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"In vitro" effects of different arsenic compounds on PBMC (preliminary study)

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RIASSUNTO. EFFETTI "IN VITRO" DI DIFFERENTI COMPOSTI ARSENICALI SU CELLULE MONONUCLEATE DI SANGUE PERIFERICO (STUDIO PRELIMINARE).

Scopo di questo studio era mettere a confronto gli effetti di composti arsenicali sulla proliferazione di PBMC umane, spontanea e stimolata da PHA e sul loro rilascio di IFN- γ e TNF- α . L'effetto inibitore dei sali arsenicali 10^{-4} M era nel seguente ordine: acido monometil-arsenioso (MMAs^{III}) > sodio arsenito (As(III)) > tetrafenilarsoniocloruro (As(V)) > Sodio arsenato (As(V)) > potassio e sodio esa-fluoro-arsenato (As(V)) > acido dimetil-arsenico (DMAs^V), mentre l'acido monometil-arsenico (MMAs^V) e l'arsenobetaina non esercitavano effetti immunologici. A concentrazione 10^{-7} M, MMAs^{III} stimolava la proliferazione spontanea delle PBMC, mentre l'As(III) e il DMAs^V aumentavano la proliferazione delle PBMC stimolata da PHA. Questo studio dimostra che gli effetti immunologici dei composti arsenicali dipendono dalla speciazione; inoltre, l'immunotossicità dell'arsenico inorganico in parte dipende dalla biosintesi intracellulare di MMAs^{III} da MMAs^V.

Parole chiave: arsenico, composti arsenicali, PBMC, immunotossicità.

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Aim of this investigation was to compare the effects of 10^{-4} M and 10^{-7} M As compounds on spontaneous and PHA stimulated PBMC proliferation and IFN- γ and TNF- α release. The inhibitory effect of the 10^{-4} M As salts was in the following order: momo-methyl-arsinous acic (MMAs^{III}) > sodium arsenite (As(III)) > tetraphenyl arsonium chloride (As(V)) > sodium arsenate (As(V)) > potassium- and sodium-hexa-fluorur arsenate (As(V)) > dimethyl arsine acid (DMAs^V), while monomethyl-arsenic-acid (MMAs^V) and arsenobetaine did not exert immune effects. 10^{-7} M MMAs^{III} stimulated the spontaneous PBMC proliferation, while As(III) and DMAs^V enhanced the PHA stimulated PBMC proliferation. This study shows that the immune effects of As salts depends on speciation; moreover, the immunotoxicity of inorganic arsenic in part depends on the intracellular bio-synthesis of MMAs^{III} from MMAs^V.

Key words: arsenic, arsenic compounds, PBMC, immunotoxicity.

Introduction

Arsenic (As) compounds are an environmental and occupational hazard. Many organisms reduce the toxicity of inorganic As species through gradual methylation and/or processing of the element into organic compounds (1). Marine organisms synthesise complex As salts; arsenobetaine is the main As compound present in fish, mussel and oyster with lower amount of other compounds such arsenocholine, tetramethylarsonium ion, monomethyl arsinic acid (MAAs^V) and dimethylarsonic acid (DMAs^V) (2).

Inorganic As is predominantly transformed in mammals into MMAs^V and DMAs^V. In humans, the toxicity of inorganic As seems to be reduced only through transformation of a As(V) in As(III), methylation of As(III) (mainly in the liver) into MAAs^V and DMAs^V and further excretion (1, 2). However, the capacity for As methylation varies among individuals (3, 4). This was confirmed by the finding that Japanese populations show about 200 genetic variations in six methyltransferase genes (5); moreover, it was reported that the marmoset, a new world animal, is deficient in liver arsenite and MMAs^V transferase activities on the contrary of the Rhesus monkey, an Old World animal (6).

It was recently reported (in "in vitro" studies on rat and human cells) that the toxicity of As compounds and their capacity to methylate As are not correlated suggesting that trivalent methylated As compounds exert cytotoxic effects (1, 2). Moreover, methylarsine and iodomethylarsine, two methylated trivalent arsenicals, were found to exert a potent genotoxic effect on human peripheral blood lymphocytes (7). Not only methylated As compounds, but also dimethylated As compounds containing As(III), were found more cytotoxic and genotoxic than inorganic As compounds (8). It was thus suggested that methylation of As was not a pathway for detoxification but for activation of As with synthesis of intermediary toxic products.

There are no comparative studies on immune effects of different As compounds; object of this research was to compare immune effects of trivalent and pentavalent inorganic and organic As compounds on peripheral blood mononuclear cells (PBMC).

Material and Methods

PBMC were obtained from 9 healthy male volunteers by Ficoll-Hypaque (BioSpa, Milan, Italy) density gradient centrifugation.

