (V), vanadium (V); LDH, lactate dehydrogenase.

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demonstrated. Furthermore, V (V)'s role in the disease process and its underlying mechanisms of action have not been clearly delineated.

Although apoptosis has been implicated as a homeostatic mechanism, increasing evidence indicates that it also plays a role in disease pathogenesis. Failure to clear unwanted cells by apoptosis will prolong or exacerbate inflammation because of the release of their toxic and inflammatory contents (Haslett, 1999). Repair after tissue injury requires the elimination of inflammatory and damaged cells from the inflamed tissue. Excessive apoptosis may also cause disease. Administration of a known apoptosis inducer, agonistic Fas (CD95/APO1) antibody, causes inflammatory cell influx and apoptosis, which subsequently leads to tissue inflammation and fibrosis (Ogasawara et al., 1993; Hagimoto et al., 1997). Lung cell apoptosis induced by silica appears to be through Fas ligand (FasL) signaling pathway, since FasL knockout mice do not develop pulmonary inflammation and fibrosis response to silica exposure in contrast to control mice (Borges et al., 2001). Apoptosis has also been observed in a variety of inflammatory lung disorders, including acute lung injury (Cox et al., 1995; Bardales et al., 1996; Hussain et al., 1998), diffuse alveolar damage (Guinee et al., 1996), and idiopathic pulmonary fibrosis (Kuwano et al., 1996, 1999). Thus, clear evidence supports a role for apoptosis in inflammatory lung disorders.

While the mechanisms of apoptosis induced by vanadium and resultant vanadium-induced lung disorders are incompletely understood, the involvement of ROS has been suggested. ROS produced by macrophages and neutrophils are meant to kill microbes, but can damage bystander cells as well (Szatrowski and Nathan, 1991). Both apoptotic and necrotic modes of cell death have been reported as a result of ROS stimulation (Sato et al., 1995; Jabs, 1999), and antioxidants can inhibit or delay ROS-induced cell death (Jabs, 1999). While numerous studies have reported the involvement of ROS in cell death, there are several other reports that show that ROS are not involved in the apoptotic process. For examples, hypoxia has been shown to induce apoptosis (Muschel et al., 1995) and Fas-induced apoptosis does not require ROS generation (Schulze-Osthoff et al., 1994). Similarly, apoptosis of lung epithelial cells induced by neutrophils was reported to be independent of ROS (Serrao et al., 2001). Furthermore, apoptosis induced by different stimuli, such as anti-Fas antibody, IL-3 withdrawal (Jacobson and Raff, 1995), dexamethasone and serum deprivation (Muschel et al., 1995), can occur in near-anaerobic conditions where ROS generation is unlikely. Together, these results suggest that while ROS can induce apoptosis they are not always strictly required.

The present study was undertaken to determine whether or not pulmonary administration of V (V) can result in apoptosis (or necrosis) of lung cells and whether or not this process is dependent on ROS generation. Experiments were also designed to identity specific cell types and key oxidative species that are involved in this process. The role of apoptosis and clearance of apoptotic cells by lung macrophages during the resolution of V (V)-induced lung inflammation were also investigated.

MATERIALS AND METHODS

Animals and reagents

Male BALB/cJ mice (4–6 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). They were acclimated in an AAALAC-accredited facility for at least 1 week before use. The mice were housed in HEPA-filtered ventilated cages on hardwood Beta-chip bedding and were given water and food ad libitum. Pulmonary administration of test agents was performed by aspiration. The animals were anesthetizing with a mixture of ketamine and xylazine (45 and 8 mg/kg, i.p., respectively) and placed on a board in a supine position. The animal's tongue was extended with padded forceps, and 50 μl of test solution containing the indicated amount of V (V) or ROS scavenger was placed on the back of the tongue, from where it was aspirated rapidly. Control animals were given an equal volume of saline vehicle. Sodium metavanadate [V (V)] and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were obtained from Aldrich (Milwaukee, WI). Catalase, superoxide dismutase (SOD), deferoxamine, diphenylene iodonium (DPI), and rotenone were purchased from Sigma Chemical Co. (St. Louis, MO).

Bronchoalveolar lavage (BAL)

At selected time intervals, treated mice were euthanized by exsanguinations under anesthesia with 0.2 ml of 6 grains/ml sodium pentobarbital (Pentosol; MedPharmex, Inc., Pomona, CA). A tracheostomy tube was inserted surgically, and the lungs were lavaged with ice-cold Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS). The lungs were lavaged five times with 8 ml of PBS to collect cellular and soluble constituents. The first lavage was centrifuged and the supernatant was used to measure lactate dehydrogenase (LDH) activity. All lavage cell pellets were mixed and resuspended in 1 ml of PBS-buffered solution. Cell counts and differentials were determined using an Electronic cell counter equipped with a cell sizing attachment (Coulter Multisizer II, Coulter Electronics, Hialeah, FL) and cell apoptosis was performed using the TUNEL assay (see below).

