Thymic cytoarchitecture changes in mice exposed to vanadium

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ABSTRACT

The thymus is a vital immune system organ wherein selection of T-lymphocytes occurs in a process regulated by dendritic and epithelial thymic cells. Previously, we have reported that in a mouse model of vanadium inhalation, a decrease in CD11c dendritic cells was observed. In the present study, we report on a thymic cortex–medulla distribution distortion in these hosts due to apparent effects of the inhaled vanadium on cytokeratin-5 (K5⁻) epithelial cells in the same mouse model – after 1, 2, and 4 weeks of exposure – by immunohistochemistry. These cells, together with dendritic cells, eliminate autoreactive T-cell clones and regulate the production of regulatory T-cells in situ. Because both cell types are involved in the negative selection of autoreactive clones, a potential for an increase in development of autoimmune conditions could be a possible consequence among individuals who might be exposed often to vanadium in air pollution, including dwellers of highly polluted cities with elevated levels of particulate matter onto which vanadium is often adsorbed.

Introduction

Increased concentrations of particulate matter (PM) in the atmosphere as a consequence of the combustion of fossil fuels have gained attention because of its consequences in the health of the population, as it has been reported by different authors (Calderon-Garciduenas et al. 2012; Fortoul et al. 2014; Crouse et al. 2015). Particles with aerodynamic diameter of ≤2.5 μm (PM₂.⁵) are especially noxious as they can penetrate deeper into the lungs and then translocate to the bloodstream, as well as interact directly with the respiratory and alveolar epithelium (Kim & Kim 2015). PM₂.⁵ are composed mainly of a carbon core, metals, and other trace elements (Kleeman & Cass 2001). One element is vanadium (V), which has increased its presence in the atmosphere as a consequence of combustion of fossil fuels derived from Mexican, Venezuelan, and Kuwaiti petroleum that are rich in V; other sources of V are industrial activities and natural events including volcanic activity, forest fires, etc. (IPCS, 2000; Fortoul et al. 2002; Rodriguez-Mercado & Altamirano-Lozano 2006). Vanadium is most often released into the atmosphere as vanadium pentoxide (V₂O₅) – the most toxic compound of this element – although several other oxides are also generated/present in the air (WHO 1998).

In previous studies, effects from inhalation of V (as V₂O₅) on a variety of systems and organs have been demonstrated (Fortoul et al. 2014). Changes in spleen morphology and a decrease in humoral immune responses have been reported (Gonzalez-Villalva et al. 2006; Piñon-Zarate et al. 2008), as well as a decrease in numbers of thymic dendritic cells (DC) and in expression of biomarkers CD11c and MHC-II (Ustarroz-Cano et al. 2012). Each of these changes suggest that inhalation of V might induce modifications in the immune response as previously reported (Gonzalez-Villalva et al. 2006; Piñon-Zarate et al. 2008; Ustarroz-Cano et al. 2012), but in this case, by causing effects at a key immune system organ, the thymus.

Thymic epithelial cells (TEC) are antigen-presenting cells (APC) that are distributed in the thymic cortex (cortical thymic epithelial cells; cTEC) and in the medulla (medullary thymic epithelial cells; mTEC). Both cell types are functionally and phenotypically different. The mTEC together with DC have a special role in eliminating auto-reactive T-cell clones through negative selection, as well as helping to regulate the production of regulatory T (Treg) cells involved in prevention of autoimmune responses (Osada et al. 2006; Mouri et al. 2014; Ucar & Rattay 2015). Preliminary studies from our group have shown the thymus cortex–medulla ratio was modified in mice exposed to V by inhalation, i.e. the medulla was smaller and its distribution erratic compared with in tissues from control mice (Fortoul et al. 2011, 2014). Because of the observed changes in the cortex–medulla ratio, the present study was undertaken to explore – using cytokeratin-5 (K5) as a biomarker – if mTEC were involved in the histologic modification.

Materials and methods

Mice

CD1 mice (male, 33 ± 2 g, 8-week-old) were obtained from the vivarium of the School of Medicine at the National University of Mexico (UNAM, Mexico City). A total of 30 mice were housed in hanging plastic cages (20/cage) kept in a certified pathogen-free animal facility maintained at 21°C, with a 57% relative humidity and controlled light conditions (12-h light/dark regime). All mice were provided ad libitum access to Purina rat Chow and filtered water. All experimental protocols were in accordance with the Guide for Care and Use of Laboratory...
Animals from the Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council (2010) and the Mexican Guideline for Animal Welfare (NOM-062-200-1999).

