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Pulmonary Reactivity to Vanadium Pentoxide Following Subchronic Inhalation Exposure in a Non-human Primate Animal Model*

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An experimental study was conducted to evaluate changes in pulmonary reactivity resulting from repeated vanadium pentoxide (V_2O_5) dust inhalation. The study assessed pulmonary reactivity to V_2O_5 through the use of provocation challenges, and compared V_2O_5 reactivity before and after subchronic V_2O_5 exposure. A total of 24 adult, male cynomolgus monkeys (*Macaca fascicularis*) were exposed by inhalation for 6 h per day, 5 days per week, for 26 weeks. Two V_2O_5-exposed groups (n=8 each) received equal weekly V_2O_5 exposures (concentration × time) with different exposure profiles. One V_2O_5-exposed group received 0.1 mg V_2O_5 m^{-3} on Mondays, Wednesdays and Fridays, with a twice-weekly peak exposure of 1.1 mg V_2O_5 m^{-3} on Tuesdays and Thursdays, and was included to investigate the influence of an exposure regimen with peaks on the development of pulmonary hyper-reactivity. The other V_2O_5-exposed group received a constant daily concentration of 0.5 mg V_2O_5 m^{-3}. A control group (n=8) received filtered, conditioned air. Pre-exposure challenges with V_2O_5 produced a concentration-dependent impairment in pulmonary function, characterized by airway obstructive changes (increased resistance and decreased flow). Analysis of respiratory cells recovered from the lung by bronchoalveolar lavage demonstrated that airway obstruction was accompanied by a significant influx of inflammatory cells into the lung. Subchronic V_2O_5 inhalation did not produce an increase in V_2O_5 reactivity in comparison to the control group, and cytological, immunological and skin test results indicate the absence of allergic sensitization. Instead, a trend toward decreased pulmonary reactivity was found following subchronic V_2O_5 inhalation. Pulmonary reactivity to V_2O_5 (both functional and cellular responses) was affected, as well as non-specific reactivity to methacholine. This finding suggests that the absence of increased pulmonary reactivity to V_2O_5 following subchronic inhalation may be related to the induction of tolerance under the exposure conditions used in the study.

INTRODUCTION

Vanadium pentoxide (V_2O_5) is the most commercially important chemical compound of vanadium. It is used as a catalyst for a variety of reactions, and in the production of high-strength steel alloys.[1] Occupational exposure presents an inhalation hazard, causing irritation of the respiratory system. Pulmonary irritation is characterized primarily by asthmatic signs and symptoms, including chest tightness, wheezing and impaired pulmonary functions.[2–9]

An important issue still remaining with regard to occupational V_2O_5 exposure relates to evidence indicating that an increase in pulmonary reactivity to V_2O_5 can be produced by repeated inhalation. Zenz *et al.*[10] studied a group of asthmatic workers employed in a plant pelletizing purified V_2O_2 powder. A striking feature of their asthmatic reaction was an increased severity of symptoms following repeated exposures of lesser time and intensity. Similar findings have been reported also by Roshchin *et al.*[11] among Russian vanadium workers. The asthmatic condition of these workers improved when contact with vanadium was discontinued. However, their asthmatic condition quickly returned when contact with the dust was renewed. A recent article[12] has also reported an increase in non-specific pulmonary reactivity among workers employed at a V_2O_5 refinery. Hyper-reactivity to provocation challenge with histamine was observed for up to 4 weeks following the last vanadium exposure. This finding suggests that a persistent increase in non-specific pulmonary reactivity may also develop in response to repeated V_2O_5 inhalation, similar to that reported for other occupational agents such as isocyanate[13] and western red cedar.[14]. Despite the publication of these reports describing increased pulmonary reactivity following repeated V_2O_5 exposure, little information is available regarding the possible underlying mechanisms. Sjoberg's[6] study of workers employed in a V_2O_5 processing plant suggests that the development of allergic reactions to V_2O_5 may play an important role. Chronic hyperplastic changes of the nasal mucosa of an allergic type were described. Positive skin reactions and blood eosinophilia were

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also observed among workers with allergic rhinitis. However, no increase in the number of eosinophils or other signs of allergic inflammation was found in a more recent study conducted on the upper respiratory tract of workers employed in a vanadium factory.[3]

The purpose of the present study was to evaluate further the changes in pulmonary reactivity resulting from repeated V_2O_5 inhalation and investigate the etiopathogenic mechanisms that could be associated with increased pulmonary reactivity. A comprehensive laboratory investigation was conducted using cynomolgus monkeys as an animal model because of their similarity to humans, including upright posture and allergic reactions mediated by class E immunoglobulins (IgE). The study assessed pulmonary reactivity to V_2O_5 through the use of provocation challenges, as performed in clinical studies, and compared V_2O_5 reactivity before and after a subchronic 26-week exposure period. Because the etiology of occupational asthma may be related to periodic higher level of 'peak' exposures,[15] one V_2O_5-exposed group received an exposure regimen with peaks superimposed on a lower background concentration. Non-specific pulmonary reactivity was evaluated by provocation challenges with methacholine. The occurrence of allergic/immunological effects and skin sensitization were also investigated before and after subchronic V_2O_5 exposures.