PBMC were placed in well standard well microtiter plates (Falcon, Oxnard, CA, USA) for determining PBMC proliferation and IFN- γ and TNF- α release under the following conditions:

- no other reagent added or with 10 or 20 $\mu\text{g/ml}$ phytoemagglutinin (PHA) (control samples)
- without PHA (for determining spontaneous stimulation) or stimulated by 20 $\mu\text{g/ml}$ PHA (for determining PBMC proliferation) or 10 $\mu\text{g/ml}$ PHA (for determining cytokine release) in presence of 10^{-4} M or 10^{-7} M sodium arsenite (As(III)), sodium arsenate (As(V)), tetraphenyl arsonium chloride (As(V)) > sodium arsenate (As(V)) > potassium- and sodium-esa-fluorum arsenate (As(V)), MMAs^V, mono-methyl-arsonous acid (MMAs^{III}), DMAs^V and arsenobetaine (As(V)).

All the As salts were provided by the ECVAM Unit (Ispra, Italy).

The cells were incubated in a humidified atmosphere at 37°C with 5% CO₂ for 72 hr for determining PBMC proliferation and for 32 hours for analyzing IFN- γ and TNF- α release from PBMC. Cells were then checked for viability by trypan blue dye exclusion by inverted Leica microscope analysis.

Enzymatic immunoassay for the quantification of PBMC proliferation

Proliferation was evaluated by using 5'-bromo-2'-deoxyuridine (BrdU) cell proliferation assay (Oncogene Research Products, Darmstadt, Germany). BrdU was added to wells of the microtiter plate during the final 24 hours of culture. Cells were fixed and permeabilized as well as DNA was denaturated by treatment for 30 min at room temperature with a fixative/denaturing solution. Anti-BrdU monoclonal antibody was pipetted into the wells and allowed to incubate for 1 hour. Unbound antibody was washed away and horseradish 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 solution tetramethylbenzidine (TMB) was added to each well and incubated in the dark at room temperature for 15 min. Stop solution was added to each well in the same order of the previously added substrate solution. All reagents were provided with the kit and used following the manufacturer's instructions. Experiments were made in triplicate. The absorbance of the content of each well was measured using a spectrophotometric plate reader at dual wavelengths of 450-540 nm. The colour intensity was proportional to the amount of incorporated BrdU in the cells and this to the degree of cell proliferation.

Measurement of Cytokines

Aliquots of supernatants were collected and stored at -70 °C until analysis. Interferon (IFN)- γ , IL-5 and Tumor-

Necrosis-Factor (TNF)- α levels in culture supernatants were determined by Quantikine colorimetric ELISA kits (Benfer-Scheller, Key-Stone Laboratories, USA) following the manufacturer's instructions. All experiments were performed in triplicate.

Statistical analyses were performed by Statistica, Release 4.5. Kolmogorov-Smirnov test was used to show the data distribution.

Results

Kolmogorov-Smirnov test showed that most of the data did not conform to parametric distributions. The values of spontaneous PBMC proliferation of control cultures were (mean \pm S.D) 169 \pm 25 absorbance units of BrdU incorporated in PBMC, while those of PHA stimulated PBMC proliferation were (mean \pm S.D) 1485 \pm 223. The units (pg/ml) of IFN- γ and TNF- α release from PHA stimulated PBMC of the control cultures were 1194 \pm 232 and 1692 \pm 309, respectively. Data were reported as % change in relation to control cultures since these values conformed better to parametric distributions than those reported as units of PBMC proliferation and cytokine release.

The following observations can be made:

10⁻⁴ M MMAs^{III} (on the contrary of the other As salts) altered the PBMC viability.

All the 10⁻⁴ M and 10⁻⁷ M As salts did not exert effects on spontaneous (without stimulation by PHA) PBMC proliferation with the exception of 10⁻⁷ M MMAs^{III} which stimulated PBMC proliferation (mean \pm S.D. : 118.4 \pm 11.9%; p<0.05 in relation to the controls without MMAs^{III}).

PHA stimulated PBMC proliferation was inhibited more by 10⁻⁴ M tetra-phenyl-arsonium-chloride and sodium arsenite than by 10⁻⁴ M sodium arsenate (table I). The other 10⁻⁴ M As compounds did not significantly affect PBMC proliferation.

PHA stimulated PBMC proliferation was significantly increased only by 10⁻⁷ M sodium arsenite and DMAs^V (table I).

Table I. % of PHA stimulated PBMC proliferation in presence of 10⁻⁴ M and 10⁻⁷ M As compounds in relation to control samples

	10 ⁻⁴ M	10 ⁻⁷ M
Sodium arsenate	61.5 \pm 16.7***	96.9 \pm 9.6
Sodium arsenite	26.5 \pm 8.2***	110.1 \pm 6.6**
MMAs ^{III}	n.d	109.8 \pm 19.7
MMAs ^V	102.7 \pm 5.2	101.3 \pm 6.5
DMAs ^V	91.3 \pm 22.4	128.4 \pm 21.1**
Arsenobetaine	98.2 \pm 13.3	100.7 \pm 6.8
Tetra-phenyl-arsonium-chloride	13.3 \pm 3.1***	107.3 \pm 13.7
Sodium-esa-fluorum-arsenate	93.4 \pm 10.7	100.3 \pm 4.9
Potassium-esa-fluorum-arsenate	104.6 \pm 11.7	100.7 \pm 11.4

Values are means \pm S. D.

