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CYTOTOXICITY-RELATED ALTERATIONS OF SELECTED CELLULAR FUNCTIONS AFTER *IN VITRO* VANADATE EXPOSURE*

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Abstract—A bovine kidney cell line was used to monitor select cellular functions for toxicity-dependent alterations in an effort to examine the cellular response to vanadium insult. The vanadium concentrations utilized ranged between 20 and 500 μM Na_3VO_4 (V) and elicited 15–60% cytotoxicity. Cytotoxicity-related decreases in thymidine incorporation into DNA and leucine incorporation into protein were noted. Paradoxically, V-treated cultures exhibited increased protein and DNA content, suggestive of a decrease in precursor transport. K^+-dependent phosphatase (KP), acid phosphatase (AP) and succinate dehydrogenase (SDH) were monitored in surviving cells and in a cell-free system. Significant inhibitions were detected for KP and AP; SDH exhibited slight enhancement. In the cell-free system, KP was inhibited significantly at 10^{-7} M V, while AP and SDH were either unchanged or sensitive only at concentrations of 10^{-4} M V or greater. Measurement of reduced glutathione (GSH) in surviving cells revealed toxicity-dependent increases of up to 500% of control values. When compared to the cellular V content, the GSH:V molar ratio decreased from 1.7 to 0.5 as cell survival decreased.

Vanadium is a ubiquitous element detectable in air, water and soil [1]. It is both a physiologically and pharmacologically active substance [2]. Recently, the essentiality of vanadium as a trace element has been suggested for microorganisms, plants and some animal species [3]; a functional role, however, has not been identified. In contradistinction to the proposed essentiality of the element are the toxic properties ascribed to vanadium for humans following occupational and experimental exposures [4–6]. The toxicity of several vanadium compounds in animals, which varied depending on route of administration, species and the nature of the compound used, has also been reviewed [5].

Although a considerable amount of information has been reported detailing the cellular and biochemical responses to vanadium [5, 7–9], much less is known about the importance of these effects to the development of vanadium-induced cytotoxicity. In preliminary studies, however, we have determined that vanadate cytotoxicity in MDBK cultures is the result of a complex, multifaceted mechanism where a 5-fold increase in cytotoxicity (15–75% cell death) results in a concomitant 50-fold increase in cellular vanadium content. It was thought that this may have resulted from a cellular balance between the suggested regulatory role of vanadate [7, 10, 11] and the toxic interactions resulting from excessive cellular vanadium. To more fully understand the nature of this toxicity, it was the objective of this study to examine the biochemical function of cultures expressing a graded cytotoxic response. Such an examination would allow for the identification of biochemical lesions which may serve a significant role in the cytotoxic expression.

MATERIALS AND METHODS

Chemicals. The following chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO): dichlorophenolindolphenol, phenazine methosulfate, *p*-nitrophenylphosphate, *o*-phthaldehyde, reduced glutathione, 3-*o*-methylfluorescein, 3-*o*-methylfluorescein phosphate and 3,5-diaminobenzoic acid. The radiolabeled compounds L-[4,5-^{3}H(N)]leucine (50 μCi/mmole) and [*methyl*-^{3}H]thymidine (2.0 Ci/mmole) were obtained from the New England Nuclear Corp. (Boston, MA). Additionally, sodium ortho-vanadate was purchased from the Fisher Scientific Co. (Pittsburg, PA).

Culture conditions. Madin Darby bovine kidney cells (MDBK) were cultured in a 3% CO_2 in air atmosphere in Eagle's minimum essential medium (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (Hyclone Products, Logan, UT). MDBK cultures originated from the American Type Culture Collection (Rockville, MD).

Twenty-four hours prior to treatment, stock cultures were subcultured and seeded in 100 mm plastic culture dishes at a density of 0.17×10^6 cells/ml, for a total cell content of 1×10^6 cells/dish. Just prior to treatment, the media in monolayer cultures were removed and fresh media added. Stock vanadate solutions were prepared in sterile phosphate-buffered saline (PBS, pH 7.2), and aliquots of 0.1 ml or less were added to appropriate cultures. For all

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treatment groups, 24-hr incubations were used. Cell numbers were quantitated from nuclei counts [12].