Vanadium exposures

For the experiments, mice were randomly allocated into two groups: 15 exposed and 15 controls. Inhalation exposure was performed as described in Fortoul et al. (2011). In brief, the whole-body exposures were performed with V$_2$O$_5$ (99.99%, Sigma, St. Louis, MO) as a 0.02-M suspension in saline. The aerosol inhalation chamber was an acrylic box measuring 45 cm $\times$ 21 cm $\times$ 35 cm that had 3.3 L total volume, and could house 20 mice/session. A DeVilbiss Ultraneb 99 (Somerset, PA) system was used to nebulize the V solution at a flow rate of 10 L/min; according to the manufacturer, $\approx$ 80% of the aerosolized particles reaching the mice would be expected to have a mass median aerodynamic diameter (MMAD) of 0.5–5 $\mu$m. The total V concentration in the chamber was quantified as follows: a filter was placed at the external outlet of the nebulizer during the entire exposure period and samples collected at a flow rate or 10 L/min. After each exposure, the filter was removed and weighed; V on each was quantified following the same protocol as with tissues. Six-filters/each inhalation exposure period were evaluated. The source of the V delivery was located at the top of the chamber to assure a homogeneous exposure. Throughout each exposure, the status of the mice was monitored to detect any behavioral modifications.

As reported in earlier studies, the use of this system again resulted in a final concentration in the chamber of 1.4 mg V/m$^3$. (Fortoul et al. 2002). As noted previously (Gonzalez-Villalva et al. 2011), the concentration selected for this experiment was greater than the concentration reported in workplaces (0.2–0.5 mg/m$^3$) by IARC (2006), but in the range of those noted by the WHO (2000) (0.01–60 mg/m$^3$). High V concentrations (600 mg/kg) have also been detected in soils near petrochemical plants in Mexico as a consequence of airborne particulate emissions (Hernandez & Rodriguez 2011). Thus, here, it was deemed that specific V concentrations were needed to determine any biological effects due to exposure. The total exposure period was 6 weeks (Tuesday and Thursday) for up to a total of 4 weeks. The 15 exposed and 15 controls were exposed to an atmosphere containing only 0.9% V$_2$O$_5$. The 15 exposed and 15 controls were exposed to an atmosphere containing only 0.9% V$_2$O$_5$. The 15 exposed and 15 controls went hematoxylin and eosin (H&E) staining. Each other half was then processed for conventional immunohistochemistry to analyze K5 expression.

For these analyses, frozen sections were quenched of endogenous peroxidase activity using immersion in a 30% H$_2$O$_2$ (in distilled water) solution for 10 min. Following three PBS rinses, nonspecific antigenic sites were blocked for 1 h at 4°C using 1% bovine serum albumin (BSA) in PBS containing 0.01% Triton X-100 (pH 7.2). The sections were then coated with a 1:100 dilution of anti-cytokeratin 5 (ab24647, Abcam, Cambridge, MA) solution (in PBS) and incubated overnight at 4°C in a humidified chamber. The next day, the samples were rinsed three times with PBS, and then incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (H+L) ready-to-use solution (ab64256; Abcam). After three washes in PBS, each section was then incubated for 30 min at room temperature with streptavidin peroxidase ready-to-use solution (4plus HRP Universal Detection, Biocare, Concord, CA). Samples were then quenched in 0.3% H$_2$O$_2$ in 0.1 M HCl. Then, the samples were incubated with 0.05% 3,3’-diaminobenzidine (DAB) followed by hematoxylin counter-staining (both Biocare). Slides were then covered with synthetic mounting resin and observed using a light Olympus CX 31 microscope coupled with a digital camera (Olympus C-7070, Tokyo, Japan). Images were analyzed using image-analyzer software (Image Pro Plus v.6.0, Silver Spring, MD). Five fields from each animal thymus were analyzed, considering as positive a presence of an ochre color. Color intensity was reported in pixel units.

To measure differences in cortex–medulla percentages, the H&E-stained tissues were analyzed with a MATLAB application “Densify” that helps to quantify the percentage nuclear density. For each group, five slides of each thymus half were evaluated (one from each animal).

Statistical analyses

All data are reported as mean ± SD. A one-way analysis of variance (ANOVA) with a Tukey’s or Dunnnett’s post-hoc test was performed to identify differences through the three exposures times. Outcomes were considered significant at $p < 0.05$. All analyses were performed using Prism Software, V 6.0, GraphPad, San Diego, CA).

Figure 1. Body weight. Weights (g) of control and V-exposed mice are shown. Data shown are means ± SD ($n = 5$/regimen/timepoint). There were no significant differences due to the exposures at each timepoint.
Results

Body and thymuses weights

There were no significant changes in either body or thymic weights between control and exposed mice over the course of all the exposures (Figures 1 and 2).