Lung histology

To obtain lung tissues for histologic examination, a separate group of animals were treated with V (V) but not subjected to BAL. After sacrifice, the lungs were inflated with 10% formalin solution instilled through the trachea for at least 5 min and then fixed with buffered 10% formalin solution for 24 h. After embedding in paraffin, the samples were sectioned, mounted on glass slides, and stained with hematoxylin and eosin for light microscopic examination.

TUNEL assay

Analysis of lung cell apoptosis was performed, using a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) kit (Promega, Madison, WI), according to the manufacturer's protocols. Briefly, after incubation with the equilibration buffer provided, the fixed tissue section slides were immersed in terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP and incubated for 60 min at 37°C. The fragmented

DNA of the apoptotic cell nuclei catalytically incorporates with fluorescein-12-dUTP at the 3′-OH DNA polymeric tail and then can be visualized as a yellow-green fluorescent signal using a fluorescence microscope. Non-apoptotic cell nuclei were counterstained with propidium iodide (Molecular Probes, Eugene, OR) and were identified by red fluorescence. For analysis of BAL cell apoptosis, cytospin preparations of BAL cells were used. The cells were fixed in 4% paraformaldehyde for 30 min and then stained with TUNEL and propidium iodide as described.

Fluorescence microscopy

After staining, the slides were examined under a fluorescence microscope using standard fluorescein excitation and emission wavelengths of 495 and 525 nm, respectively. A bright green fluorescence signal in the nucleus indicates TUNEL-positive apoptotic cells. Propidium iodide was employed to aid in the identification of cell types. The signal was viewed with excitation at 595 nm, and a bright red emission was seen in all non-apoptotic nuclei present. The number of apoptotic cells was determined using standard morphometric methods as previously described (Stone et al., 1992; Mercer et al., 1994). A minimum of 20 views were analyzed for each lung tissue section slide or BAL cell preparation at a magnification of $\times 200$. The total number of normal and apoptotic cells was determined using standard morphometric methods.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity was monitored to determine lung cell necrosis (lysis). LDH is a cytoplasmic enzyme that is released when the cell membrane is lysed or disrupted. Analysis of LDH release was performed using the supernatants from the first acellular BAL fluids obtained after specific treatments. LDH activity was determined by monitoring the oxidation of pyruvate coupled with the reduction of NAD at 340 nm using an LDH assay kit (Roche Diagnostic Systems, Montclair, NJ). The assay was performed using a Cobas Fara II Analyzer (Roche Diagnostic Systems). One unit per liter of LDH activity is defined as the amount of enzyme that converts 1 μmol of lactate to 1 μmol of pyruvate with the concomitant reduction of 1 μmol of NAD to 1 μmol of NADH per minute per liter of sample in the assay procedure.

Electron spin resonance

The electron spin resonance (ESR) spin trapping technique was used to detect short-lived free radical intermediates generated in BAL cells after V (V) treatment. This technique involves an addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived compound, the so-called spin adduct, which can be studied by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of free radicals trapped, and the hyperfine splittings of the spin adduct are characteristic of the trapped radical. All measurements were conducted with a Varian E9 ESR spectrometer with a flat cell assembly. Hyperfine splittings were measured (to 0.1 G) directly from magnetic field separations using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as standards. Reactants were mixed in test tubes in a total volume of 0.5 ml containing 1×10^6 BAL cells/ml. The reaction mixture was then transferred to a flat cell for ESR measurement. All measurements were carried out using pre-purified DMPO as a spin trap.

Statistical analyses

Analysis of variance and Duncan's multiple comparison test (Duncan, 1955) were used to evaluate the significance between measurements. All tests were 2-sided tests, and $P < 0.05$ was considered to be significant. Data are given as mean ± SEM.

RESULTS

Pulmonary inflammatory response to vanadium

The effect of vanadium on pulmonary inflammation was studied by monitoring inflammatory cell influx following instillation of V (V). Figure 1 shows that V (V) administration caused a time-dependent increase in the cell number of neutrophils and macrophages in the BAL

