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Chemico-Biological Interactions 156 (2005) 55–68
Chemico-Biological Interactions
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Characteristics of in vivo murine erythropoietic response to sodium orthovanadate

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Received 8 April 2005; received in revised form 8 July 2005; accepted 8 July 2005
Available online 30 August 2005

Abstract

Current knowledge about the effects of vanadium compounds on erythropoiesis is still reduced and even contradictory.

The aim of this work was to evaluate the in vivo effects of a single dose of sodium orthovanadate (OV, 33 mg/kg i.p.) on CF-1 mice in a time course study (0–8 days).

Murine erythropoiesis was assessed through a combinatory of experimental approaches. Classical peripheral and bone marrow (BM) hematological parameters were determined. Erythroid maturation in blood stream and hemopoietic tissues (^{59}Fe uptake assays), BM erythroid progenitor frequency (clonogenic assays) and erythroid crucial protein expressions for commitment and survival: GATA-1, erythropoietin receptor (Epo-R) and Bcl-x_L (immunoblottings) were evaluated.

Neither BM cellularities nor BM viabilities changed noticeably during the study. Peripheral reticulocytes showed a biphasic increment on days 2 and 8 post-OV. hematocrits enhanced transiently between days 2 and 4. ^{59}Fe uptake percentages enhanced in peripheral blood nearly two-fold over control values between 4 and 8 days ($p < 0.01$) without changes in BM and spleen. Additionally, mature erythroid BM compartments: polychromatophilic erythroblasts and orthochromatic normoblasts increased by the eighth day. BFU-E colonies remained near basal values during the whole experience, whilst CFU-E colonies raised 60% over control at 8 days post-OV ($p < 0.05$). GATA-1 and Epo-R were significantly over-expressed from the third until the end of the experimental protocol ($p < 0.01$). Surprisingly, Bcl-x_L showed a constitutive expression pattern without changes during the experience.

Experimental data let us suggest that OV does not to cause bone marrow cytotoxicity and that it accelerates maturation of BM committed erythroid precursors. Moreover, there are significant correlations among erythroid-related protein expressions: GATA-1 and Epo-R and the frequency of CFU-E. In addition, Bcl-x_L expression invariance during the time course study would indicate that the stimulatory effect of OV treatment on erythropoiesis was mainly exerted on the maturation of red cell precursors rather than on the antiapoptosis of erythroid terminal progenitors.

Keywords: Sodium orthovanadate; Erythropoiesis; GATA-1; Epo-R; Antiapoptosis

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doi:10.1016/j.cbi.2005.07.003

1. Introduction

Initially, vanadium has been reported as a toxic [1–3], genotoxic [4] and potential carcinogenic element, as it was classified by IARC. Afterwards, it was eventually ratified as an essential oligoelement involved in maintaining normal biological functions [5]. The biological effects of this transition element are variable, upon several factors including the dose and the oxidative state of the test drug. Thus, there are controversial viewpoints, from toxicity to essentiality, about vanadium and its related compounds.

Furthermore, many studies were carried out to investigate its potential pharmacological applications; the best known possibility is as a glucose-lowering agent [6–9]. Currently, vanadium salts are used as body mass builder in athletes [10]. It was also found that vanadium reduces tumor sizes and tumor incidences in various carcinogenic models [11–13].

At molecular level, it is well known that salts of vanadium interfere with enzymes such as different ATPases [14], protein kinases [15], phosphatases [16], ribonucleases [17] and ornithine decarboxylase [18]. Its ability to increase the expression or activation of proto-oncogenes and their products [19] justify its role as tumor promoter [20], co-carcinogen and mitogen [21]. Moreover, vanadium influences the regulation of several genes in normal and immortal cell lines, such as the genes codifying for pro-inflammatory proteins (e.g. TNF-α, IL-8, MIP-2) [22–24], nuclear transcription factors (e.g. NF-KB, AP-1), kinases (e.g. MAPK, $p70^{56k}$), proto-oncogen products (ras, c-raf-1) [25] and apoptotic related proteins (p53) [26]. However, to date, there has been no detailed study on how vanadium affects erythropoiesis in mammals.

Erythropoiesis is the complex process of red blood cell production through the promotion of the survival, proliferation and differentiation of the erythroid progenitors in the bone marrow. The balance between erythroid cell division and maturation requires coordinate expression of lineage-specific transcription factors, structural proteins, and growth factor receptors which confer the unique physiology of the erythroid cell [27,28]. Among them, GATA-1 and the erythropoietin receptor (EPO-R) play crucial roles in erythropoiesis. While GATA-1 help to establish and maintain the erythroid phenotype by activating erythroid-expressed genes [29], the gene encoding the EPO-R contains functional GATA-1 binding sites, suggesting that the gene is a downstream target of GATA-1 [30]. GATA-1 could also modulate, either directly or indirectly, the expression of EPO-R during terminal stages of erythroid differentiation [31]. Both, EPO and GATA-1, also induce the expression of EPO-R and Bcl-X_L, an antiapoptotic protein of *bcl-2* family, crucial for the survival of erythroid cells [32,33].

Focusing the attention on vanadium effects on erythropoiesis, available information is contradictory. It has been reported that circulating erythrocytes and hemoglobin concentration decreased in rats which consumed vanadium orally [34]. Conversely, studies in rats under comparable experimental conditions failed to show discernable hematological variations [35]. Hogan [36] reported the induction of a transient lowering of circulating red blood cells and the further stimulation of erythropoiesis in mice after a single i.p. administration of three vanadium compounds of different valance states. On the other hand, orally administered vanadyl sulphate did not affect blood viscosity, hematological indices (RBC, WBC, platelet counts, erythrocyte mean cell volume and hemoglobin level) in weight training athletes [37].