^{48}V uptake studies. For the uptake studies, a ^{48}V solution ($^{48}VOCl_3$, Amersham, Arlington Heights, IL) was made by adding $^{48}VOCl_3$ to a stock Na_3VO_4 solution brought up to volume in sterile PBS (pH 7.3). The oxychlorides are converted to vanadates at this pH [13]. The final specific activity was adjusted so that approximately 0.2 μCi was added per sample dish. Three to five dishes of cells were used for each concentration of the chemical per experiment. At the appropriate time, test media were removed, and the dishes were washed three times with PBS containing 10 mM ethylenediaminetetraacetic acid (EDTA). After washing, 0.1 M citric acid was used to osmotically lyse the cells. The dishes were then scraped and the cells were collected for ^{48}V counting in a Packard gamma spectrometer (model 3320). Counting efficiency for ^{48}V was determined to be 58.5%. Parallel cultures were used for cell number determinations.

Incorporation of radiolabeled leucine and thymidine. For determinations of the amount of [^{3}H]leucine incorporated into cellular protein, test cultures were pulsed with 1.0 μCi of the radiolabeled leucine 4 hr prior to terminating the incubation. At the end of the test period, the media were removed and the cultures were washed with PBS + EDTA. Two milliliters of 0.1 M citric acid was then added for 15 min for cell lysis. The dishes were then scraped and the suspension was pipeted into 3 ml of 0.4 N perchloric acid (PCA) and centrifuged at 1000 *g* for 10 min. The supernatant fraction was then discarded, and the pellet was resuspended with 2 ml of 0.4 N PCA and centrifuged at 1000 *g* for 10 min. The second supernatant fraction was also discarded, and the pellet was resuspended with 2 ml of 95% ethanol and then centrifuged at 1000 *g* for 10 min, after which the supernatant fraction was discarded and the pellet saved. To the pellet, 1 ml of 1.0 N NaOH was added, and the suspension was heated at 80° for 30 min in a mineral oil bath to solubilize the protein. After solubilization the NaOH was neutralized with 1 ml of 1.0 N HCl. Aliquots were then taken for protein determinations [14] and for liquid scintillation counting (Packard 2600 scintillation counter; toluene base, 2a70 scintillation fluid, RPI, Mount Prospect, IL). For thymidine incorporation studies, test cultures had 2.0 μCi [^{3}H]thymidine added 5 hr prior to terminating the incubation. At the end of the test period, DNA was extracted according to the method outlined by Patterson [12]. Following the selective extraction procedures, the DNA was resuspended with 1 ml of 0.5 N PCA and hydrolyzed at 90° for 15 min in a mineral oil bath. Duplicate aliquots were then taken for liquid scintillation counting, as above. Quantitation of DNA was achieved with the fluorometric 3,5-diaminobenzoic acid method outlined in Setaro and Morley [15] and Johnson-Wint and Hollis [16].

Biochemical assays. For the analysis of succinate dehydrogenase (SDH), acid phosphatase (AP), K^+-dependent phosphatase (KP) and reduced glutathione (GSH), cells were prepared as follows. Test cultures were rinsed three times with PBS + EDTA (10 mM). PBS without EDTA was then added, and the cells were scraped and pooled. Suspended cells were pelleted at 1000 *g* for 5 min, after which all procedures were performed at 0–4°, unless indicated otherwise. To the pellet, 1 ml citric acid (0.1 M) was added for cell lysis. After 15 min PBS was added to provide 3 mg protein/ml. Aliquots of the cell suspension were then taken as needed for the specific assay; in all cases the specific analyses were completed within 1 hr after final dilution.

For biochemical assays in a cell-free system, the above preparation was used with untreated control cells and vanadium was added directly into the assay medium.

The reactivity of vanadium with GSH was measured by incubating 33 μM GSH with 0.1 to 100 μM Na_3VO_4 for 15 min at room temperature, in a PBS-buffered system (pH 7.3). The quantity of reduced GSH was then measured as indicated below.