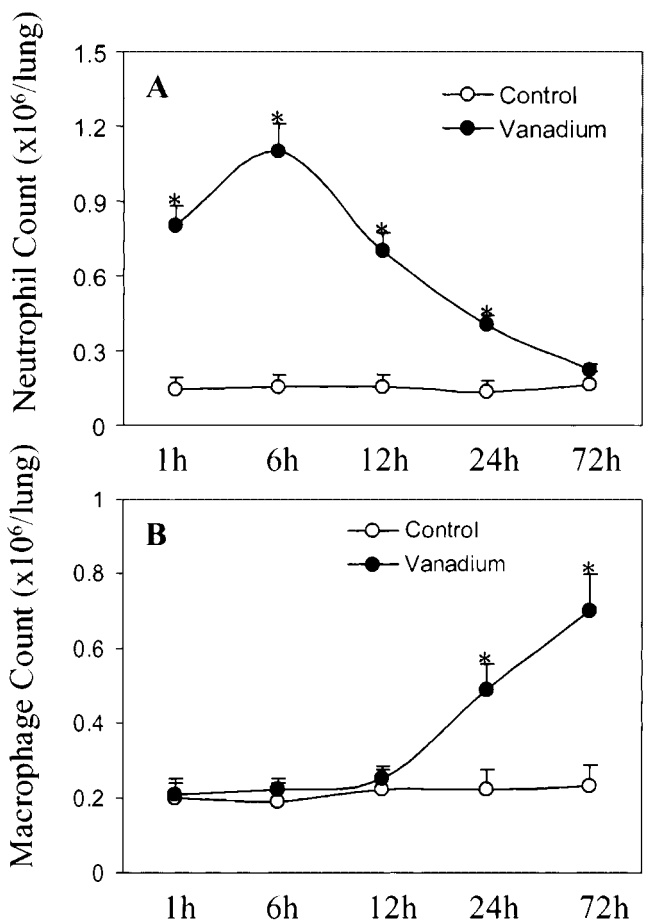

Fig. 1. BAL cell number analysis after vanadium treatment. Mice were treated with V (V) (50 μg/mouse) or saline via aspiration. BAL was performed at indicated times post-exposure and analyzed for neutrophil (**A**) and macrophage (**B**) cell numbers. Data are shown as the mean ± SEM, n = 4 mice/group. *$P < 0.05$ versus saline-treated control groups.

fluid. At early time points, the recruited cells were predominantly neutrophils with a peak cellular response observed at 6 h post-exposure (Fig. 1A). After 6 h, the neutrophil cell number gradually declined and returned to the background level by 72 h post-exposure. The macrophage cell number was relatively unchanged during the early phase of inflammation, significantly increased by 24 h post-exposure, and continued to increase at 72 h (Fig. 1B). Treatment of mice with saline vehicle had no significant effects on either neutrophil nor macrophage cell counts at any time post-exposure.

Apoptosis and necrosis of lung cells

Apoptosis of lung cells was determined by TUNEL assay using tissue sections at 6 h, 12 h, 1 day, 3 day, and 7 days after V (V) administration. Figure 2A shows that exposure of the lung to V (V) caused a time-dependent increase in the number of TUNEL-positive cells, whereas aspiration of saline vehicle had no effect. V (V)-induced pulmonary apoptosis increased significantly within 6 h, peaking around 1 day, and decreasing to the baseline level by 7 day. In contrast, a similar treatment of mice with V (V) caused minimum necrosis (lysis) of lung cells as determined by measurement of LDH activity of acellular BAL fluid samples (Fig. 2B). LDH activity was relatively unchanged at all post-exposure times except at 1 day where a small but significant increase in the LDH release was observed. These results indicate that apoptosis was the primary mode of cell death following pulmonary administration of V (V).

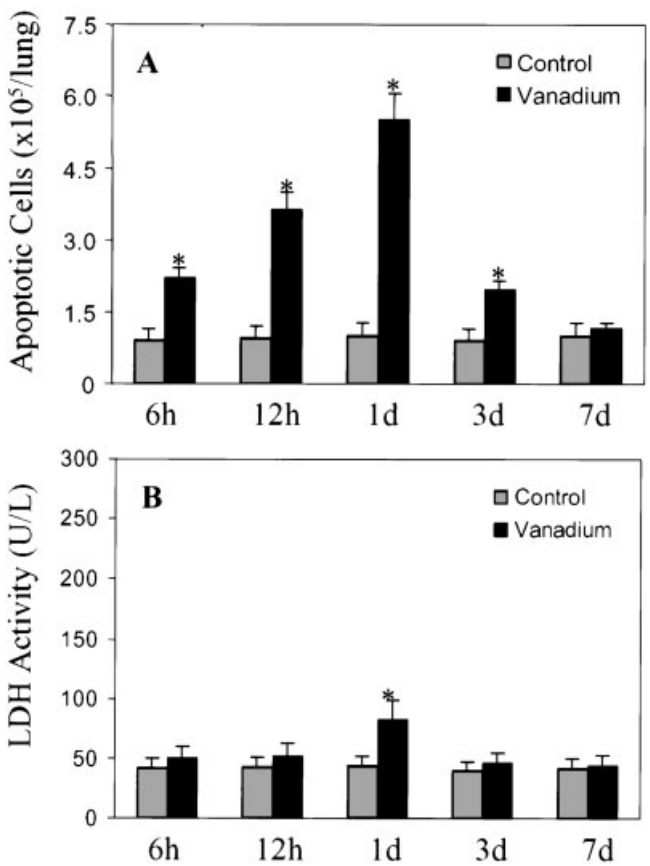

Fig. 2. Time courses of vanadium-induced pulmonary apoptosis and necrosis. Mice were treated with V (V) (50 μg/mouse) or saline, and were sacrificed at 6 h, 12 h, 1 day, 3 day, and 7 day after exposure. **A**: Apoptosis determined by TUNEL assay using lung tissue sections. **B**: Necrosis determined as LDH activity of the acellular BAL fluid. Data are shown as the mean ± SEM, n = 4 mice/group. *$P < 0.05$ versus saline-treated control groups.