Pre-exposure testing

Weeks 1–2 Phlebotomy for cytological and immunological analyses, skin testing, baseline pulmonary function testing (PFT) and baseline bronchoalveolar lavage (BAL) fluid collected for cytological and immunological analyses.

Weeks 3–4 Provocation challenge with 0.5 mg V_2O_5 m^{-3}, followed by PFT.

Weeks 5–6 Provocation challenge with 3.0 mg V_2O_5 m^{-3}, followed by PFT and BAL.

Weeks 7–8 Provocation challenge with methacholine followed by PFT, to assess non-specific bronchial reactivity.

- Assign 26 monkeys to three groups, based on pre-exposure V_2O_5 reactivity.

Subchronic exposures (weeks 9–34)

- Control group: filtered, conditioned air.
- Peak group: 0.1 mg V_2O_5 m^{-3} Mon, Wed, Fri and 1.1 mg V_2O_5 m^{-3} on Tues & Thurs.
- Constant group: 0.5 mg V_2O_5 m^{-3}.
- Followed by a 2-week post-exposure recovery period.

Post-exposure testing

Weeks 37–38 Phlebotomy for cytological and immunological analyses, skin testing, baseline pulmonary function testing (PFT) and baseline bronchoalveolar lavage (BAL) fluid collected for cytological and immunological analyses.

Weeks 39–40 Provocation challenge with 0.5 mg V_2O_5 m^{-3}, followed by PFT.

Weeks 41–42 Provocation challenge with 3.0 mg V_2O_5 m^{-3}, followed by PFT and BAL.

Weeks 43–44 Provocation challenge with methacholine followed by PFT, to assess non-specific bronchial reactivity.

Figure 1. Experimental design and time schedule.

METHODS AND MATERIALS

Animals and experimental design

Adult, male cynomolgus monkeys (*Macaca fascicularis*) were purchased from Charles River Research Primate Corp. (Port Washington, NY). Monkeys were caged individually in stainless-steel mesh cages and were fed a standard diet (Monkey Chow Jumbo®; Ralston Purina Co., St. Louis, MO) with fresh fruit (apples and bananas). Water was available *ad libitum*. Prior to the start of the study, 32 monkeys were challenged with methacholine, as described below, to assess their pulmonary reactivity. A pulmonary flow resistance (*RL*) measurement of 85 cm H_2Ol^{-1} s^{-1} in response to methacholine challenge at a concentration of 6.25 mg ml^{-1} was established as an upper acceptable limit for naive animals.

A repeated measures design was used for the study, in which the 26 monkeys selected were tested before and after subchronic inhalation exposures (Fig. 1). The pre-exposure and post-exposure testing elements were identical, and utilized provocation challenges with V_2O_5 dust to detect differences in pulmonary reactivity to V_2O_5 following subchronic exposure. Three to four monkeys were tested each day, and a 2-week interval was established between testing periods. A 2-week period was also provided between the last exposure day and the beginning of the post-exposure testing to investigate the development of a persistent, acquired increase in V_2O_5 reactivity following repeated exposure.

Exposures were conducted for 6 h per day, 5 days per week, for 26 weeks (excluding legal holidays). Two V_2O_5-exposed groups (n = 9 each) received equal weekly exposures (concentration × time, $C \times T$) with different exposure profiles. One V_2O_5-exposed group received a constant concentration of 0.1 mg V_2O_5 m^{-3} for 3 days per week (on Monday, Wednesday and Friday) and a peak exposure at a constant concentration of 1.1 mg V_2O_5 m^{-3} for 2 days per week (on Tuesday and Thursday), and was included to investigate the influence of an exposure regimen with peaks on the development of pulmonary hyper-reactivity. The other V_2O_5-exposed group received a constant daily concentration of 0.5 mg V_2O_5 m^{-3}. A control group (n = 8) received filtered, conditioned air. These groups are referred to as 'Peak', 'Constant' and 'Control', respectively. During the daily exposures and V_2O_5 challenges, monkeys were caged individually in stainless-steel mesh cages without access to food or water.