Enzyme activities were assayed either spectrophotometrically, on a Perkin–Elmer Coleman 124 double beam spectrophotometer, or fluorometrically with an Aminco–Bowman spectrofluorometer; specific methods follow. SDH activity was determined by the phenazine methosulfate-2,6-dichlorophenolindolphenol method outlined by King [17]. For KP the production of 3-*o*-methylfluorescein was monitored fluorometrically [18]. The *p*-nitrophenylphosphate method from Bergmeyer [19] was used to monitor AP, while GSH was determined fluorometrically [20]. Protein was determined by the method of Lowry *et al.* [14].

Statistical analysis. The statistical analysis for the variation of treated groups from controls was by a two-tailed *t*-test with a preset significance level of $P < 0.05$.

RESULTS

In conjunction with the decreased cell numbers recovered following vanadate treatment, a graded and progressive decrease occurred in the incorporation of radiolabeled thymidine and leucine into the cell DNA and protein respectively (Figs. 1 and 2). For both leucine and thymidine, the level of incorporation was normalized to recovered cell numbers, as well as to the cell protein and DNA respectively. Leucine incorporation exhibited a greater sensitivity to vanadate treatment than did the thymidine incorporation. At all levels of cytotoxicity (based on cell survival), the leucine incorporation was inhibited to a greater extent than the corresponding thymidine incorporation into DNA.

For thymidine incorporation (Fig. 1), normalization of the data to the recovered cell numbers was a less sensitive indicator of the nuclear effect than was the normalization to DNA content. A significant decrease in incorporation was only detected in cultures treated with vanadate concentrations of 100 μM or greater. Thus, a 50% decrease in recovered cells was necessary before significant inhibition ($P < 0.05$) was detected. This was in contrast to the actual DNA content in the recovered cells which showed a progressive increase following all levels of vanadate treatment (Table 1).

When leucine incorporation was normalized to cell protein content, a distinctly different profile from

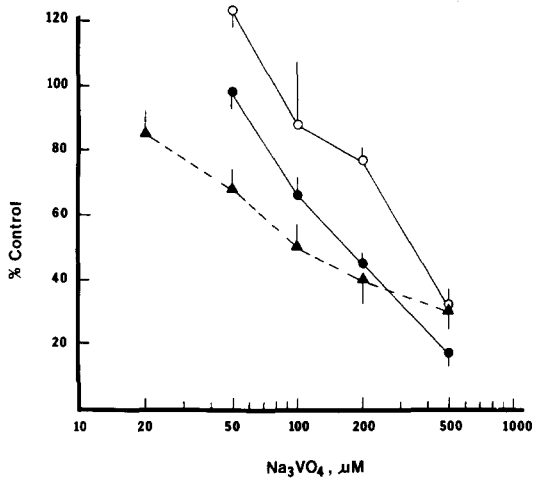

Fig. 1. Comparison of [^{3}H]thymidine incorporation into cellular DNA with cell survival following 24-hr Na_3VO_4 incubation. Thymidine incorporation was normalized to both DNA content (●) and cell number (○); cell survival is indicated by the closed triangle (▲). Each point represents the mean ± S.D. for three determinations. Control incorporation values were: 24,026 ± 749 dpm/μg DNA and 482,765 ± 9,284 dpm/10^6 cells. The cell number in control cultures was 2×10^6 cells/dish.

that reported for the thymidine studies was observed. When normalized in this manner, leucine incorporation exhibited a sensitivity to vanadate treatment that was directly comparable to the decreased cell survival following 20 and 50 μM Na_3VO_4 treatment (Fig. 2). At vanadate concentrations greater than 50 μM, however, the percent decrease in leucine incorporation was much greater than the decreased