The aim of the present study was to elucidate the effect of a single dose (33 mg/kg i.p.) of sodium orthovanadate (OV) on the erythropoiesis using an "in vivo" murine model in a time-course protocol (0–8 days post-administration) through several experimental approaches.

Murine erythropoiesis was assessed through a combinatory of experimental methods, from the classical hematological determinations to assays for evaluating: the maturation of the red cells (^{59}Fe uptake), the frequency and nature of erythroid progenitors in bone marrow (clonogenic cultures) to the commitment, differentiation and antiapoptosis of erythroid lineage (GATA-1, EPO-R and Bcl-x_L expressions by Western blotting).

Here, we report for first time that OV alters murine erythropoiesis in a transient way by inducing a deeply acceleration of bone marrow erythroblasts maturation, without cytotoxicity of hematological precursors, at least in this experimental condition. Additionally, OV affects the expression of GATA-1 and Epo-R, erythroid-related proteins that regulate the commitment and maturation of red blood cells. The invariability of Bcl-x_L expression during the study,

suggests that OV affects erythropoiesis by accelerating red blood cell maturation rather than by exerting anti-apoptosis on erythroid precursors.

2. Material and methods

2.1. Animals

Adult female CF-1 Swiss mice (average weight: 26–28 g, $n = 6$–10 mice/lot) were used. They were allowed to free access to pelleted food and water. We used two sets of murine lots for whole experience. Experiments were conducted according to the principles outlined in the Guide for the Care and Use of Laboratory Animals of the National Northeast University.

2.2. Drugs

Sodium orthovanadate (Sigma®) was dissolved in triple distilled sterile water. The total dose, 33 mg/kg, was adjusted to pH 7.4 and injected i.p. Control mice were injected i.p. with saline solution (NaCl 0.9%). Samples were taken at 0, 1, 2, 3, 4, 5, 6, 7 and 8 days post-OV.

Recombinant human erythropoietin (Epo) – Hemax 2000, Biosidus, Argentina – was used for clonogenic assays.

2.3. Hematological peripheral parameters

At scheduled days post-OV injection, samples of peripheral blood were obtained. Blood was withdrawn by cardiac puncture under anesthesia (pentobarbital 100 mg/kg i.p.). Circulating erythrocytes (RBC $\times 10^6/\mu$l) were determined using a Multisizer 3 Coulter Counter (Beckman).

Reticulocyte counts were determined using Brilliant Blue Cresyl (1%) staining and hematocrits were determined by standard methods. Results are expressed as the RBC $\times 10^6/\mu$l and percentages of reticulocytes and hematocrits. Three different assays were done in triplicates. All results are expressed as the mean ± S.E.M.

2.4. Hematological bone marrow profiles

Mice were sacrificed under anesthesia, as described before, by cervical dislocation. Bone marrows from right femora were expelled by flushing with Minimum Essential Medium (MEM, Alpha Modification. Sigma Co., USA). Differential cell determinations were performed counting 500–1000 cells in May Gründwald Giemsa (MGG) stained bone marrow smears. Three independent assays were done in triplicates at each day of the experimental protocol. Cells were then classified as erythroid, myeloid and lymphoid. Results are expressed as percentage ± S.E.M. for each lineage.

2.5. Determination of mitotic bone marrow indexes

Mitotic indexes were determined by light microscopy counting 500–1000 cells in MGG stained bone marrow (BM) smears from three different assays in triplicates. Dividing cells were recognized following standard criteria. Mitotic indexes were obtained by dividing the number of mitosis to 1000 cells counted in random fields (400×). Results are expressed as mean percentage of mitosis ± S.E.M. at each day of the experimental protocol.

2.6. Determination of apoptotic bone marrow indexes

Apoptotic indexes were determined by light microscopy in MGG stained bone marrow smears from three different assays in triplicates [38] by dividing the number of recognizable apoptotic cells to 1000 cells counted randomly (400×). Apoptotic cells were identified morphologically. Typical apoptosis findings consist of nuclear condensation, vacuolation, fragmented nuclei, blebbing of plasma membranes and cell shrinkage [39]. Results are expressed as mean apoptotic percentage ± S.E.M. at each day of the experimental protocol.

2.7. Determination of bone marrow cellularity and viability

Both parameters were determined in cell suspensions from three independent assays in triplicates at each day of the study.

Femora were flushed with 500 μl of MEM and the cells were counted using a hemocytometer. BM cellularities are expressed as total nucleated cells $\times 10^6$/femur (mean ± S.E.M.). BM cellular

viability was determined by the Trypan Blue (0.2%) exclusion assay. Results are expressed as mean percentage of viable cells ± S.E.M.

2.8. ^{59}Fe uptake assay

Radioisotopic iron was obtained as a sterile ferrous citrate solution (0.15 mCi/ml) from Du Pont® NEN Products, Boston, USA. Control and OV-treated mice were weighted and i.p. injected with 0.5 μCi diluted in 0.2 ml of saline solution. After 24 h, samples of peripheral blood, BM and spleens were obtained. Blood samples were withdrawn by cardiac puncture. Afterwards, mice were euthanized by cervical dislocation. Femora and spleens were excised for further measurement of radioactivity.

We measured the radioactivity in 0.5 ml of each blood sample.