The 26 monkeys used in the study were assigned to the control and two V_2O_5-exposed groups such that the V_2O_5 pulmonary reactivity of the three groups was approximately equal. This was accomplished by using results of the pulmonary function tests performed following the pre-exposure V_2O_5 challenge at the 3.0 mg m^{-3} level. Based on a listing of values for *RL* and FEF_{50}, three strata of pulmonary reactivity to V_2O_5 were identified, and monkeys in each of the three strata were then randomly assigned to the three treatment groups.

V_2O_5 challenge and methacholine challenge

The V_2O_5 challenges consisted of 6-h whole-body dust inhalation exposures performed at two aerosol concentrations separated by a 2-week time interval (Fig. 1). The first V_2O_5 challenge was performed at

an aerosol concentration of 0.5 mg V_2O_5 m^{-3}, while the second was performed at 3.0 mg V_2O_5 m^{-3}. Because results of previous work had shown that pulmonary function changes occur 18–21 h after acute V_2O_5 exposure,[16] pulmonary function tests were conducted on the day following each dust challenge.

Methacholine challenges were performed 2 weeks after the 3.0 mg V_2O_5 m^{-3} challenges in order to preclude any pulmonary effects of recent V_2O_5 inhalation (Fig. 1). The methods used were similar to those described by Biagini *et al.*[17] Aerosols for methacholine challenges were generated with a micronebulizer and a positive pressure ventilator respirator (Bird Mark 7, Bird Inc., Palm Springs, CA). Methacholine (acetyl-β-methacholine chloride, 99% pure; Sigma Chemical Co., St. Louis, MO) challenges were performed for 1 min (15 breaths min^{-1}) at 10-min intervals in the following sequence: phosphate-buffered saline (PBS) (0.9% NaCl and 0.02 M PO_4^{-3}) and 0.1, 0.5, 1.0 and 6.25 mg methacholine ml^{-1} PBS. Pulmonary function tests were conducted after each challenge level.

Pulmonary function testing

Pulmonary function tests were conducted while the monkeys were anesthetized, using methods reported elsewhere.[16,18] Mechanical properties (RL and CL_{dyn}) and maximal expiratory performance (flow-volume analysis) were measured after V_2O_5 and methacholine challenges. Lung volumes were also measured after V_2O_5 challenges. Standard abbreviations for all pulmonary function test measurements are used throughout the report.

V_2O_5 challenge/exposure chambers

The V_2O_5 challenges and the subchronic exposures were conducted in two separate inhalation chambers. These chambers are 2-m (length, width and height) stainless-steel and glass cubes with a common middle wall and pyramidal cones attached to their tops and bottoms.[19] Vanadium pentoxide dust aerosols were generated by packing V_2O_5, > 99.6% pure (Aldrich Chemical Co., Milwaukee, WI), into three separate variable-speed Wright Dust Feeders (BGI Inc., Waltham, MA) and introducing the dust at controlled rates into the two chambers through tangential airfeed manifolds mounted on the upper pyramidal cones. Exposures were conducted under dynamic airflow conditions of 14 or 15 air changes per hour. Adjustments were made to maintain the exposure concentrations at planned levels by continuous monitoring with a light-scattering photometer (RAS-1; M.I.E. Inc., Bedford, MA). Chamber concentrations of V_2O_5 were measured by colorimetric analysis of aerosol samples collected at least once daily by drawing chamber air through membrane filters, and were verified by an independent gravimetric method. The particle size of the V_2O_5 dust aerosols was normally determined on a weekly schedule during challenges and biweekly during exposures, using a cascade impactor (model no. 20-801, Andersen Samplers Inc., Atlanta, GA).

Cytological and immunological analyses

Cytological (hematological) and immunological data were obtained from blood samples collected during the pre-exposure and post-exposure baseline testing (Fig. 1). A complete blood count (CBC) and differential count were performed on each blood sample. The CBCs were performed on EDTA-treated blood with an automatic counter (Coulter Electronics, Hialeah, FL), and the differential counts were made by microscopic examination of blood smears. An additional 15 ml of blood was collected in Serum Separator Tubes, allowed to clot and centrifuged for 20 min. The resulting serum samples were stored at −70°C for later immunological analysis.

