In Vitro Effects of Vanadate on Human Immune Functions

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Abstract. Vanadium (V) is an element with wide industrial applications and environmental release. The object of this study was to determine the in vitro effects of high (10^-4 M) and low (10^-7 M) concentrations of sodium metavanadate (NaVO_3) on cultured peripheral blood mononuclear cell (PBMC) proliferation, cytokine release, CD expression, and granulocyte O_2^- production. At 10^-4 and 10^-7 M, NaVO_3 did not modify PBMC proliferation in the absence of phytohemagglutinin (PHA). On the other hand, 10^-4 M NaVO_3 reduced by ~25% the PBMC proliferation in PHA-stimulated cultures, with a significant reduction of the stimulation index (SI) of blastogenesis. Moreover, 10^-4 M NaVO_3 significantly reduced the release of IFN-γ by PHA-stimulated PBMCs, and 10^-7 M NaVO_3 significantly enhanced the release of TNF-α. In addition, IL-5 release was significantly inhibited by high concentration of sodium metavanadate and significantly enhanced by low concentration of NaVO_3. Neither 10^-4 nor 10^-7 M NaVO_3 modified the expression of CD3+, CD4+, CD8+, or CD56+ in PHA-stimulated and unstimulated lymphocytes. Finally, 10^-4 M NaVO_3 reduced the granulocyte production of O_2^- by about 70%, while 10^-7 vanadate reduced its production to a lesser extent. These results show that 10^-4 M NaVO_3 exerts inhibitory effects on PBMCs, while at 10^-7 M it exerts a stimulatory action with a slight shift of the immune response towards a Th2-type response. This investigation suggests that environmental V can have important effects on the human immune system. (received 25 October 2001, accepted 18 November 2001)

Keywords: vanadium, mononuclear cells, IFN-γ, TNF-α, IL-5, CD expression, granulocyte O_2^- production

Introduction

Vanadium (V) is an element of environmental interest because of its wide industrial applications and large release into the environment [1]. V is used in the production of alloys (including those for aircraft construction, space technology, and the atomic energy industry), in the manufacture of glass, pigments, paints, and printing inks. V is accumulated in some mineral oils, possibly being involved in the early photosynthetic processes. Therefore, oil-fired power plants are a source of V exposure for the population [2]. High levels of occupational V exposure (inducing lung diseases) occur in workers during cleaning and maintenance operations of boilers of these plants [3].

V exerts effects on biological systems with a narrow range between the essential and toxic levels [4]. It is present in many oxidation states exerting effects on enzymatic activities (eg, V strongly inhibits Na+, K+ ATPase and Ca++ ATPase) [4].
Pentavalent vanadate is more metabolically active than tetravalent vanadyl in exerting mutagenic effects [5], causing an insulin-like action [6], and affecting cardiovascular regulatory mechanisms [6,7]. In particular, V is present almost completely as vanadyl in the tissues of rats chronically exposed to vanadate [7,8].

Depression of phagocytic activity of peripheral blood granulocytes was found in V-exposed rats [9,10]. Addition of V to murine thymocyte cultures inhibited the mitogenic response induced by concavalin A [11]. Mouse macrophage-like WEHI-3 cells treated with ammonium metavanadate or vanadium pentoxide had reduced capacity for synthesis and release of cytokines such as TNF-α [12]. Cohen et al [13], utilizing WEHI-3 cells, also showed that ammonium metavanadate or vanadium pentoxide altered INF-γ production and its inducible responses.

A study was performed over a period of 3 years on Czech children exposed to V emitted from a plant producing V₂O₅ by a hydrometallurgical processing of V-rich slag [2]. V-exposed children showed a higher incidence of viral and bacterial infections, as well as changes of several blood immune parameters, including lymphocyte proliferation.

The present study is part of a project aimed at evaluating the immune effects of metals. In this paper we report our results on vanadium. The in vitro effects of low (10⁻⁷ M) and high (10⁻⁴ M) concentrations of the metal on human peripheral blood mononuclear cells (PBMC) were determined, evaluating its influence on lymphocyte proliferation and expression of CDs, cytokine release by PBMCs, and O₂⁻ production by granulocytes. Future studies will evaluate the influence of vanadon on cytokine production and expression, and the uptake, transmembrane transport, and intracellular distribution of V in the immune cells.