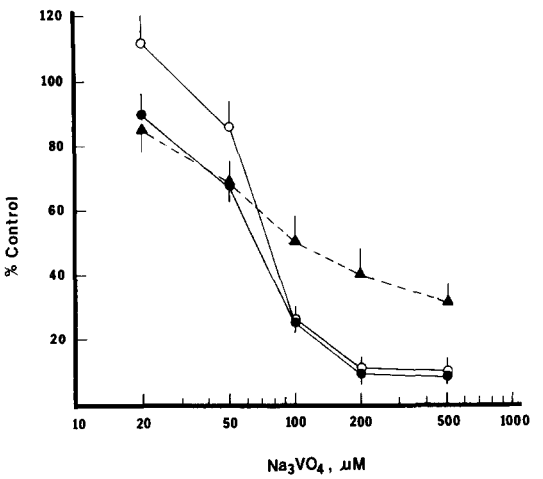

Fig. 2. Comparison of [^{3}H]leucine incorporation into cellular protein with cell survival following 24-hr Na_3VO_4 incubation. Leucine recovery in the acid-insoluble precipitate was normalized to both protein content (●) and cell number (○); cell survival is indicated by the closed triangle (▲). Each point represents the mean ± S.D. for three determinations. Control incorporation values were: 26,054 ± 1,097 dpm/mg protein and 9,440 ± 627 dpm/10^6 cells.

Table 1. Cellular protein and DNA content following a 24-hr incubation of MDBK cultures with Na_3VO_4*

Na_3VO_4 (μM)	Protein (mg/10^6 cells)	DNA (μg/10^6 cells)
0	0.32	20.2
20	0.40 (125)†	ND‡
50	0.41 (128)	25.2 (125)
100	0.42 (131)	30.8 (153)
200	0.39 (122)	34.2 (169)
500	0.40 (125)	38.1 (189)

* Values are expressed as the mean of two determinations.

† Values in parentheses represent the percentage of control protein or DNA content for the specific treatment group.

‡ Not determined.

cell survival. Furthermore, when normalized to recovered cell number, the leucine incorporation was identical to the level observed when incorporation was normalized to the protein content of the recovered cells. Interestingly, the decreased leucine incorporation was in contrast to the protein content of the recovered cells, which was found to be increased in treated cultures (Table 1).

Pursuing further the influence vanadate treatment had on cell function, the activities of several cellular enzymes were measured (Table 2). K^+-dependent phosphatase (KP) and acid phosphatase (AP) were most sensitive to the 24-hr vanadate treatment, while succinate dehydrogenase (SDH) activity was enhanced slightly. Although the AP and KP activities were decreased at all levels of cytotoxicity studied, the inhibition plateaued between the 200 and 500 μM Na_3VO_4 treatments. This corresponded with the relatively small change in cell survival observed between these vanadate concentrations (Figs. 1 and 2).

To assess whether the enzyme effects were the result of direct vanadate intervention, untreated MDBK cells were lysed and then resuspended, after

Table 2. Effect of Na_3VO_4 on the activity of various cellular enzymes following a 24-hr incubation period

	% of Control cultures*		
Na_3VO_4 (μM)	Succinate dehydrogenase	Acid phosphatase	K^+-dependent phosphatase
100	76 ± 13.2	61 ± 3.8†	71 ± 16.6†
200	115 ± 12.5	49 ± 5.6†	19 ± 12.0†
500	141 ± 9.4†	42 ± 4.9†	21 ± 13.0†

* Values are expressed as mean ± S.D. for at least three determinations. Control activities were: succinate dehydrogenase, 9.2 ± 3.4 μmoles succinate oxidized/mg protein/min; acid phosphatase, 8.3 ± 3.5 μmoles phosphate liberated/mg protein/min; and K^+-dependent phosphatase, 38.4 ± 5.3 nmoles phosphate liberated/mg protein/hr.

† Statistically significant difference from control activity ($P < 0.05$).