Morphologic studies of lung inflammation and apoptosis

Figure 3A,B shows the lung histology of control mice versus V (V)-exposed mice, respectively, using H&E stained lung sections. Increased cellularity in the airspaces can be clearly seen at day 1 post-V (V) exposure, which corresponds to the peak time of neutrophil influx (Fig. 1A). Inflammation decreased in subsequent days and returned to a baseline level by day 7 (result not shown). Morphologic evidence of lung cell apoptosis is shown in Figure 3C,F. In this study, TUNEL-stained lung sections were examined at different times after V (V) treatment using fluorescence microscopy. Figure 3C shows a representative result of control lung sections. No TUNEL-positive apoptotic cells (green fluorescence) and relatively few infiltrated cells were observed. At 6 h after V (V) treatment, increased numbers of TUNEL-positive cells and infiltrated cells became apparent (Fig. 3D). The maximum number of TUNEL-positive cells was reached at 1 day post-exposure, most of which appeared to be associated with infiltrated cells presumably neutrophils (Fig. 3E). At 3 day, the number of infiltrated cells was substantially reduced but some TUNEL-positive cells can still be seen (Fig. 3F).

BAL cell apoptosis

Due to the difficulty in identifying TUNEL-positive cells in the lung tissue sections; we performed further TUNEL experiments using BAL cells from treated animals. Figure 4A,B show representative TUNEL results from BAL cells of V (V)-treated mice at 1 day and 3 day post-exposure. At 1 day, BAL cells were predominantly neutrophils (N) and macrophages (M) as identified by their characteristic nuclear morphology and size (neutrophils exhibiting typical nuclear lobes and being smaller in size than macrophages). At this time, apoptotic neutrophils (AN) and macrophages containing numerous apoptotic bodies in cytoplasm were observed (Fig. 4A). At 3 day post-exposure, neutrophils were virtually absent but remnants of apoptotic bodies, presumably fragmented AN, can still be observed in the cytoplasm of macrophages (Fig. 4B). The above results indicate that V (V) treatment can induce neutrophil infiltration and apoptosis, and that macrophages play an important role in the clearance of these apoptotic cells.

Effects of ROS scavengers on vanadium-induced apoptosis and lung inflammation

ROS have been implicated in the cellular toxicity and augmented biological activity induced by vanadium (Duncan, 1955; Szatrowski and Nathan, 1991; Stone et al., 1992; Ogasawara et al., 1993; Mercer et al., 1994; Schulze-Osthoff et al., 1994; Cox et al., 1995; Jacobson and Raff, 1995; Muschel et al., 1995; Sato et al., 1995; Bardales et al., 1996; Guinee et al., 1996; Kuwano et al.,