Materials and Methods

Isolation of human peripheral blood mononuclear cells. Eight healthy men with mean age of 34 yr (range 24-58 yr) were recruited for the study. They were unaffected by any disease and did not take any medications. Venous blood samples were obtained from each subject in fasting condition at 8 a.m. Peripheral blood mononuclear cells (PBMC) were purified from EDTA-treated whole blood by density gradient centrifugation (20 min, 400 x G) in Ficoll-Hypaque (BioSpa, Milan). After 3 washings, the PBMC were resuspended in RPMI-1640 medium, containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 25 mM HEPES buffer, 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma Chemical Co.). All experiments were performed in triplicate.

Cell proliferation. PBMC were suspended at 10⁶ cells/ml in complete medium. Aliquots (100 µl) of cell suspension were placed in the 96 wells of a microtiter plate (Falcon, St Louis, MO). The cells were incubated at 37°C in humidified atmosphere with 5% CO₂ for 78 hr at the following conditions: (a) without phytohemagglutin (PHA) (control samples); (b) with 20 mg/ml PHA (control samples); (c) containing 10⁻⁴ or 10⁻⁷ M sodium metavanadate without PHA; (d) containing 10⁻⁴ or 10⁻⁷ M sodium metavanadate with PHA.

The sodium metavanadate was added from 10⁻³ and 10⁻⁷ M stock solutions that had previously been prepared. None of the reagents contained endotoxin, based on Limulus amebocyte assays (detection limit, 0.1 µg/ml).

Immunoreactivity to quantify cell proliferation. Cell proliferation was evaluated using the BrdU cell proliferation assay (Oncogene Research Products, Darmstadt, Germany). During the final 24 hr of culture, BrdU was added to wells of the microplate. Cells were fixed and permeabilized and the DNA was denaturated by treatment for 30 min at room temperature with fixative/denaturing solution. Anti-BrdU monoclonal antibody was pipetted into the wells and incubated for 1 hr. Unbound antibody was washed away and horse-radish peroxidase-conjugated goat anti-mouse antibody was added for 30 min at room temperature. Contents of wells were removed by inverting over sink and tapping on paper towels. Chromogenic substrate (tetramethylbenzidine (TMB) solution) was added to each well.
After incubation in the dark at room temperature for 15 min, “stop solution” was added to each well in the same order as the previously added substrate solution. All reagents were provided with the kit and used following the manufacturer’s instructions. Absorbance of each well was measured with a spectrophotometric plate reader at dual wavelengths (450 nm, 540 nm). Color intensity was proportional to the amount of BrdU incorporated in the cells, and thus to the degree of cell proliferation.

**Cultures to assay cytokines and surface marker expression.** Cultures were set up in 1 ml/well 24-well Costar plastic plates, using 0.8 ml of PBMC containing 1 million cells/well in complete medium, with the following conditions:
(a) control wells replenished with 0.2 ml of complete medium without phytohemagglutinin (PHA);
(b) control wells replenished with 0.2 ml of phytohemagglutinin (PHA) at a final concentration of 10 µg/ml;
(c) wells replenished with medium containing 10^{-4} and 10^{-7} M sodium metavanadate without PHA;
(d) wells replenished with 10^{-4} and 10^{-7} M sodium metavanadate stimulated with PHA.
Cultures were incubated at 37°C in humidified atmosphere with 5% CO₂ for 48 hr; cells were then checked for vitality by trypan blue dye exclusion, examined with an inverted Leica microscope.

**Determination of cytokines.** Supernatants were collected and stored at -70°C in aliquots until analysis. Interferon-γ (IFN-γ), interleukin-5 (IL-5), and TNF-α levels in culture supernatants were determined by Quantikine ELISA kits (RRD Systems, Minneapolis, MN), according to the manufacturer’s instructions.

**Immunofluorescence staining of cells.** All the procedures were carried out after washing the cells that had been cultured for 48 hr and stimulated with PHA in presence of 10^{-4} and 10^{-7} M sodium metavanadate and resuspending them in PBS; each culture was divided in two aliquots, dispensed in 75 x 12 mm tubes (PBI, Milan), and incubated with the following mix of conjugated monoclonal antibodies (mAbs) to CD4-PE, CD3-FITC, CD8-PC5 and CD56-PE (all purchased from Beckman-Coulter). Briefly, 10 µl of conjugated mAbs were incubated with 100 µl of resuspended stimulated cultured cells at room temperature for 20 min, then washed once in PBS and resuspended. After addition of 500 µl of fixing reagent (4% formaldeyde) and further incubation for 10 min, 500 µl of fresh PBS was added.