Cytological and immunological analyses were also performed on respiratory cells and fluid recovered from the lung by bronchoalveolar lavage (BAL). Lavages were performed immediately after the pulmonary function tests were completed, following the 3.0 mg V_2O_5 m^{-3} challenges (pre-exposure and post-exposure), and compared to respective baseline values (Fig. 1). While the anesthetized monkeys were still intubated from pulmonary function testing, a small plastic catheter was inserted through the endotracheal tube. One hundred milliliters of sterile Hank's Balanced Salt Solution (HBSS), warmed to 37°C, was instilled into the lung and recovered by aspiration and percussion. The recovered lavage fluid was then centrifuged at 70 *g* for 10 min to sediment the cells. The cell pellets were resuspended in 5 ml of warm HBSS and a total nucleated cell count was done with an automatic counter (Coulter Electronics, Hialeah, FL). Differential cell counts [polymorphonuclear leukocytes (PMNs), lymphocytes, eosinophils, macrophages, and mast cells] of 400 cells were made from slide-mounts prepared with a cytocentrifuge (model 7 Cytospin; Shandon Scientific Co., Sewickley, PA) and stained with May-Grunwald–Giemsa stain. In preparation for leukotriene C_4 (LTC_4) analysis, 10 ml of the lavage fluid supernatant was mixed with 40 ml of ethanol and centrifuged at 600 *g* for 20 min at 0°C. The LTC_4 sample and the remaining lavage fluid supernatant were stored at −70°C for later analysis.

The immunological analyses performed on blood serum samples and BAL fluid supernatant included measurements of total IgE and total IgG. Albumin and total protein were also measured in lavage fluid supernatant. Total IgG and albumin were measured by nephelometry with a Beckman Auto Immunochemistry System (Beckman Instruments Inc., Fullerton, CA). Total IgE was measured by radioimmunoassay with a Kallestad Quantitope kit (Kallestad Labs., Austin, TX). Total protein was measured by the Lowry method,[20] and LTC_4 concentrations were determined with a radioimmunoassay kit (NEN Research Products, Boston, MA).

Skin testing

Cutaneous sensitivity tests were conducted before the V_2O_5 challenges (Fig. 1). Graded concentrations of sodium vanadate solution and V_2O_5–Monkey Serum Albumin (MSA) conjugate were injected intracutaneously in 100 μl aliquots into shaved areas of the chest

of the anesthetized animals, following the intravenous injection of 5 ml of 0.5% Evans blue. The graded concentrations of sodium vanadate solution were made by tenfold dilutions from a stock solution that was 0.76 M for vanadium, and contained a mixture of sodium vanadate ions ($H_2VO_4^-$ and HVO_4^{2-}) and related polymers, including trivanadate, tetravanadate and decavanadate.[21] The graded concentrations of V_2O_5–MSA conjugate were made by tenfold dilutions from a stock solution prepared by passive saturation with V_2O_5 (99.99% pure; Aldrich Chemical Co., Milwaukee, WI) and chromatographically purified MSA (Cappel Worthington, Cooper Biomedical, Malvern, PA). The conjugate contained 11 mol vanadium mol^{-1} MSA, and the concentration of the stock solution was 10^{-2} g ml^{-1}. To control for the possibility that the conjugate carrier protein (MSA) may have some skin reactivity, control intradermal injections of MSA were incorporated into the procedure. Skin test sites were observed 30 min after injection for an immediate reaction and again ca. 24 h later for a delayed reaction. Cutaneous reactivity was determined by recording the negative logarithm of the maximum dilution of skin test solution producing a cutaneous blueing reaction. If a positive reaction to V_2O_5–MSA conjugate (or MSA carrier protein) was not observed at the most highly concentrated solution injected (1×10^{-2} g ml^{-1}), then the response was given a value of 1.

Data analyses

The pulmonary function, BAL cytological and BAL immunological data were analyzed statistically by an analysis of variance (ANOVA) using logarithmic transformations of ratios calculated by dividing the challenge values (pre-exposure and post-exposure) by their respective baseline values (pre-exposure and post-exposure). These ratios, termed challenge reactivity, were used as dependent variables. To analyze effects occurring equally during the pre-exposure testing when the monkeys were ungrouped, challenge reactivities for all monkeys were combined for each measurement parameter and tested for a significant difference from 100%. Changes in the three treatment groups occurring after the subchronic exposures were investigated using the exposure groups (Control, Peak, and Constant) as an independent variable. One-tailed probabilities were calculated for the ungrouped comparisons because specific challenge responses were expected. Two-tailed probabilities were calculated for comparisons made between the three exposure groups.

A different method was used to analyze the hematology, serum immunology and skin sensitivity data. Because these data were not collected in a challenge-type design where challenge responses were compared to a baseline value, the dependent variables were the actual cytological, immunological and skin test measurements recorded during the pre-exposure and post-exposure testing. Two-tailed probabilities were calculated for comparisons made between the three exposure groups.