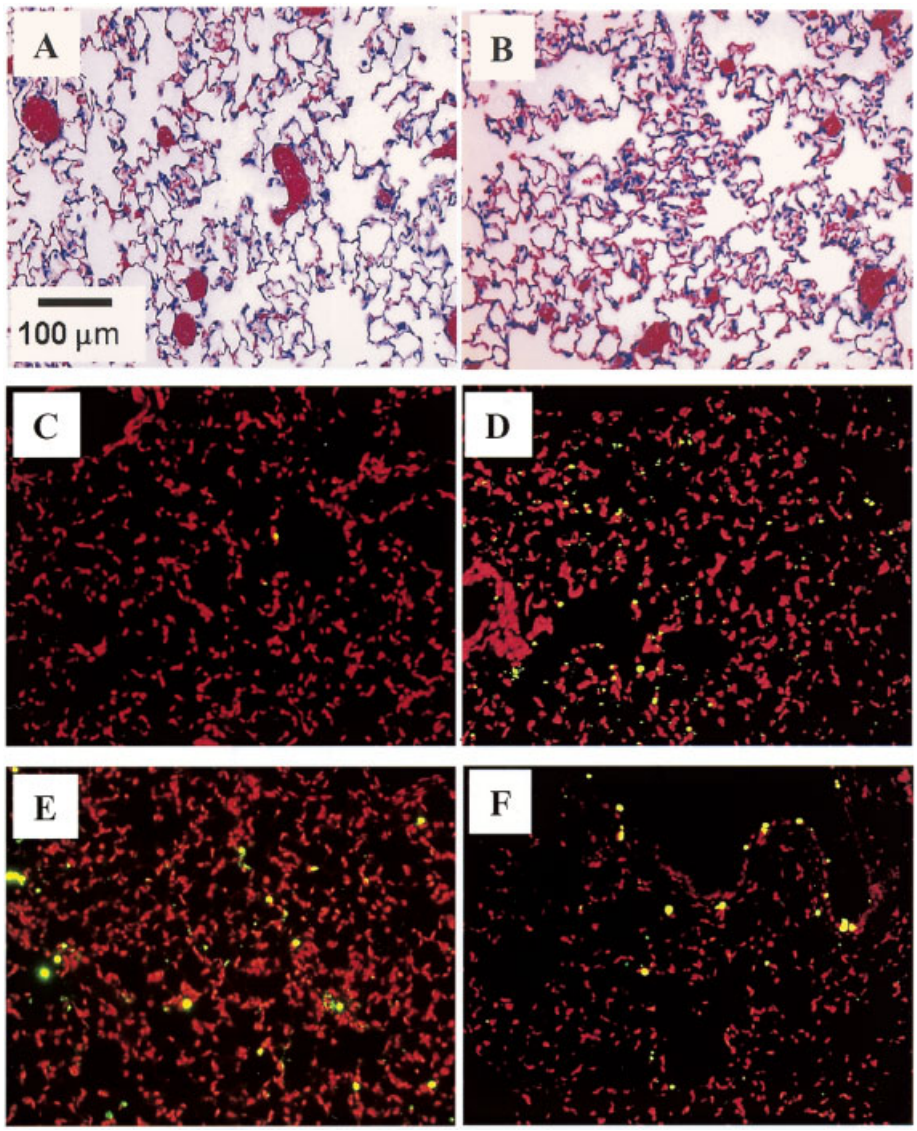

Fig. 3. Microscopic studies of vanadium-induced lung inflammation and apoptosis. Mice were treated with V (V) (50 μg/mouse) or saline, and lung sections were prepared for H&E staining (**A**, **B**) or TUNEL (**C**–**F**). A: Saline-treated control mice at 1 day. B: V (V)-treated mice at 1 day post-exposure. Note the accumulation of inflammatory cells in the airspaces. C: Control mice showing no TUNEL-positive cells. D–F: V (V)-treated mice at 6 h, 1 day, and 3 day, respectively. Note the increasing and declining presence of TUNEL-positive cells at 1 day and 3 day, respectively.

1996, 1999; Hagimoto et al., 1997; Hussain et al., 1998; Haslett, 1999; Jabs, 1999; Huang et al., 2000, 2001; Borges et al., 2001; Serrao et al., 2001), but their role in lung cell apoptosis and pulmonary inflammation has not been demonstrated. To test this possibility, mice were exposed to V (V) with or without various known ROS scavengers, and the effects on lung cell apoptosis and inflammatory cell influx monitored. Results show that catalase (an H_2O_2 scavenger) effectively inhibited both V (V)-induced apoptosis (Fig. 5A) and neutrophil influx (Fig. 5B). SOD (an $O_2^{\cdot-}$ scavenger) and deferoxamine (an inhibitor of $^{\cdot}OH$ generation by a Fenton-like reaction) had smaller but significant inhibitory effects. Treatment of saline-treated control mice with individual ROS scavengers alone had no effect on either apoptosis or neutrophil levels. Thus, these results support a role for ROS in V (V)-induced pulmonary apoptotic and neutrophilic responses. Subsequent ESR studies of ROS generation (see below) further confirm these results.

Vanadium-induced ROS generation and its inhibition by ROS scavengers

To provide the evidence for ROS formation and its inhibition by ROS scavengers, ESR studies using the spin trap DMPO were carried out. The ESR spin-trapping technique was used since it allows detection and identification of specific ROS involved. BAL cells from naive mice were treated with V (V) with or without

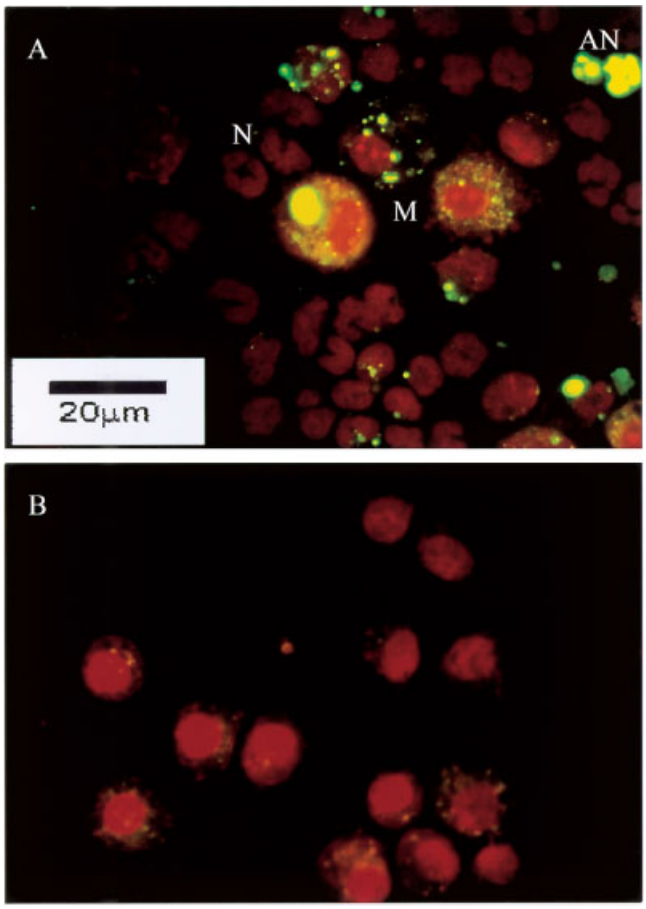

Fig. 4. BAL cell apoptosis and phagocytosis by macrophages. Mice were treated with V (V) (50 μg/mouse), and BAL cells were isolated and processed for TUNEL at 1 day (**A**) and 3 day (**B**) post-exposure. Note the presence of neutrophils (N), which are characterized by their distinct nuclear lobes, at 1 day and their disappearance at 3 day post-exposure. Note also the presence of AN and macrophages (M) ingesting apoptotic bodies.