The cells were immediately analyzed by flow cytometry using an EPICS XL instrument (Beckman-Coulter) equipped with software for triple fluorescence analysis. Data were recorded for side-scatter, forward-scatter, three fluorescentities, and mean fluorescence intensity (MFI). The data were normalized for acquisition time, number of cells, scatter, and MFI. Comparisons were made to the PHA cultures that did not receive any addition of sodium metavanadate.

**Phagocyte function test.** The “Fago-Test” (FAR S.r.l., Verona, Italy) was performed according to Rossi et al [14] and the manufacturer’s recommendations. Whole blood was incubated with zymosan and cytochrome c. In this assay, the zymosan is opsonized by plasma components and, during opsonization, the blood phagocytes produce O₂ that reduces cytochrome c. Phagocyte function is evaluated by colorimetric measurement of reduced cytochrome c, and is expressed as O₂⁻ nM /10⁷ granulocytes.

**Statistics.** Statistical analyses were performed with Statistica software (Release 4.5). Kolmogorov-Smirnov tests showed that results for most immune parameters conformed best to a normal distribution when they were expressed as percentages, in relation to the corresponding controls.

**Results**

**Cell proliferation.** Cell proliferation assays without NaVO₃ addition (baseline profile) showed a mean adsorbance of 121 (SD±54) units, which increased to 1345±315 units in PHA-stimulated cultures. Addition of 10^{-4} or 10^{-7} M NaVO₃ did not modify PBMC proliferation in PHA-unstimulated cells. Non-significant changes were found in PHA-
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Table 1. Proliferation of human PBMCs incubated with or without PHA and also with or without NaVO3. Absorbance data (mean ± SD) are expressed as units and as ratios of the basal values, with/without NaVO3.

<table>
<thead>
<tr>
<th>NaVO3 conc.</th>
<th>Without PHA</th>
<th>With PHA</th>
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<tbody>
<tr>
<td></td>
<td>Absorb.</td>
<td>Ratio (%)</td>
</tr>
<tr>
<td>none</td>
<td>121 ± 54</td>
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<tr>
<td>10^-4 M</td>
<td>152 ± 72</td>
<td>123 ± 49</td>
</tr>
<tr>
<td>10^-7 M</td>
<td>165 ± 88</td>
<td>125 ± 28</td>
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* p <0.01 vs the PHA-stimulated cells without NaVO3

stimulated PBMCs in the presence of 10^-7 M NaVO3 (Table 1). On the other hand, 10^-4 M NaVO3 reduced the lymphocyte proliferation in PHA stimulated cultures, averaging 67 ± 25% of the basal values (without NaVO3).

The blastogenesis stimulation index (SI) (ie, the ratio of lymphocyte proliferation stimulated and unstimulated by PHA) of cultures containing 10^-4 M NaVO3 was significantly (p <0.01) reduced in comparison to cultures without NaVO3 (Fig. 1). A modest reduction was also observed in cultures containing 10^-7 M NaVO3, but was not statistically significant.

Cytokine production. Table 2 shows the baseline levels of IFN-γ, TNF-α, and IL-5 in supernatants of PHA-stimulated and unstimulated PBMC cultures, in the absence of NaVO3. Low levels (in some cases undetectable) of IL-5 were observed in supernatants of PHA-unstimulated cultures. Significant increases of all cytokine levels were found in the supernatants of PHA-stimulated cultures.

Addition of 10^-4 M NaVO3 to PHA-stimulated cultures caused a reduction of IFN-γ release in supernatants to 1082 ±121 pg/ml, which averaged 76 ±9% compared to the baseline values without NaVO3 (p <0.01) (Fig. 2). No significant changes were found with the addition of 10^-7 M NaVO3.

Addition of 10^-4 M NaVO3 induced little change in the production of TNF-α by PBMC, while 10^-7 M NaVO3 significantly enhanced the production of this cytokine to 2083 ±104 pg/ml, which averaged 118±7 % compared to the baseline values without NaVO3 (p <0.05) (Fig. 3).

Finally, 10^-4 M sodium metavanadate significantly inhibited the production of IL-5 by PBMC, with an average of 77 ± 9% of the basal values (p <0.01), whereas 10^-7 M sodium metavanadate significantly stimulated its production, averaging 143 ±21% of the basal values (p <0.01) (Fig. 4).