Significant differences between the three treatment groups were analyzed further using Dunnett's test.[22] The probability level for Type 1 error ($P \leq 0.05$) was adjusted by the Bonferroni method[23] to control for multiple-dependent variables. The pulmonary function variables *RL* and FEF_{50}/FVC were selected as hypothesis-testing variables, as were the BAL cytological variables (total nucleated cells, PMNs and eosinophils), the BAL fluid variables (albumin, total protein and LTC_4), serum neutrophil and eosinophil counts and immediate skin test reactivity to the sodium vanadate and V_2O_5–MSA conjugate solutions. The additional measurement variables were used to assist in the interpretation of the findings.

All ratios were multiplied by 100 to yield percentages for presentation in the tables. Mean values for the provocation challenge responses are reported as the geometric mean and range. Other mean values are reported as the arithmetic mean and standard error of the mean (SEM).

Table 1. V_2O_5 Dust aerosol concentrations and particle sizes

Targeted concentrations	Aerosol concentration[a] (mg m^{-3})	Particle size[b] (μm)
Pre-exposure challenges		
0.5 mg V_2O_5 m^{-3}	0.59 ± 0.03	0.84 ± N.D.[c]
3.0 mg V_2O_5 m^{-3}	3.33 ± 0.24	1.48 ± N.D.
Subchronic exposures		
0.1 mg V_2O_5 m^{-3}	0.16 ± 0.01	3.17 ± 2.48
0.5 mg V_2O_5 m^{-3}	0.57 ± 0.03	3.15 ± 3.25
1.1 mg V_2O_5 m^{-3}	1.38 ± 0.07	3.10 ± 2.45
Post-exposure challenges		
0.5 mg V_2O_5 m^{-3}	0.52 ± 0.02	2.61 ± 1.92
3.0 mg V_2O_5 m^{-3}	3.01 ± 0.07	3.06 ± N.D.

[a] Aerosol concentrations are presented as means ± SEM.
[b] Particle sizes are reported as mass median aerodynamic diameter (MMAD), and are presented as the mean particle size ± geometric standard deviation.
[c] Geometric standard deviation not determined because plots were not linear.

RESULTS

Atmospheric analyses

Average V_2O_5 aerosol concentrations and particle sizes are presented in Table 1. At the completion of the 26 weeks of inhalation exposure, the overall $C \times T$ values were within 10.2% for the two V_2O_5-exposed groups. Particle sizes measured during the V_2O_5 challenges and subchronic exposure were generally within the 1–5 μm range, producing tracheobronchial deposition.[24] Pre-exposure particle sizes for both challenge concentrations were lower than those measured during the post-exposure challenges. However, these pre- vs. post-differences in particle size did not impair our ability to test for treatment-related changes in V_2O_5 challenge reactivity because a concurrent control group was included in the experimental design.

Table 2. Pulmonary reactivity (as per cent of baseline) to V_2O_5 provocation challenge before and after subchronic (26 weeks) exposure

Pulmonary function measurements and V_2O_5 challenge conc.	Pre-exposure Ungrouped (n = 24)	Post-exposure Control group (n = 8)	Peak group (n = 8)	Constant group (n = 8)
RL				
0.5 mg V_2O_5 m^{-3}	103 (67–163)[a]	101 (80–110)	106 (86–120)	102 (82–133)
3.0 mg V_2O_5 m^{-3}	114 (65–370)[b]	153 (99–317)	115 (86–161)	121 (89–213)
FEF_{50}/FVC				
0.5 mg V_2O_5 m^{-3}	99 (81–125)	98 (87–101)	99 (84–102)	101 (55–185)
3.0 mg V_2O_5 m^{-3}	87 (53–113)[b]	92 (83–98)	94 (62–114)	97 (79–112)
FVC				
0.5 mg V_2O_5 m^{-3}	96 (88–100)[b]	100 (99–101)	101 (98–107)	100 (98–104)
3.0 mg V_2O_5 m^{-3}	97 (80–104)[b]	97 (92–99)	100 (93–104)	100 (95–106)
RV				
0.5 mg V_2O_5 m^{-3}	105 (75–133)	101 (80–123)	105 (77–131)	109 (89–124)
3.0 mg V_2O_5 m^{-3}	114 (77–293)[b]	125 (99–139)	118 (83–149)	131 (91–348)
CL_{dyn}				
0.5 mg V_2O_5 m^{-3}	105 (64–182)	107 (78–177)	99 (82–123)	98 (80–122)
3.0 mg V_2O_5 m^{-3}	104 (49–182)	85 (48–141)	104 (65–143)	113 (79–145)

[a] Data are presented as the geometric mean and range.
[b] Significantly different from 100% (adjusted alpha level, $P \leq 0.025$).