Table 3. Effect of Na_3VO_4 on various cellular enzymes in a cell-free system

Na_3VO_4 (μM)	% of Control*		
	Succinate dehydrogenase	Acid phosphatase	K^+-dependent phosphatase
0.1	ND†	ND	67 ± 9.6†
1.0	103 ± 9.4	100 ± 3.3	71 ± 7.4‡
10	103 ± 9.4	97 ± 2.8	41 ± 3.8‡
100	109 ± 23.5	80 ± 5.3‡	48 ± 5.3‡
1000	79 ± 14.6‡	57 ± 8.7‡	ND

* Values are expressed as mean ± S.D. for at least three determinations. Control activities were: succinate dehydrogenase, 26.8 ± 1.7 μmoles succinate oxidized/mg protein/min; acid phosphatase, 3.0 ± 0.6 μmoles phosphate liberated/mg protein/min; and K^+-dependent phosphatase, 94 ± 14.8 nmoles phosphate liberated/mg protein/hr.

† Not determined.

‡ Statistically significant difference from control activity ($P < 0.05$).

Table 4. Cellular GSH levels following a 24-hr incubation of MDBK cultures with Na_3VO_4

Na_3VO_4 (μM)	GSH* (μg/mg protein)	% Control
0	0.94 ± 0.22	100
50	1.51 ± 0.23†	161
100	1.39 ± 0.21†	148
200	1.83 ± 0.38†	195
500	4.62 ± 1.50†	493

* Values are expressed as mean ± S.D. for three to five determinations.

† Statistically significant difference from control ($P < 0.05$).

which enzyme activities were monitored in the presence of various vanadate concentrations (Table 3). The KP activity again was found to be the most sensitive to vanadate, with significant inhibition (33%) detected with concentrations as low as 0.1 μM Na_3VO_4; however, the enzyme remained at 50% of the control value even in the presence of 100 μM vanadate. Although AP activity was depressed significantly, concentrations greater than 100 μM Na_3VO_4 were required for inhibition. The SDH activity was essentially insensitive to vanadate.

Since cellular GSH has been proposed as a redox regulating molecule for endogenous vanadium [21], it was of interest to determine the cellular GSH status following vanadium exposure. In the cell-free system vanadate had no effect on GSH concentration (data not shown). In contrast, in cells recovered following 24-hr vanadate incubation cellular GSH content was found to be systematically enhanced over that of the control cultures (Table 4). When compared to the cellular vanadium content, it was noted that the GSH-to-vanadium molar ratio decreased from 1.7 to 0.52 as cell survival decreased (Table 5).

DISCUSSION

In contrast to the many investigations which have examined the cellular and enzymatic effects of vanadium following short-term incubation [7, 9, 11, 22], the present study was designed to monitor the biochemical viability of cells surviving a cytotoxic vanadate exposure. It was felt that such an examination would allow for the identification of biochemical lesions which may have served a significant role in the cytotoxic expression.

As indicated in Results, leucine incorporation into cellular protein was decreased significantly in cells surviving vanadate treatment. Since the decreased synthesis of the macromolecules was in contrast to the increased cell protein content, the possibility that the depressed incorporation resulted from a decreased intracellular transport of the radioactive percursor could not be ruled out. With the demonstrated vanadate sensitivity of enzyme-mediated protein degradation [23], however, decreased protein turnover may have been responsible for the enhanced protein content. Moreover, if it were assumed that inhibition of protein synthesis occurred subsequent to an inhibition of protein degradation, then the increase in cellular protein would have occurred simultaneously with the decreased protein synthesis, thus the appearance of a transport-related effect. To further understand the nature and relevance of these effects to the cytotoxicity, monitoring of amino acid transport as well as protein synthesis and degradation over the time course of the exposure would be necessary.

It should also be noted that protein synthesis was more sensitive to vanadate exposure than was DNA synthesis. In contrast to the influence of vanadate on DNA synthesis in MDBK cells is the mitogenic activity of non-toxic vanadate concentrations observed in a human fibroblast culture [24]. Although stimulated DNA synthesis was not evi-

Table 5. Comparison of cellular vanadium and GSH content in MDBK cells following a 24-hr exposure to various concentrations of Na_3VO_4*

Na_3VO_4 (μM)	V (nmoles/mg protein)	GSH (nmoles/mg protein)	GSH/V
50	3.0 ± 1.0	5.0 ± 0.76	1.7
100	4.7 ± 1.2	4.5 ± 0.68	0.96
200	11.0 ± 1.1	6.0 ± 1.3	0.55
500	29.0 ± 4.0	15.0 ± 4.9	0.52

* Values are expressed as mean ± S.D. for three to five determinations.

denced in the MDBK cultures, a progressive increase in the cellular DNA content was observed after the vanadate treatment. The apparent paradox cannot be readily explained; however, the possibility of either decreased thymidine uptake or decreased cell division of the MDBK cells may provide a partial explanation.