Surface marker expression. Neither 10^-4 nor 10^-7 M NaVO3 modified the expression of CD3+, CD4+, CD8+, or CD56+ in PHA-stimulated and unstimulated PBMCs (data not shown).
Granulocyte production of $O_2^-$. The production of $O_2^-$ by human peripheral blood granulocytes in the absence of NaVO$_3$ averaged $44 \pm 12$ nmol/10$^7$ cells (basal profile). Addition of 10$^{-4}$ M NaVO$_3$ inhibited $O_2^-$ production by granulocytes ($p < 0.01$). The inhibition caused by 10$^{-7}$ M NaVO$_3$ was less significant ($p > 0.05$) (Fig. 5).

Discussion

This study demonstrates that 10$^{-4}$ M vanadate exerts inhibitory effects on the proliferation of human PBMC, on their release of cytokines, and on their phagocytic activity. These findings agree with previous in vitro studies, which showed that V inhibits the mitogenic response to concanavalin A in murine thymocyte cultures [15] and (as vanadium pentoxide) reduced the mitotic index of cultured lymphocytes [11]. Mouse myelocytic macrophages treated with ammonium metavanadate or ammonium pentoxide prior to stimulation with lipopolysaccharide endotoxin (LPS) showed reduced capacities for synthesis and release of cytokines [12]. A previous study also showed that V exposure may produce changes in immune functions, in part by modifying cell interactions with INF-$\gamma$ and associated INF-$\gamma$ inducible responses [13].

In this study, 10$^{-7}$ M sodium metavanadate stimulated the production of IL-5 and TNF-$\alpha$ and slightly stimulated PBMC proliferation. In V-exposed Czech children, the stimulation index (SI) of lymphocyte mitotic activity increased in response to PHA, concanavalin A and pokeweed mitogen [14]. In the area of V exposure, children had higher incidence of viral and bacterial infections, indicating that the increased lymphocyte proliferation did not correlate with an improvement of immune defenses. On the other hand, our data show that sodium metavanadate reduces the release of IFN-$\gamma$ (a marker of Th-1 type immune response), and $O_2^-$ production by granulocytes. These inhibitions may favor a susceptibility to infections.
Moreover, 10^{-4} M vanadate inhibits the release of IL-5 by PBMC, while the 10^{-7} M concentration stimulates the production of IL-5 (a marker of Th-2 response) and TNF-\alpha, but not of IFN-\gamma. This suggests that exposure to low levels of vanadate may modulate the balance of type 1 vs type 2 cytokine production by immune cells, inducing a shift of the cytokine response from type 1 to type 2. This agrees with results in brown Norway rats sensitized to house dust mite and exposed to residual oil fly ash (ROFA) containing V [16]. In these rats, ROFA increased the immediate immune response (including bronchoconstriction) to antigen challenge. A similar pattern of immune responses was also induced in experimental animals and humans by exposures to lead [17-20].

The reduced production of O_2^- by human blood granulocytes exposed to NaVO_3 agrees in part with the results of previous studies on V effects on the leukocyte system. Depressed phagocytic activity of peripheral blood granulocytes was observed in V-exposed rats [9,10]. Eosinophilia was observed in workers occupationally exposed to V compounds [24]. The mechanism of action of V on phagocytosis is difficult to explain; it was suggested that V causes a decrease of L-ascorbic acid, which stimulates both phagocytosis and chemotaxis [10].

From all of these observations, V appears to be an important environmental pollutant, which by interacting with other toxic agents may modify the immune response of exposed populations, increasing the incidence of allergic and immunologic diseases.

Fig. 4. Effects of sodium metavanadate on IL-5 release by PHA-stimulated PBMCs. The primary Y axis shows IL-5 concentrations (pg/ml, mean ± SE); the secondary Y axis shows the ratio of IL-5 release with/without vanadate (%); * p <0.01 vs controls (Mann-Whitney U-test).

Fig. 5. Effects of sodium metavanadate on O_2^- production by granulocytes. The primary Y axis shows O_2^- levels (nmol/10^7 cells, mean ± SD); the secondary Y axis shows the ratio of O_2^- production with/without vanadate (%); * p <0.01, ** p <0.01 vs controls (Mann-Whitney U-test).
Acknowledgement

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References