Animal observations

Twenty-six of the 32 monkeys initially screened for participation in the study met the pre-established criteria for pulmonary reactivity in response to methacholine challenge and were included in the study. One monkey in the Constant group was eliminated from the study after he was found to have a parasitic infestation, thus reducing that group size to eight. The number of monkeys in the Peak group was also reduced to eight when one monkey in that group died unexpectedly of an effect (acute gastric dilatation) unrelated to V_2O_5 exposure.

Respiratory distress developed in three monkeys from the Peak group during the subchronic V_2O_5 exposures. The pattern of effect was characterized by audible wheezing and coughing, and occurred only on peak exposure days during the first few weeks of exposure. Responses developed within 3–4 h of exposure, and occasionally required early removal of the affected monkeys from the exposure chamber. Through careful animal monitoring, these three monkeys continued to receive daily exposures for the entire study. All post-exposure testing was completed as scheduled for these animals, and their test measurements were included in the data analyses.

V_2O_5 and methacholine challenge reactivity

The pre-exposure provocation challenges with V_2O_5 produced statistically significant impairments in pulmonary function at an aerosol concentration of 3.0 mg V_2O_5 m^{-3}, but not at 0.5 mg V_2O_5 m^{-3} (Table 2). Significant changes in the hypothesis-testing variables (*RL* and FEF_{50}/FVC) were observed. The increase in *RL* and the decrease in FEF_{50}/FVC were accompanied by a significant increase in *RV* and a significant decrease in *FVC*, thereby demonstrating an obstructive pattern of impaired pulmonary function. The pattern of pulmonary function observed when the monkeys were rechallenged following subchronic exposure resembled the obstructive pattern measured in response to the pre-exposure V_2O_5 challenges. Post-exposure reactivity was not significantly different between the three exposure groups at either challenge concentration. However, a trend (not statistically significant) toward decreased post-exposure challenge reactivity in *RL* and FEF_{50}/FVC was noted in the V_2O_5-exposed groups as compared to the control group at the 3.0 mg V_2O_5 m^{-3} level.

The respiratory system was highly reactive to provocation challenges with methacholine (Table 3). Dramatic increases in *RL* ($P \leq 0.001$) and decreases in FEF_{50}/FVC ($P \leq 0.001$) were measured at the 6.25 mg ml^{-1} methacholine concentration. Methacholine reactivity was not significantly increased by subchronic V_2O_5 exposure; however, a trend toward a post-exposure decrease in *RL* was observed in the V_2O_5-exposed groups that was similar to that observed for post-exposure V_2O_5 reactivity.

Cytological/immunological and skin test results

Just as the pre-exposure challenge with V_2O_5 had produced a significant impairment in pulmonary function, pre-exposure challenge also produced a cellular response characterized by a significant increase in the total number of respiratory cells recovered from the lung by BAL (Table 4). The increase in the total number of cells occurred through a highly significant increase ($P \leq 0.001$) in the number of PMNs. The number of eosinophils recovered from the lung was also increased, while the numbers of lymphocytes, macrophages and mast cells were not. Significant challenge responses were not observed for total protein,

Table 3. Pulmonary reactivity (as per cent of baseline) to methacholine challenge before and after subchronic (26 weeks) exposure[a]

Pulmonary function measurements	Pre-exposure ungrouped ($n = 24$)	Post-exposure Control group ($n = 8$)	Peak group ($n = 8$)	Constant group ($n = 8$)
RL	411 (170–1259)[b,c]	444 (142–2646)	428 (283–1247)	385 (208–1309)
FEF_{50}/FVC	77 (54–98)[c]	77 (61–95)	82 (56–94)	74 (60–93)

[a] Provocation challenges with methacholine were conducted 2 weeks following the 3.0 mg V_2O_5 m^{-3} challenges. Results are from the 6.25 mg methacholine ml^{-1} concentration.
[b] Data are presented as the geometric mean and range.
[c] Significantly different from 100% (adjusted alpha level, $P \leq 0.025$).