The percentage of ^{59}Fe incorporation to the circulating RBC was calculated as:

$$\%\,^{59}\text{Fe in circulating RBC} = \frac{\text{total blood volume} \times \text{sample (cpm)}}{\text{injected cpm} \times 0.5} \times 100$$

The value for total blood volume for normocytemic mouse was considered as 5 ml/100 g body weight [40]. The percentages of radio iron incorporation into spleens and femoral bone marrows were determined by the method described by Bozzini et al. [41]. Background cpm was discounted in all sample measurements. Experimental data are expressed as mean percentage of ^{59}Fe uptake ± S.E.M. of the initial dose injected.

2.9. Clonogenic assay

In vitro semisolid cultures of hematopoietic progenitor cells from OV-treated mice were performed as described previously [42]. Briefly, 2×10^5 BM cells/ml were cultured in 24-well dishes containing 0.5 ml of Iscove's modified Dulbecco's medium (IMDM, Sigma, St. Louis, MO, USA) and 1% methylcellulose, 20% fetal bovine serum (FBS), 1% deionized bovine serum albumin (BSA, Sigma Co., USA), 0.1 mM 2-mercaptoethanol, 300 μg/ml glutamine, 150 μg/ml transferrin, penicillin and streptomycin. Three different assays were performed in quadruplicate with 1 U/ml Epo. Cell cultures were incubated for 2 and 7 days at 37 °C in a humidified atmosphere with 5% CO_2. Colonies were counted under an Olympus IMT-2 (Olympus, Japan) inverted microscope after 48 h of incubation and they were classified as late erythroid colony forming units (CFU-E). Colonies containing more than 40 cells were counted at 7 days of incubation and they were classified as burst-forming units-erythroid (BFU-E). Results are expressed as colonies per femur (mean ± S.E.M.).

2.10. Immunoblotting

Briefly, BM single cell suspensions were obtained at each day of the protocol and treated with RIPA buffer (50 mM Tris, 150 mM NaCl, 2.5 mg/ml deoxycholic acid, 1 mM EGTA, 10 μg/ml Nonidet-40, pH 7.4, supplemented with protease inhibitors: 2.5 μg/ml leupeptin, 0.95 μg/ml aprotinin and 2.5 mM phenylmethylsulfonyl fluoride).

Proteins from whole cell lysates (40 μg) were loaded per well onto 12% SDS-PAGE. Proteins in gels were either stained with Coomassie Blue R-250 or transferred to nitrocellulose membranes to perform Western blots. Five percent low fat milk in TBS-T buffer (50 mM tris(hydroxymethyl)aminomethane, 175 mM NaCl, adjusted to pH 7.5 with HCl and supplemented with 0.1% Tween 20) was used as blocking solution. Primary Antibodies: GATA-1 rat monoclonal, Epo-R rabbit polyclonal, and Bcl-x_L goat polyclonal were used. All antibodies were diluted 1/500 in blocking solution, except tubulin rabbit polyclonal antibody, which was diluted 1/1000 in the same solution. (Primary antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.) Conditions of incubation were optimized for each antibody. Secondary antibodies: IgG goat anti-rabbit and IgG goat anti-rat, horseradish peroxidase (HRP) labeled (Jackson Immunoresearch Inc.) were diluted 1/2000 in blocking solution. Incubation was performed for 1 h at room temperature. Bound HRP was detected by Opti4CN kit (Bio-Rad). Four different Western blots were performed.

Results were expressed as arbitrary units (AU) considering the ratio between the protein band optical density under study to corresponding tubulin band optical density. As bone marrow whole lysates contain proteins from a heterogeneous cell population, AU values

of GATA-1, Epo-R, and Bcl-x_L were related to absolute erythroid cell numbers present in samples at each particular day, as described before [42].

2.11. Image and statistical analysis

Data were statistically analyzed using Instat and Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA). Studies with $p < 0.05$ values were considered statistically significant. Graphics were obtained using a Graph Pad Prism software and Adobe Photoshop 8.0. Correlation analysis between erythroid-related proteins and erythroid colonies were performed using the Spearman rank correlation test. Optical densities of immunoblot images were obtained and further analyzed using Scion Image 3.0 software.

3. Results

3.1. Peripheral hematological parameters and bone marrow profiles

In order to evaluate whether this single dose of OV affects murine erythropoiesis, we designed a time course study (0–8 days) after a single i.p. injection of 33 mg/kg of this salt. The dose used in this study was selected according to the Hogan's report [36].

We determined RBC/μl, hematocrits and percentages of circulating reticulocytes in blood stream. RBC showed a biphasic pattern with a significant decrease on day 1 (about 33.3% below control, $p < 0.01$) followed by an enhancement on day 4 post-OV (1.5-fold over control, $p < 0.01$). Red blood cell counts returned to control values by the end of the experience ($7.5\ 10^6$/μl).

Data are represented in Fig. 1A. These changes were also reflected in hematocrits (see Fig. 1B). They showed a significant increment between 2 ($43.3 \pm 0.75\%$) and 4 days post-OV ($44.4 \pm 0.78\%$, $p < 0.01$) compared to control values ($39.0 \pm 0.26\%$).

Peripheral reticulocyte percentages also showed a biphasic pattern. We noticed two-fold of enhancement on days 2 and 8 post-OV ($p < 0.05$), as it can be seen in Fig. 2A. Meanwhile, BM reticulocyte percentages only increased about 10% over basal values on the fourth day post salt administration ($p < 0.01$), as shown in Fig. 2B. In spite of BM reticulocyte percentages returned to normality, blood stream reticulocytes remained elevated until the end of the experience.