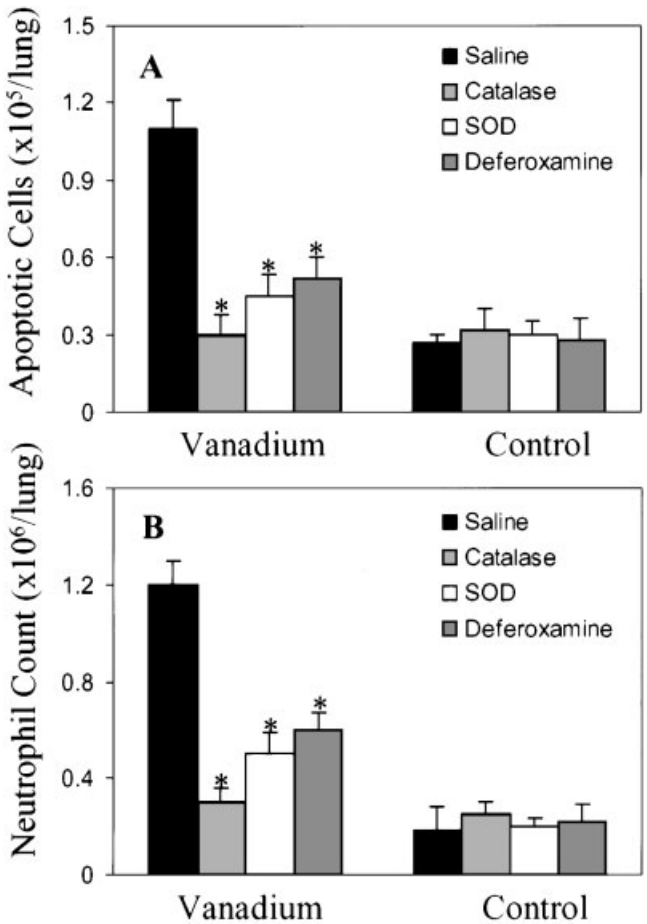

Fig. 5. Effects of ROS scavengers on vanadium-induced apoptosis and inflammation. Mice were treated with V (V) (50 μg/mouse) in the presence or absence of different ROS scavengers as indicated. **A**: Apoptosis was determined at 1 day post-exposure by TUNEL assay. **B**: Neutrophil number in BAL fluids was determined at 6 h post-exposure. The concentrations of ROS scavengers in 50 μl dosing solutions were: catalase, 2,500 U/ml; SOD, 2,500 U/ml; and deferoxamine, 5 μmol/ml. Data are shown as the mean ± SEM, n = 4 mice/group. $^*P < 0.05$ versus V(V)-treated control group.

ROS scavengers in vitro for indicated times. Figure 6 shows the time course of ROS formation after V (V) treatment. In the absence of V (V), no ROS generation was detected. In the presence of V (V), increased levels of ROS were generated with a peak response at 10–30 min of exposure. The ESR spectrum consisted of a 1:2:2:1 quartet with hyperfine splittings of $a_H = a_N = 14.9$ G, where a_N and a_H denote hyperfine splittings of the nitroxyl nitrogens and α-hydrogen, respectively. Based on these splittings and the 1:2:2:1 line shape, the spectrum was assigned to the DMPO-$^{\cdot}$OH adduct, which is evidence of $^{\cdot}$OH generation. Addition of catalase completely inhibited the signal (Fig. 7), indicating that H_2O_2 was generated in V (V)-treated cells and that this oxidative species was a precursor for $^{\cdot}$OH generation, possibly through a Fenton-like reaction such as V (IV) + $H_2O_2 \rightarrow$ V (V) + OH + OH^-. Addition of deferoxamine, which would chelate V (IV) and block its reaction toward H_2O_2, decreased the signal intensity, further showing that the $^{\cdot}$OH radicals were generated. Similarly, SOD decreased the signal intensity, presumably due to its inhibitory effect on $O_2^{\cdot-}$-mediated V (IV) generation (i.e., $O_2^{\cdot-}$ + V (V) → V (IV) + O_2), which is required for $^{\cdot}$OH formation via a Fenton-like reaction. It is possible that NADPH oxidase or the mitochondrial electron transport chain plays a role in this V (V)-induced ROS generation. To examine this possibility, DPI, a known NADPH oxidase inhibitor (Irani et al., 1997), or rotenone, a mitochondrial electron transport interrupter (Huang et al., 2000) was used. The results show that both DPI and rotenone were able to partially reduce $^{\cdot}$OH generation induced by V (V), suggesting that both NADPH oxidase and the mitochondrial electron transport chain may be involved in the production of ROS induced by V (V).