Table 4. Cytological and immunological effects (as per cent of baseline) measured in bronchoalveolar lavage fluid[a]

BAL variables	Pre-exposure Ungrouped ($n = 24$)	Post-exposure Control group ($n = 8$)	Peak group ($n = 8$)	Constant group ($n = 8$)
Total nucleated cells	141 (72–452)[b,c]	197 (49–346)	143 (74–451)	124 (91–186)
PMNs	393 (45–4969)[c]	780 (145–3408)	508 (62–2485)	462 (151–1321)
Eosinophils	170 (45–881)[c]	452 (58–2943)	142 (74–475)	101 (69–159)[d]
Lymphocytes	106 (25–490)	145 (48–260)	81 (32–151)	82 (31–186)
Macrophages	104 (63–353)	129 (24–255)	119 (54–230)	106 (73–148)
Mast cells	54 (6–929)	88 (11–307)	123 (25–591)	115 (27–1200)
Albumin	103 (25–433)	128 (77–249)	93 (30–215)	94 (44–177)
Total protein	104 (66–182)	141 (78–217)	129 (60–335)	99 (54–137)
Leukotriene C_4	102 (55–228)	92 (24–180)	91 (29–262)	80 (29–156)
Total IgG	97 (16–1013)	141 (40–256)	81 (16–263)	79 (26–158)
Total IgE	47 (25–120)	102 (50–168)	91 (57–133)	95 (50–153)

[a] Bronchoalveolar lavage fluid was collected following provocation challenges with 3.0 mg V_2O_5 m^{-3}.
[b] Data are presented as the geometric mean and range.
[c] Significantly increased from 100% (adjusted alpha level, $P \leq 0.017$).
[d] Significant difference between the three exposure groups (adjusted alpha level, $P \leq 0.017$).

albumin, LTC_4 or the immunoglobulins, despite the significant cellular response to V_2O_5 challenge. A similar pattern of cellular and immunological response was observed after subchronic exposure. Post-exposure challenge responses for PMNs were > 400% of baseline values. A post-exposure trend (statistically significant for eosinophils) toward decreased responses was observed in the V_2O_5-exposed groups as compared to the control group.

The number of circulating neutrophils and eosinophils in venous blood was not affected by subchronic V_2O_5 exposure. The number of circulating neutrophils during pre-exposure testing was 6.6 ± 0.5, and 6.6 ± 0.8, 6.1 ± 1.2 and $8.6 \pm 2.6 \times 10^3$ cells mm^{-3} for the Control, Peak and Constant groups, respectively, during post-exposure testing. The number of eosinophils was 0.22 ± 0.05, and 0.24 ± 0.09, 0.14 ± 0.06 and $0.26 \pm 0.09 \times 10^3$ cells mm^{-3}, respectively. Similarly, serum immunoglobulins were unchanged throughout the study, averaging 1070 ± 61, and 1107 ± 81, 1095 ± 122 and 1159 ± 111 mg dl^{-1} for IgG; and 37.4 ± 1.5, and 42.7 ± 3.5, 43.0 ± 3.7 and 44.5 ± 2.4 ng ml^{-1} for IgE.

Results of the skin tests with sodium vanadate and V_2O_5–MSA conjugate are presented in Table 5. Skin sensitivity to both skin test solutions averaging < 1 was observed for both the immediate and delayed responses, and remained unchanged after subchronic exposure.

DISCUSSION

Pulmonary reactivity to V_2O_5 was studied experimentally in non-human primates using provocation challenges with V_2O_5 conducted before and after subchronic V_2O_5 exposure. Pre-exposure V_2O_5 challenges produced statistically significant responses that were characteristic of an obstructive pattern of impaired pulmonary function. Pulmonary obstructive changes were accompanied by a significant increase in the total number of inflammatory cells present in the lung. Pulmonary reactivity to V_2O_5 was not increased by subchronic V_2O_5 exposure in comparison to the control group, and cytological/immunological and skin test results indicate the absence of allergic sensitization. Instead, a trend toward decreased pulmonary reactivity

Table 5. Cutaneous reactivity to sodium vanadate and V_2O_5-MSA conjugate[a]

Parameter	Pre-exposure Ungrouped (n = 24)	Post-exposure Control group (n = 8)	Peak group (n = 8)	Constant group (n = 8)
Vanadate				
Immediate	0.13 ± 0.07[b]	0.50 ± 0.19	0.88 ± 0.30	0.50 ± 0.27
Delayed	0.33 ± 0.10	0.50 ± 0.19	0.38 ± 0.18	1.00 ± 0.33
Conjugate				
Immediate	0.92 ± 0.21	0.38 ± 0.18	0.88 ± 0.48	0.38 ± 0.18
Delayed	0.66 ± 0.22	0.13 ± 0.35	0.63 ± 0.26	0.63 ± 0.26

[a] Cutaneous reactivity was determined by recording the negative logarithm of the maximum dilution of skin test solution to produce a blueing effect.
[b] Data are presented as the mean ±SEM.

to V_2O_5 was observed following subchronic inhalation exposure. Pulmonary function and cellular responses, as well as non-specific reactivity to methacholine, were affected. These findings suggest that the absence of increased pulmonary reactivity to V_2O_5 following subchronic inhalation may be related to the induction of tolerance under the exposure conditions used.