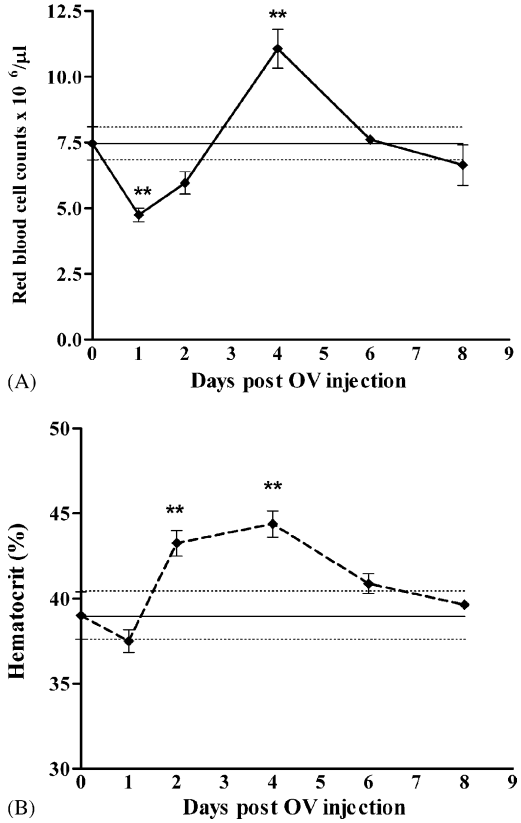

Fig. 1. Changes in red blood cell counts and hematocrits after OV injection: (A) shows variations of erythrocyte counts during the time course study. Each point represents the mean RBC counts $\times 10^6$/μl from three different assays in triplicates. The decrease of RBC counts observed on the first day was followed by a compensatory enhancement of this hematological parameter on the fourth day post-OV; (B) illustrates the changes of hematocrits during the time course study. Each point represents the mean percentage from three different assays in triplicates. A transitory increment of the hematocrits was noticed between days 2 and 4 post-OV. The horizontal lines show the control values ± S.E.M. All other values are the mean ± S.E.M. $^{**}p < 0.01$ indicates significant differences between control and OV-treated animals.

3.2. Mitosis in bone marrow

On the first day post-OV, mitotic percentages enhanced about 3.6 times over control values from $2.5 \pm 0.65\%$ to $9.5 \pm 0.6\%$ ($p < 0.01$). This increment

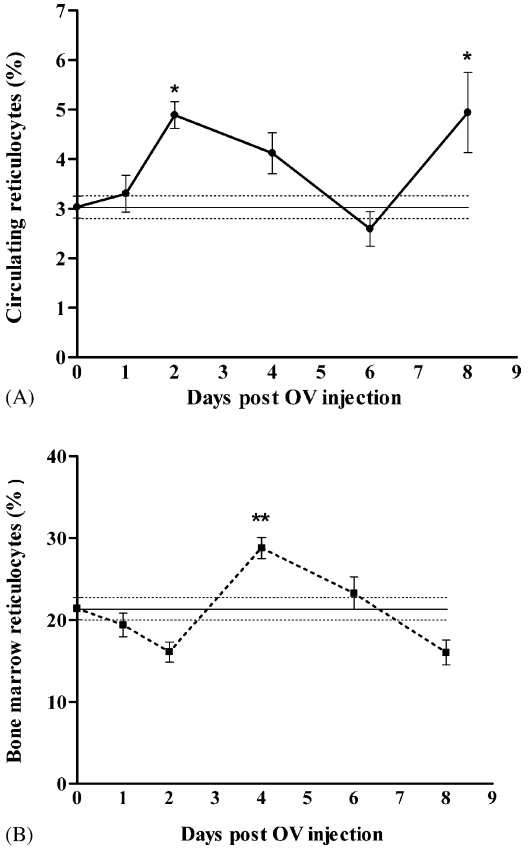

Fig. 2. Changes in peripheral and bone marrow reticulocytes after OV injection: (A) illustrates variations of peripheral reticulocyte counts during the time course study. Each point represents the mean reticulocyte percentage from three different assays in triplicates. A biphasic pattern of blood stream reticulocytes was noticed. Two waves of significant increment in this hematological parameter were observed on days 2 and 8 post-salt injection; (B) shows variations of bone marrow reticulocyte counts during the time course study. Each point represents the mean BM reticulocyte percentage from three different assays in triplicates. An increment of this parameter was noticed on the fourth day post-OV. The horizontal lines show the control values ± S.E.M. $^{**}p < 0.01$ indicates significant differences between control and OV-treated animals.

remained on the second day and decreased progressively until the end of the experience.

3.3. Apoptosis in bone marrow

Experimental data from apoptosis were almost comparable with the previous reported in mitotic variations. The maximum apoptotic percentage was noticed at day 2 post-OV ($11.8 \pm 1.11\%$, $p < 0.01$) over control mean value ($2.0 \pm 0.86\%$).

It may be little tempting here to speculate that the changes in mitotic and apoptotic percentages, produced by the effect of OV might be restricted to certain stages of erythroid maturation, responsible for erythroid triggering.

These observations allow us to presume that OV somehow alters erythropoiesis in a transient way. In order to examine the nature of this influence on red blood cell production, we combined additional experimental approaches.

3.4. Bone marrow cellularity and viability

Neither BM cellularities nor BM viabilities changed noticeably during the time course study. They were near basal values: $(15.5 \pm 3.78) \times 10^6$ total BM cells/femur and $91.9 \pm 1.24\%$ of BM viable cells, respectively. These findings suggest that OV did not cause cytotoxicity in BM cells in the present experimental conditions.

3.5. Bone marrow hematological profiles

After classifying BM cells in different hematological lineages according to standard criteria, myeloid and lymphoid compartments were not altered by the treatment. However, erythroid lineage showed significant enhancement by the end of the experience (Table 1).