Vanadate has been described as a highly specific modulator of phosphohydrolases in mammalian cells [11] and, as reported here, appeared to directly inhibit the acid phosphatase (AP) and K^+-dependent phosphatase (KP) from MDBK cells in a cell-free system. The sensitivity of KP, which is a component of the Na^+,K^+-dependent ATPase [25] coincided with the reported high affinity inhibition of Na^+,K^+-ATPase by vanadate [10, 26]. Much less sensitive was the AP activity of MDBK cells under the cell-free assay conditions. The sensitivity of AP has been reported previously [8].

In MDBK cells recovered following a 24-hr vanadate incubation, an extensive inhibition of both KP and AP was detected. Enzyme activities were progressively depressed as the cytotoxicity became more severe. It should also be noted that the enzyme inhibition apparently plateaued following the 200 and 500 μM vanadate exposure (corresponding to 60 and 69% toxicity respectively). This contrasted with the cellular vanadium content at these levels of cytotoxicity which was found to be increased 3-fold over the 200–500 μM Na_3VO_4 exposure range; the AP and KP enzyme inhibition remained relatively constant at 50 and 80% respectively. Furthermore, cytotoxicity was only marginally increased, from 60 to 69%. Therefore, above a critical vanadium burden any subsequent increase in cellular vanadium content appeared not to contribute significantly to a further decrease in cell viability.

The influence of GSH on the cellular actions of vanadium has not been well studied. It has been reported, however, that vanadate initiated a rapid depletion of cellular GSH in isolated rat hepatocytes prior to the initiation of extensive membrane peroxidation [27]. A mechanism for the depletion was not suggested but, as reported here, the inability of vanadate to oxidize GSH in a buffered cell-free solution would seem to suggest an indirect mechanism.

In contrast to the rapid depletion of GSH from hepatocytes was the elevated GSH content measured in MDBK cells following the 24-hr vanadate exposure. As has been shown previously *in vivo*, chemical depletion of rat liver GSH can be followed by compensatory enhancement of the cellular GSH concentration [28]. Thus, the elevated GSH content of surviving MDBK cells may have represented a similar compensatory enhancement. Furthermore, if, as proposed by Macara *et al.* [21], GSH mediates the cellular reduction of vanadate ions, then the enhanced GSH levels in MDBK cells may have resulted from the need to produce cellular reducing equivalent.

The importance of the cellular GSH levels to the vanadium cytotoxicity can only be determined after further research. As the GSH-to-vanadium ratio progressively decreased in treated MDBK cultures, however, the cellular vanadium content increased. With the greater sensitivity of cellular enzymes to vanadate, as opposed to vanadyl ions [29, 30], the importance of the oxidation state of the intracellular vanadium to the cytotoxicity becomes apparent. Interestingly, we have found that the degree of MDBK cytotoxicity stabilized at 40% cell survival even when vanadium uptake continued to be enhanced (data to be reported elsewhere). It may be that these apparently contradictory effects were the result of vanadium detoxification via intracellular polymerization of vanadate ions. Although it is known that vanadium will start to polymerize at concentrations greater than 10^{-4} M [13], the feasibility of this happening intracellularly has not been investigated; nonetheless, its proposal underscores the complexity of the vanadium cytotoxicity.

In summary, data have been presented which selectively outline the response of select biochemical functions in cells surviving a cytotoxic vanadate exposure. Though the markers examined did not necessarily bear a direct relation to vanadate-induced biochemical lesions, their toxicity-related alterations provide a starting point from which future studies can be designed to more accurately assess the biochemical mechanism of vanadate cytotoxicity.

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