Thymic histological changes

Well-preserved cortex–medulla areas characterized the thymuses of control animals. The cortex (c) was characterized by a dense nuclei presence, with few nuclei observed in the medulla (m) (Figure 3(A)). After 1 week of exposure (Figure 3(B)), the area occupied by the medulla showed a different distribution (m). After 2 and 4 weeks of exposures, the nuclear densities were similar to the tissues from time-matched controls (Figure 3(C,D)), but did not present with a pattern like that seen in the control mice tissues. It is noteworthy that the less dense areas continued with an irregular distribution. The differences in nuclear density were statistically different between the controls and each time-matched exposed set of mice and between the 1-week exposure and the other V groups (Figure 4). The mean percentages measured for each cortex/group were: 31.0 (control), 19.3 (1 week), 25.9 (2 weeks), and 27.9% (4 weeks) (Figure 4).

Immunohistochemistry

Cytokeratin 5-bearing (K5⁰) cells were observed in the thymus of control, 1-, 2-, and 4-week V-exposed mice. The K5⁰ cells location was noticed mainly in the medulla in control mice tissues (Figure 5(A)), while in the 1, 2 and 4-week-exposed mice, K5⁰ cells were observed in the medulla and some cortical areas (Figure 5(B–D)). Diffuse distribution of K5⁰ cells in the cortex of exposed mice tissues was also clearly evident.

Figure 2. Thymus weight. Weights (g) of organs from control (c) and V-exposed (V) mice are shown. Data shown are means ± SD (n = 5/regimen/timepoint). There were no significant differences due to the exposures at each timepoint.

Figure 3. Thymuses histology. In control mouse, the difference between the cortex (c) and medulla (m) is clearly delineated as nuclear density is more evident in the cortex (A). After 1 week of V exposure, the medulla increased and has blurred limits (B). With 2- and 4-week exposures (C and D), nuclear density was similar to that in the controls, but the less dense areas (m) had an irregular distribution. (H&E)

Figure 4. Thymuses histology. In control mouse, the difference between the cortex (c) and medulla (m) is clearly delineated as nuclear density is more evident in the cortex (A). After 1 week of V exposure, the medulla increased and has blurred limits (B). With 2- and 4-week exposures (C and D), nuclear density was similar to that in the controls, but the less dense areas (m) had an irregular distribution. (H&E)
As shown in Figure 6, analyses of the staining in the tissues showed that the density mean (a + ochre color) for K5 \(^+\) stain increased in the thymic tissues of mice during the first week of V-exposure compared with levels in tissues from controls \(p < 0.05\); Figure 6(B). In comparison, a decrease in the ochre color was noticed in tissues of mice exposed for 2 and 4 weeks; however, the values never reached those observed in controls. Significant differences between the 2- and 4-week-exposed mice compared with the 1-week V-exposed hosts were not observed, most likely due to variability in the former groups’ results.

**Discussion**

The present study showed that inhalation of vanadium (as V\(_2\)O\(_5\)) disrupted the cytoarchitecture of mice thymuses. Most generally, this was demonstrated by K5 \(^+\) expression in inappropriate areas of the organ. In particular, this modification was noted in the thymus medulla wherein an erratic distribution of the marker was noted; interestingly, these changes did not impact upon the weight of the individual thymuses.

A thymus in an adult mouse has two well-defined areas, i.e. the cortex and the medulla, and each has different types of TEC (Blackburn & Manley 2004; Osada et al. 2006). These cells create small-specialized microenvironments to differentiate immature T-cells (Assarsson et al. 2007). Immature T-cells exit the bone marrow and enter into the thymus in the cortico-medullar region, where thereafter they can interact with TEC and DC to initiate a programed organized differentiation process. In the organ, T-cells migrate through four well-identified cortico-medullar zones: the first region is cortico-medullar where T-cells are double-negative

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DN1 (CD4<sup>+</sup>CD8<sup>−</sup>) to the cortex; second region, DN2 (CD4<sup>−</sup>CD8<sup>−</sup>), is where T-cells lose their possibility to become B cells or NK cells; third region is where the T-Cell Receptor chain (TCR) undergoes reorganization; and, finally, the fourth region (subcapsular) is where change to double-positive [DP, CD4<sup>+</sup>CD8<sup>+</sup>] cells take place. The DP T-cells return to the medulla to become single positive cells (SP) CD4<sup>+</sup> or CD8<sup>+</sup>T-cells. Positive selection occurs mainly in the cortex, with DP T-cells [after differentiation to either CD4<sup>+</sup>or CD8<sup>+</sup>cells] interacting with the cTEC. Negative selection occurs mainly in the medulla with involvement of mTEC or DC that interact with DP T-cells that are, in turn, then eliminated by apoptosis to prevent liberation of autoreactive clones (Blackburn & Manley 2004).