As shown in Fig. 3, OV injection caused a significantly increment of the polychromatophilic erythroblast percentages on days 2 and 8 post-injection ($p < 0.01$), while orthochromatic normoblast percentages enhanced on day 8 ($p < 0.01$). Pronormoblast and basophilic erythroblast percentages failed to show significant variations throughout the study. Data show maximal contribution of the mature erythroid compartments associated to the biphasic pattern of reticulocytes percentages in blood stream.

These observations allow us to suggest that OV promotes differentiation of bone marrow mature erythroid precursors in a time dependent way.

3.6. Fe ^{59}uptake assay

In attempt to confirm whether OV promotes acceleration of erythroid maturation or not, we performed

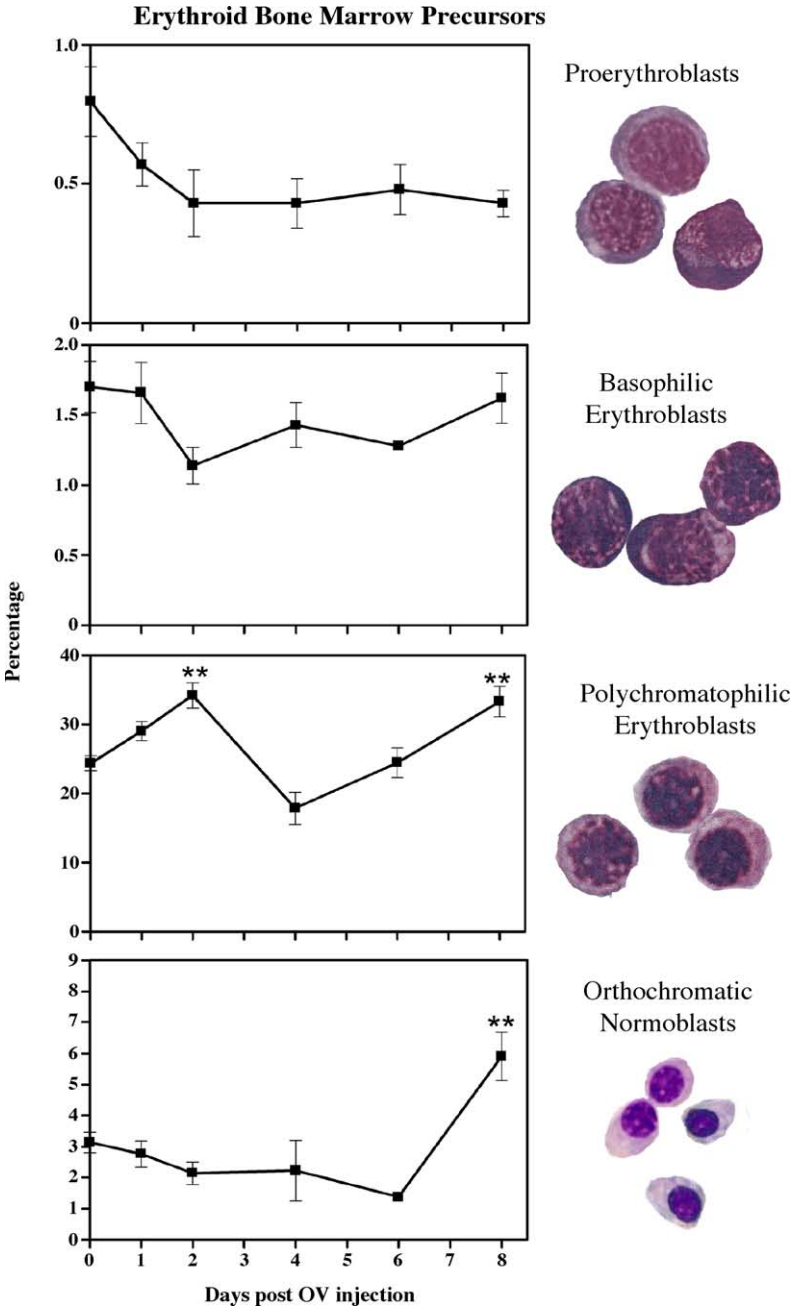

Fig. 3. Changes in erythroid bone marrow precursors after OV injection. Erythroid precursors were morphologically identified following standard criteria at each day of the experimental protocol counting 500–1000 cells in May Gründwald Giemsa (MGG) stained bone marrow smears done in triplicates. Cells were classified as proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts and orthochromatic normoblasts. Data were obtained from three different assays. Results are represented as mean percentage ± S.E.M. of each BM red cell compartment. Representative images of each kind of erythroid BM precursors are shown beside each graph (400×). OV caused a significantly increment of the polychromatophilic erythroblast percentages on days 2 and 8 post-injection while orthochromatic normoblast percentages enhanced on day 8. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences between control and OV-treated animals.

Table 1
Changes in erythroid, myeloid and lymphoid bone marrow compartments after OV injection

	Days post-OV								
	0	1	2	3	4	5	6	7	8
Erythroid lineage (%)	30.0 ± 1.68	34.0 ± 2.11	37.9 ± 2.44	25.7 ± 3.14	22.0 ± 3.54	26.8 ± 3.91	27.6 ± 2.35	26.9 ± 2.87	41.3 ± 3.18*
Myeloid lineage (%)	37.2 ± 5.90	39.1 ± 3.56	33.3 ± 2.55	41.1 ± 3.15	47.1 ± 5.60	43.7 ± 3.50	39.7 ± 3.25	38.3 ± 3.70	39.9 ± 2.46
Lymphoid lineage (%)	32.8 ± 3.70	26.9 ± 3.06	28.7 ± 2.48	33.2 ± 4.13	31.0 ± 3.55	29.5 ± 5.44	32.7 ± 1.50	34.8 ± 2.90	18.8 ± 7.90

Differential cell determinations were performed at each day of the experimental protocol counting 500–1000 cells in May Gründwald Giemsa (MGG) stained bone marrow smears done in triplicates. Data were obtained from three different assays. Cells were classified morphologically as erythroid, myeloid and lymphoid following standard criteria. Results are expressed as mean percentage ± S.E.M. for each lineage.
* $p < 0.05$ indicates significant differences between control and OV-injected lots.

the assay of ^{59}Fe uptake. Isotopic incorporation in BM and spleen remained invariable during the time course study (data not shown). However, experimental data in circulating RBC showed a remarkably increment of the radio iron incorporation percentages. This enhancement was nearly two-fold over control values between 4 and 8 days post-OV ($p < 0.01$). Fig. 4 illustrates these observations.