DISCUSSION

The present study provides evidence that V (V), one of the most common forms of vanadium found in tissues and cells after exposure (Cantley and Aisen, 1979;

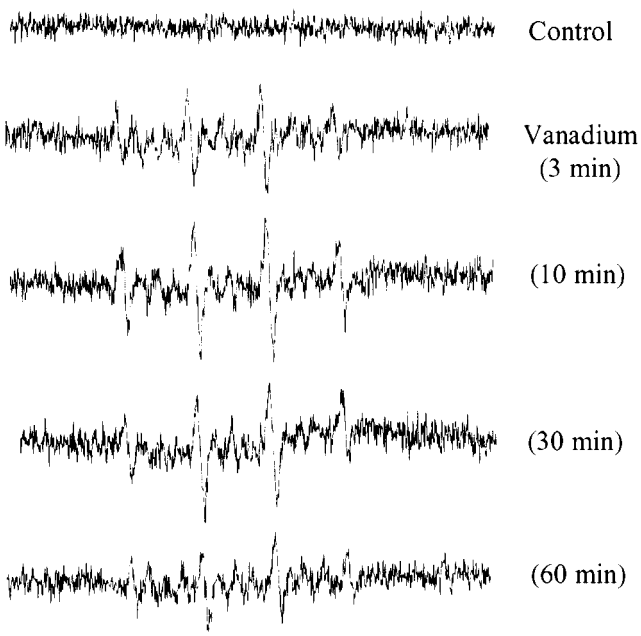

Fig. 6. ESR measurements of vanadium-induced ROS generation. ESR spectra were recorded at different time points after the addition of V (V) (100 μM) to BAL cells (10^6/ml) from naive mice in the presence of the spin trap, DMPO (100 mM). The spectrometer settings were as follows: receiver gain, 1.5×10^5; time constants 0.3 sec; modulation amplitude, 1.0 G; scan time, 4 min; magnetic field, 3470 ± 100 G.

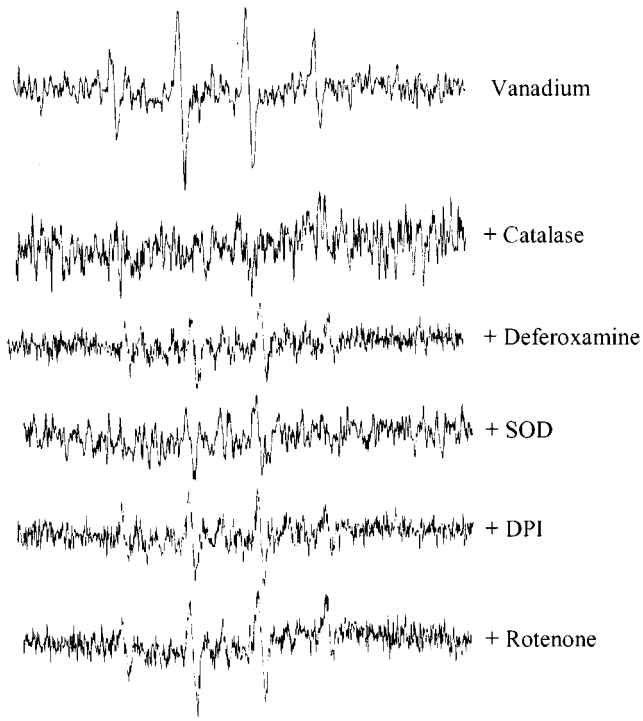

Fig. 7. Effect of ROS scavengers on vanadium-induced ROS generation. ESR spectra were recorded 15 min after the addition of V (V) (100 μM) and DMPO (100 mM) to BAL cells from naive mice (10^6/ml) in the presence or absence of one of the following agents: catalase (1,000 U/ml), SOD (1,000 U/ml), deferoxamine (1 mM), DPI (20 μM), rotenone (50 μM). Spectrometer settings were the same as note for Figure 6.

Zychinski and Byczkowski, 1990), is capable of inducing influx of inflammation cells and lung cell death in mice. Apoptosis was the primary mode of cell death induced by V (V), whereas necrosis contributed relatively little under the experimental conditions used. During the early phase of pulmonary inflammation, neutrophils were recruited to the pulmonary airspace with a peak response at 6 h post-exposure, followed by a delayed cellular infiltration by macrophages at 24–72 h post-exposure. The neutrophilic response resolved spontaneously and completely by day 3, a time at which increased macrophage influx was still observed. At 24 h, when V (V)-induced neutrophilic infiltration was in early resolution, a peak apoptotic response along with the evidence of apoptotic bodies in the cytoplasm of macrophages was observed. At day 3, remnants of apoptotic bodies can still be seen in the cytoplasm of macrophages with few neutrophils present in the BAL fluid. Collectively, these results suggest that V (V) can induce lung inflammation and apoptosis and that macrophages play an important role in the resolution of lung inflammation through a process that involves phagocytosis of apoptotic cells.