Previous studies from our group indicated medulla–cortex ratio changes in the thymuses of mice exposed to V (as V<sub>2</sub>O<sub>5</sub>) via inhalation (Fortoul et al. 2011, 2014). Those studies reported there was a significant, decrease in the medulla percentage [with a lobular variable distribution], results that were in concordance with the present findings. Specifically, here, mice that inhaled V had mTEC K5<sup>+</sup> that were also erratically distributed. As a consequence of the thymus zonal rearrangement, immature T-cells could interact with cTEC, mTEC, and DC and consequently, positive or negative T-cell selection could be interrupted. A result of all these changes could be a liberation of autoreactive clones into circulation with negative repercussions for the overall host immune responses.

Adult mice TEC have been classified in different sub-populations according to cytokeratin expression profiles. With a panel of antibodies against different cytokeratins, TEC in the thymus cortex showed as K8<sup>+</sup>K18<sup>+</sup>K5<sup>−</sup>K14<sup>+</sup>, and in the medulla as K8<sup>−</sup>K18<sup>−</sup>K5<sup>+</sup>K14<sup>−</sup>. It was also demonstrated there is a cortico-medullar zone with a sub-population of K8<sup>−</sup>K5<sup>−</sup>TEC (Klug et al. 1998, 2002); those authors also emphasized the relevance that the interaction of thymocytes with TEC has in adequate establishment of compartments in the thymus architecture. Because we previously reported expression of CD11c and a diminished presence of DC in the thymuses of mice exposed to V by inhalation (Ustarroz-Cano et al. 2012), it would be expected that signaling between thymocytes, DC, and mTEC would be disturbed.

It would also be expected that distorted cortex–medulla patterns would be seen in the thymus of these mice (as we observed with mTEC K5<sup>+</sup> populations here).

As previously stated, the thymus is relevant to secure a wide repertoire of functional T-cells. The thymic medullae are integrated by DC and mTEC that create a microenvironment conducive to induce T-cell tolerance. The mTEC express a wide repertoire of tissue-restricted self-antigens (TRA) that grant these cells the leadership in the induction of cell tolerance (Lopes et al. 2015). DC are also relevant for the negative selection of immature T-cells (Oh & Shin 2015); previous studies in mice from our group demonstrated that V inhalation decreased the number of DC as well as expression of CD11c – a DC biomarker (Ustarroz-Cano et al. 2012). These data suggest that negative selection by DC might be disrupted in the V-exposed mice. The increase in K5 expression in the thymuses in the exposed mice, we report here, could be a consequence of an increased number of mTEC or in expression of the biomarker; either of these events could be a mechanism to compensate DC damage induced as a consequence of the V. It is important to explain these events with further studies. The increase in K5 expression in exposed mice could be the consequence of the V inhalation (Zubkova et al. 2005) and the result of the oxidative stress generated by this metal (Bleck et al. 2010; Chinn et al. 2012; Fortoul et al. 2014). Going forward, it would be of great interest the use of other T-cell biomarkers, such as CD4, CD8, and CD25, to better identify changes in T-cell subsets that would lend support to any changes in negative selection processes as a consequence of V-inhalation.

**Conclusions**

Vanadium inhalation modified the cytoarchitecture of mice thymuses. Most generally, this was demonstrated by K5<sup>+</sup> expression in inappropriate areas of the organ. This modification was noted in the thymus medulla in which erratic distribution of the marker was seen. Further, in there were also distorted structures in the thymuses of the V-exposed mice. We surmise that this distortion could ultimately result in an increase in autoreactive clones entering into the circulation of the exposed host. The study also showed that inhalation of V<sub>2</sub>O<sub>5</sub> resulted in an increased expression of K5 in the thymus after 1 week; however, these levels dropped after 2 or 4 weeks of exposure, but never returned to control levels. Given the critical role of TEC (K5<sup>+</sup>) and thymic architecture in T-cell selection, the present study suggests that the disruption of the thymus and K5<sup>+</sup> cell distribution caused by V inhalation may impact the negative selection process, thereby having implications for autoimmunity. This type of change could result in a survival/expansion of autoreactive clones and a subsequent possible rise in autoimmune pathologies in V-exposed hosts.

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**Disclosure statement**

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.
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