Changes in isotopic incorporation are in agreement with the increment of BM contribution of the majority of mature erythroid compartments: polychromatophilic erythroblasts – between 2 and 8 days – and orthochromatic normoblasts—on the eighth day post-OV.

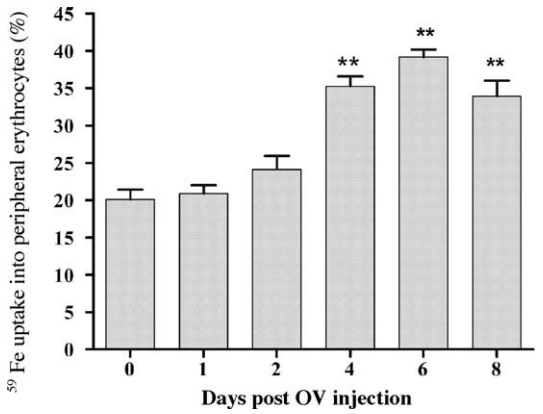

Fig. 4. ^{59}Fe uptake in peripheral red blood cells after OV injection. Percentages of radio iron incorporation in peripheral blood were obtained at each day of the experimental protocol as described in Section 2. Bars represent the mean percentage of ^{59}Fe uptake ± S.E.M. of the initial dose injected. An increment of isotopic incorporation was observed between 4 and 8 days post-OV injection. ** $p < 0.01$ indicates significant differences between control and OV-treated animals.

3.7. Clonogenic assays

Semisolid methylcellulose cultures were used to assess the frequency of BM hemopoietic progenitors throughout this time course study after OV administration.

In order to evaluate putative differences in the occurrence of the monolineage populations committed with erythropoietic pathway, immature erythroid progenitors (BFU-E) and late erythroid colony forming units (CFU-E) were identified. As shown in Fig. 5, the number of BFU-E colonies remained near basal values (529.6 ± 45.9 colonies/femur) during the whole experience, whilst the number of CFU-E colonies raised 60% over control at 8 days post-OV (1294.3 ± 88.9 versus 814.2 ± 73.4 colonies/femur, $p < 0.05$).

These results indicate that erythroid colony forming units have different responsiveness to the assayed salt (Fig. 5).

Moreover, the second wave of reticulocytosis, the highest ^{59}Fe incorporation in peripheral red blood cells and the maximum contribution of mature erythroid precursors in bone marrow are coincident at day 8 post-OV. This agreement in timing from several experimental facts, strengthens the hypothesis of the acceleration of red blood cell production through an acceleration of the maturation rate of late erythroid populations. Taken together, experimental results encourage us to

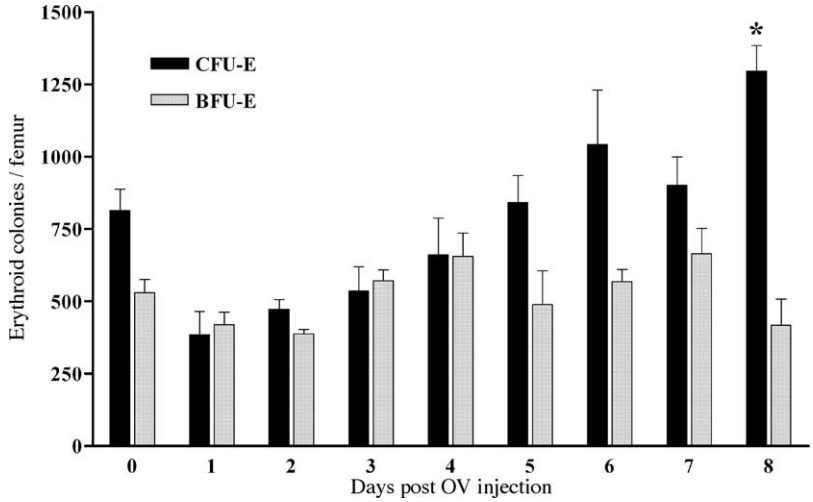

Fig. 5. Erythroid bone marrow colonies after OV injection. Single murine bone marrow cell suspensions (2×10^5 cells/ml) obtained at each time of the experimental protocol following OV injection were cultured as described in Section 2. CFU-E and BFU-E colonies were counted at 2 and 7 days of incubation, respectively. Bars represent the number of colonies per femur (mean ± S.E.M.) from three different assays in quadruplicates. BFU-E colonies frequency did not change during the time course study. CFU-E colonies enhanced significantly by the end of the experience (day 8 post-OV). $^{*}p < 0.05$ indicates significant differences between control and OV-treated animals.

suppose that OV would also cause deeply changes in critical red cell-related protein expressions in bone marrow.

3.8. *GATA-1, Epo-R and Bcl-x_L expressions*

A well-coordinated cohort of transcription factors regulates the formation, survival, proliferation and differentiation of multipotent progenitors into the erythroid lineage [28].