The respiratory distress observed during the exposures and the obstructive pattern of impaired pulmonary function measured in response to the pre-exposure challenges agree well with the effects reported in occupationally exposed workers[4,5,12] and in laboratory animals.[16] Both our current and previous studies found significant increases in the number of PMNs recovered from the lungs by BAL, and suggest that the airway obstruction resulted from the release of inflammatory mediators from PMNs. These cells are capable of releasing a variety of inflammatory mediators (i.e. leukotrienes and histamine) that have been shown to cause contraction of airway smooth muscle.[25] Inflammatory mediators can also act on capillary endothelium to increase vascular permeability,[26] as has been demonstrated following exposures to other chemical agents.[27,28] Extravascular diffusion of blood proteins into the pulmonary parenchyma could have an edematogenic effect on these tissues, which could also contribute to airway obstruction. The lack of significant increases in the concentration of LTC_4 and proteins in the BAL fluid suggests that inflammatory mechanisms may not play an important role in producing airway obstruction following V_2O_5 inhalation. *In vitro* evidence suggests that airway obstruction may occur through a direct action of vanadium on the airway smooth muscle.[29] Significant increases in smooth muscle tension have been demonstrated in isolated preparations of guinea pig central and peripheral airways when graded concentrations of sodium vanadate were added to the incubation media. Further research is needed to determine the pathophysiological mechanisms responsible for producing the airway obstructive changes induced by V_2O_5 dust inhalation.

The present study found that pulmonary reactivity to V_2O_5 was not increased by subchronic V_2O_5 inhalation. To the contrary, a trend toward reduced pulmonary reactivity to V_2O_5 was found in an *a posteriori* examination of the data. Pulmonary function and BAL responses, as well as non-specific reactivity to methacholine, generally were reduced in the V_2O_5-exposed groups as compared to the control group. Although this finding suggests that the absence of increased pulmonary reactivity to V_2O_5 following subchronic inhalation may be related to the induction of tolerance, additional experimental studies are needed to corroborate this finding.

The absence of an increase in pulmonary reactivity to V_2O_5 is supported by the cytological/immunological and skin test results, which indicate that an allergic response was not produced. Although species differences are always a concern in any study using laboratory animals, several reasons point to the cynomolgus monkey as the best animal model for this study. Macaque monkeys have been shown to have all the immunoglobulin classes present in humans,[30] and the role of IgE antibodies in monkeys appears to be similar to its role in humans.[31] Immunoglobulins IgE and IgG were measured with reagents and techniques designed for human immunoglobulin analysis, and serum levels measured in the present study agree well with those reported previously by this laboratory.[32] Non-human primates have been used successfully as an animal model in asthma research involving complete proteins[31] and haptens formed by the conjugation of low-molecular-weight compounds with protein.[19] Aerosol exposure concentrations used in the study were closely related to V_2O_5 concentrations encountered in the workplace, as regulated by the Occupational Safety and Health Administration's permissible exposure limit of 0.05 mg m^{-3}.[33] Furthermore, repeated exposures were conducted for 6 months, which is longer than the time period reported for the induction of increased pulmonary reactivity to low-molecular-weight compounds such as phthalic anhydride.[34] Taken together, these facts indicate that our experiment was conducted in the most relevant animal species, using an experimental design with provocation challenges like those performed in clinical studies, and also using exposure conditions most likely to produce a persistent increase in V_2O_2 reactivity through humoral allergic mechanisms. Although results of our study do not support the development of allergic sensitization following prolonged V_2O_5 inhalation, the possibility that such reactions do occur in specific occupational settings

or populations not represented by our experimental conditions can be excluded.

In conclusion, provocation challenges with V_2O_5 produced statistically significant pulmonary responses in monkeys, prior to a subchronic V_2O_5 exposure. Pre-exposure challenge responses were characterized by an obstructive pattern of impaired pulmonary function, and were accompanied by a significant increase in the total number of inflammatory cells present in the lung. Subchronic V_2O_5 inhalation did not produce an increase in V_2O_5 reactivity in comparison to an unexposed control group, and cytological/immunological and skin test results indicate the absence of allergic sensitization. Instead, a trend toward decreased pulmonary reactivity was found following subchronic V_2O_5 inhalation. Pulmonary reactivity to V_2O_5 (both functional and cellular responses) was affected, as well as non-specific reactivity to methacholine. This finding suggests that the absence of increased pulmonary reactivity to V_2O_5 following subchronic inhalation may be related to the induction of tolerance under the exposure conditions used in the study.

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