Neutrophils are key inflammatory cells of acute tissue injury. They perform an important function in host defense against noxious and infectious agents by producing hydrolytic and proteolytic enzymes, antimicrobial polypeptides, and ROS. They can also contribute to injury in a variety of models of acute inflammation, including injury to the lung (Cox et al., 1995; Hussain et al., 1998). Our findings of the neutrophil influx and apoptosis by V (V) are similar to those observed in other lung injury models caused by bacterial lipopolysaccharide (Cox et al., 1995), oleic acid (Hussain et al., 1998), and Fas antigen (Hagimoto et al., 1997). These results therefore suggest that the neutrophilic and apoptotic responses are not specific to a particular injurious agent but are more general responses to stimulating agents, and possibly through common mediators such as ROS.

For the lung to return to normal following the inflammation, excess neutrophils and damaged cells must be cleared from the airspaces and alveolar walls. While several possible mechanisms of lung clearance exist, apoptosis and subsequent phagocytosis by macrophages are believed to play the most crucial role. Although macrophages are known to ingest necrotic cells as part of their general clearance mechanism (Haslett, 1999), our results show that necrosis plays only a minor role in V (V)-induced lung cell death. Because the cell membranes of apoptotic cells remain intact, the release of inflammatory and toxic contents is minimized (Savill et al., 1993). Thus apoptosis provides a clearance mechanism that limits tissue injury and promotes resolution, which was observed in the present study. Lung clearance through mucociliary transport mechanisms is unlikely, since such transport systems only exist in the larger airways and not in the alveolar region. Likewise, possible emigration of lung neutrophils back into the circulation can be ruled out, since there is no evidence

of such a process after acute lung inflammation (Hughes et al., 1997). A major function of macrophages is phagocytosis and these cells have been shown to phagocytize apoptotic cells both in vitro (Haslett, 1999) and in vivo (Cox et al., 1995; Hussain et al., 1998). The results of the present investigation support a role of macrophages in apoptotic cell clearance and the subsequent resolution of lung inflammation induced by V (V).

The possible role of ROS in V (V)-induced pulmonary inflammation and lung cell apoptosis was investigated in this study by treating mice with various known ROS scavengers along with V (V). Our results showed that ROS scavengers were able to inhibit both V (V)-induced neutrophil influx and lung cell apoptosis, suggesting the involvement of ROS in these processes. Other studies have shown that neutrophil-mediated apoptosis of lung cells does not require ROS generation (Serrao et al., 2001). The results of our study, however, show that ROS are generated and are also required for V (V)-induced neutrophil influx and lung cell apoptosis. These results do not necessarily indicate that neutrophil apoptosis is dependent on ROS generation since the observed reduction in lung cell apoptosis may simply be a consequence of reduced neutrophil influx and not apoptosis. Several other studies, however, have reported the involvement of ROS in neutrophil apoptosis (Misso et al., 2000; Akgul et al., 2001; Ruiz et al., 2002). Among the ROS generated in our system, H_2O_2 appears to play the most critical role, since catalase, a specific scavenger of H_2O_2, was most effective in inhibiting both the neutrophilic inflammatory and apoptotic responses induced by V (V). The difference in results obtained in the present versus the other study (Serrao et al., 2001) may be due to the fact that the previous study was carried out in vitro using a lung epithelial cell line which may not necessarily be representative of in vivo responses. A previous study by Kazzaz et al. (1996) demonstrated that mice exposed to hyperoxia developed lung cell apoptosis, whereas a similar treatment of lung cells in culture resulted in cell necrosis. This finding emphasized differences in cellular responses under the in vivo and in vitro exposure conditions.

To further confirm the formation of ROS after V (V) treatment, ESR spin trapping studies were conducted in combination with various antioxidants. The results show that in vitro treatment with V (V) was able to stimulate BAL cells to produce ROS, including $O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$ radicals, and that ROS scavengers inhibited this ROS generation. BAL cell preparations which contain mainly macrophages were used in this experiment since they are the first major ROS producing cells that come into contact with V(V) after treatment. The sources of ROS generation induced by V (V) appear to be, at least in part, from the activation of an NADPH oxidase complex and the mitochondrial electron transport chain. This conclusion is supported by the observation that DPI, a known NADPH oxidase inhibitor, and rotenone, an inhibitor of the mitochondrial electron transport chain, inhibited ROS generation. It is proposed that molecular oxygen generation by these processes was consumed to generate $O_2^{\cdot -}$. $O_2^{\cdot -}$ may react with V (V) to form V (IV) plus molecular oxygen. In addition, $O_2^{\cdot -}$ would dismutate to produce H_2O_2. H_2O_2 further produced $^{\cdot}OH$ via a Fenton-like reaction, V (IV) + $H_2O_2 \rightarrow$ V (V) + $^{\cdot}OH + OH^-$, which was inhibited by catalase and deferoxamine.

In conclusion, the present investigation demonstrates that pulmonary administration of V (V) resulted in inflammatory cell influx and lung cell apoptosis. These processes were shown to require ROS generation. Among the various ROS generated, H_2O_2 appears to play a major role. The observed temporal relationship between cellular infiltration, apoptosis, and ingestion of apoptotic bodies by macrophages suggests a critical role of apoptosis and phagocytosis in the resolution of V (V)-induced lung inflammation.

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