We noticed that GATA-1 was over-expressed significantly from the third until the last day of the experimental protocol. This GATA-1 enhancement was coincident with the over expression of EPO-R ($p < 0.01$). Surprisingly, Bcl-x_L showed a constitutive expression pattern without changes during the experience. This observation implies that OV does not alter the erythroid cell surviving program (Fig. 6).

These results show that rearrangements of critical protein expression patterns are involved in the commitment and expansion of red cell progenitors to accelerate erythropoiesis following OV administration.

Therefore, we might suggest a novel relationship operating in vivo between the GATA-1 transcription factor, Epo-R and Bcl-x_L, triggered by OV administration.

4. Discussion

Erythropoiesis is a multi-step process involving commitment, proliferation and differentiation of hematopoietic progenitors to mature, terminally differentiating red blood cells [43]. The transformation of the erythrocyte from a stationary, extravascular nucleated cell to a non nucleated one specialized in oxygen transport, involves marked changes in the structure, composition and metabolic repertoire required for this purpose.

Erythropoietin is the major hormone regulating the proliferation of erythroid precursors and their differentiation into erythrocytes.

In the past century, it has been reported, that vanadium complexes were used in medicine for the treatment of anemia [1]. Current information about the effects of vanadium compounds on erythropoiesis is still reduced and even contradictory [22–25].

The present study focused attention on the effects of a single dose of sodium orthovanadate (OV) on murine erythropoiesis in a time course study throughout several experimental approaches.

From the experimental data resulting from BM assays, two initial observations are worth noting: first, OV treatment did not cause cytotoxicity of

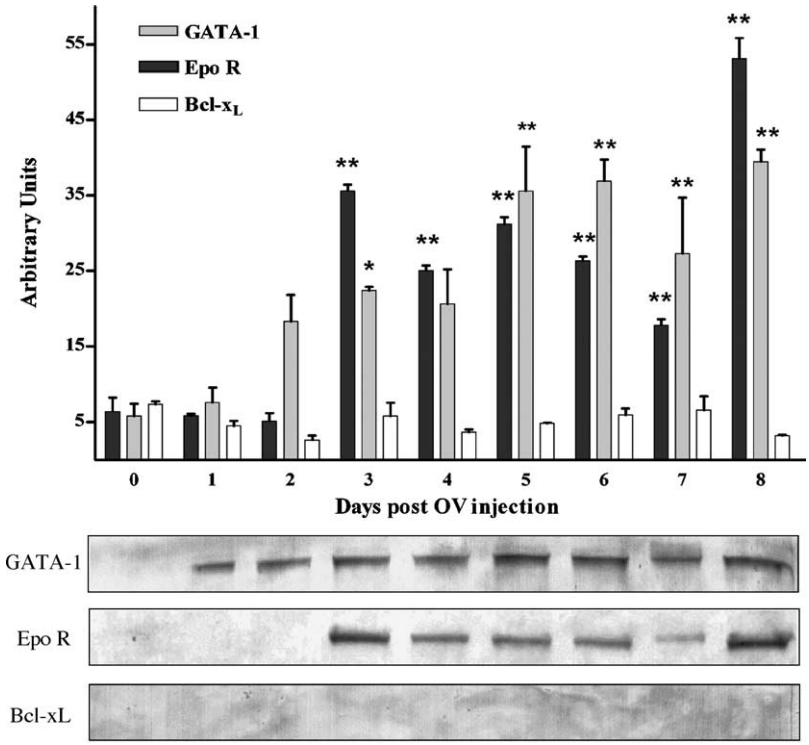

Fig. 6. Effect of OV on GATA-1, Epo-R and Bcl-x_L bone marrow expressions. Scale bars represent Arbitrary Units (mean ± S.E.M.) of GATA-1, Epo-R and Bcl-x_L expressions from four independent experiments performed at each time of the experimental protocol. Murine bone marrow cells following OV injection were isolated at each day of the experimental protocol. Proteins from whole bone marrow lysates (40 μg/lane) were loaded for Western blottings. Representative blots are shown. GATA-1 expression was noticed from the first day post-OV. However, GATA-1 and Epo-R over-expressions were significantly coincident from the third day until the end of the assay. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences between control and OV-treated animals.

hematological precursors, at least in this experimental condition. Second, OV affects erythropoiesis by inducing a great acceleration of bone marrow erythroblasts maturation.

The absence of toxicity is supported by the maintenance of BM cellularity and viability during the scheduled protocol. Moreover, mitotic and apoptotic indexes are almost coincident between the first and second day post-OV. These facts might be explained by the molecular cross-talk between cell proliferation and apoptosis involved in tisular homeostasis [44].

In agreement with Hogan [36], there appear to be no dramatic and persistent effects on peripheral blood parameters after a single i.p. OV treatment.

We found a biphasic pattern of peripheral reticulocytosis on the second and eighth days post-injection. The slight initial reticulocyte increment was followed by the hematocrit enhancement between days 2 and 4 post-OV injection. It seems probably that, the erythropoietic program continues during the time course study by accelerating the maturative stages from another pool of red cell precursors, as BM reticulocyte changes suggest. As recently reported by Kuory et al., BM nascent reticulocytes are not in developmentally synchronized populations [45]; so the first contribution of peripheral reticulocytes might be due to the release of the spare pool of cells ready to reach blood stream. Moreover, it has been reported that after a stressing stimulus, the reticulocytes released might differ in size, life span, ^{59}Fe uptake and globin genes expression, among several other features [46,47].

Additional experimental data from ^{59}Fe uptake assays along the experimental schedule reinforce these preliminary conclusions. Radioisotopic incorporation in newly hemoglobin synthesizing cells was noticed to increase in peripheral blood between 4 and 8 days

post-OV, similarly to Hogan's results. However, radio iron incorporation remained without appreciable changes in BM and spleen. Hence, erythropoietic acceleration was adequately covered by BM progenitors without splenic contribution in this transient stimulation.

The analysis from BM erythroid precursors percentages are also according with these observations since that, the erythroid compartments affected were the most mature BM red precursor cells (polychromatophilic erythroblasts and orthochromatic normoblasts) when the second wave of reticulocytosis occurred at the end of the experience.

Besides, the acquisition of the red blood cell phenotype involves the coordinate expression of regulatory and structural proteins acting in concert to direct the development of progenitor cells into mature erythrocytes. The timing of each transcriptional factor's, as well as Epo-R expressions during hematopoiesis, the cell lineages affected and the existing combination of other factors determine the erythroid cell fate. In general, as erythroid differentiation proceeds, specific genes are expressed at certain stages during red cell maturation in a coordinated and selective process rather than a random one [48,49]. Current models of hematopoiesis propose that GATA-1 helps to establish and maintain the erythroid phenotype by activating erythroid genes [29].

Studies from transgenic mouse revealed that GATA-1 is highly expressed in CFU-E but low in BFU-E, suggesting that the incremental expression of GATA-1 is required for the formation of erythroid progenitors [50]. Moreover, GATA-1 likewise Epo, induces the Epo-R and Bcl-x_L expressions, critical for erythroid cells survival [29,33]. Cell fractionation followed by Western blot analysis showed that the over-expression of GATA-1 was previous and coincident in a significant way, with Epo-R expression from the third day until the end of the experience. Additionally, results from clonogenic assays are according to these observations, since immature erythroid progenitors (BFU-E) remained almost constant while late bone marrow erythroid progenitors (CFU-E) enhanced by the end of the experience, timing concomitant with GATA-1 and Epo-R over-expressions. Correlations among GATA-1, Epo-R and CFU-E experimental data were significant (GATA-1 versus CFU-E: $r = 0.80$, $p = 0.0138$, GATA-1 versus Epo-R: $r = 0.78$, $p = 0.0172$) supporting our previous interpretations.

Additionally, it is well known that vanadium was classically used as a probe because of its unparalleled ability to selectively inhibit protein tyrosine phosphatases at submicromolar concentrations. It has been reported that pervanadate maintained Tyr phosphorylation of Epo-Receptor [51] and that Epo depending HCD 57 cells were increased in Epo sensitivity by low concentrations of vanadate, suggesting that the level of receptor phosphorylation was directly related to signal transduction [52]. Nevertheless, OV might affect erythropoiesis by triggering another pathway of signal transduction [53]. As suggested by Hulley and Davidson it seems that OV elicits a whole response in which vanadate-sensitive phosphatases act as a "gateway" to cellular response [54]. It has been reported that vanadium salts activate phosphatidylinositol-3 kinase activity, the same kinase that had been found to play a crucial role in erythropoiesis [25,55]. Moreover, OV might also influence normal murine erythropoiesis through insulin like effects [56,57] and/or by an indirect mechanism involving triiodothyronine action [58–60]. Taken together these reports, an intricate network of regulations could be involved in vanadium effects on erythropoiesis.

In order to evaluate whether an antiapoptotic mechanism has also been triggered by the OV administration, the expression of Bcl-$x_{L,}$ was examined. The antiapoptotic effect of Bcl-x_L on primitive and mature red blood cells is well known [33,61]. It has been reported that high concentrations of orthovanadate in combination with Epo triggered apoptosis in HCD 57 cells [62]. Surprisingly, we have not observed changes on Bcl-x_L expression at different times of the experimental protocol. Discrepancies of experimental data might be due to differences in sodium orthovanadate concentrations, the nature of the target cells and/or the molecular interactions operating in vitro and in vivo models. Therefore, we have detected for the first time a novel relationship between crucial proteins that govern erythroid fate: GATA-1, Epo-R and Bcl-$x_{L.}$

There are several determinants which regulate murine hemopoietic cell amplification (i.e. cycling status of stem cells and precursors cells, the transit time of dividing cell compartments, apoptotic levels and migration of primitive and progenitor cells between BM and spleen). They often occur simultaneously and

it is almost impossible consider them independently [63]. Thus, the whole erythropoietic response after one single OV administration might be caused by the confluence of several kinds of different regulatory processes operating "in vivo" at cellular and extracellular level.

Although the data presented here strongly indicate a direct involvement of OV in the induction of erythropoiesis, its relevance on normal and pathological hematopoiesis must be determined.

In conclusion, present experimental data let us suggest that OV does not cause cytotoxicity in bone marrow and that it accelerates maturation of committed erythroid precursors toward mature red blood cells production. Moreover, there are significant correlations among specific erythroid-related protein expressions: GATA-1 and Epo-R and the frequency of CFU-E throughout the experience. The amplification of terminal erythroid precursors were also reflected in ^{59}Fe peripheral red blood cells uptake. In addition, Bcl-x_L expression invariance during the time course study, would indicate that the stimulatory effect of OV treatment on erythropoiesis was mainly exerted on the maturative stages of red cell precursors rather than on the antiapoptosis of erythroid terminal progenitors.

Acknowledgments

We thank Dr. J.-M. Trifaró for the provision of the antibodies used in immunoblottings, Dr. C. Bozzini for providing us with radioiron and to Mr. O. Sanabria for the artwork of this article. This work was supported by Grants from CONICET – Argentina (PIP 2340) and SeGCyT – UNNE (PI 616).

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