Mann Whitney U test. Statistical significance: **p < 0.01; ***p < 0.001.

IFN- γ release from PBMC was inhibited by 10^{-4} M As salts in the following order: Na arsenite > tetra-phenyl-arsonium-chloride > DMAs^V > sodium arsenate > Sodium- and potassium/esa-fluorum-arsenate, while 10^{-4} MMAs^V, and arsenobetaine as well as all the 10^{-7} M As salts did not exert significant effects (table II).

TNF- α release was inhibited more by 10^{-4} M sodium arsenite than tetra-phenyl-arsonium-chloride (table III), while the others 10^{-4} M salts and all the 10^{-7} M As compounds did not significantly modify the cytokine release.

Table II. % of interferon- γ release from PHA stimulated PBMC in presence of 10^{-4} M and 10^{-7} M As compounds in relation to control samples

	10^{-4} M	10^{-7} M
Sodium arsenate	63.4 \pm 19.0**	86.4 \pm 27.4
Sodium arsenite	8.7 \pm 15.5***	93.7 \pm 14.8
MMAs ^{III}	n.d.	89.9 \pm 18.7
MMAs ^V	84.2 \pm 23.4	84.4 \pm 20.7
DMAs ^V	46.1 \pm 24.8**	106.5 \pm 23.4
Arsenobetaine	92.6 \pm 19.8	96.6 \pm 15.7
Tetra-phenyl-arsonium-chloride	36.7 \pm 27.5***	86.2 \pm 21.5
Sodium-esa-fluorum-arsenate	82.6 \pm 19.2*	84.5 \pm 22.7
Potassium-esa-fluorum-arsenate	78.0 \pm 22.6*	84.9 \pm 23.8

Values are means \pm S. D.
Mann Whitney U test. Statistical significance: *p<0.05; **p<0.01; ***p<0.001.

Table III. % of TNF- α release from PHA stimulated PBMC in presence of 10^{-4} M and 10^{-7} M As compounds in relation to control samples

	10^{-4} M	10^{-7} M
Sodium arsenate	95.4 \pm 12.1	99.7 \pm 26.1
Sodium arsenite	12.3 \pm 16.2***	97.1 \pm 13.4
MMAs ^{III}	n.d.	95.2 \pm 12.8
MMAs ^V	92.1 \pm 8.1	95.0 \pm 24.4
DMAs ^V	85.0 \pm 28.3	94.5 \pm 10.9
Arsenobetaine	91.5 \pm 20.7	94.3 \pm 21.7
Tetra-phenyl-arsonium-chloride	40.8 \pm 16.9***	89.4 \pm 11.4
Sodium-esa-fluorum-arsenate	73.4 \pm 26.8*	88.2 \pm 18.0
Potassium-esa-fluorum-arsenate	95.2 \pm 5.9	96.1 \pm 6.8

Values are means \pm S. D.
Mann Whitney U test. Statistical significance: *p<0.05; ***p<0.001.

Discussion

The results of this study show that the immunotoxicity of As compounds depends on chemical speciation. In previous investigations, the toxicity of As salts was related to the capacity of binding intracellular components: e.g. the lack of toxicity of arsenobetaine was related to inability of interacting with cell molecules (10). Recently, the me-

tabolism of As compounds was investigated in several cell cultures including those of human and rat hepatocytes, bronchial and bladder epithelial cells and epidermal keratinocytes (5, 8, 11). Primary rat hepatocytes showed the higher methylation capacity for inorganic As followed by primary human hepatocytes and human epidermal keratinocytes and bronchial epithelial cells, while cells derived from human bladder did not methylate inorganic As. The following transformation of As was shown: As(V) \rightarrow As(III) \rightarrow MMAs^V \rightarrow MMAs^{III} \rightarrow DMAs^V \rightarrow DMAs^{III} (dimethylarsinous acid).

Trivalent monomethylated and dimethylated arsenicals showed cytotoxicity and genotoxicity similar to that of inorganic As(III) salts, while pentavalent As compounds were found significantly less cytotoxic than the trivalent salts (4, 5, 8, 11). The results of this study on the immune effects of As salts are similar, with the exception of tetra-phenyl-arsonium-chloride, a pentavalent As organic salt, which exerted marked immune effects on PBMC.

As₂O₃ was used in the treatment of acute promyelocytic leukaemia by induction of partial differentiation and apoptosis (12). The apoptotic effect of MMAs^{III} was found more potent than that of As₂O₃ suggesting that the former compound could be considered for chemotherapy of haematologic malignancies (13).

The toxicity of DMAs^{III} seemed to be lower than that of MMAs^{III} since DMAs^V is excreted more efficiently from the intracellular compartment than DMAs^V (4, 5): the intracellular retention of MMAs^{III} thus contribute to the immunotoxicity of inorganic As.

In agreement with previous investigations on different cell types (5, 8, 11), this study shows that MMAs^{III} was more reactive than inorganic As(III): the effects of MMAs^{III} on viability of PBMC (lymphocytes and macrophages) is greater than that of inorganic As(III); moreover, MMAs^{III} enhanced spontaneous PBMC proliferation at 10^{-7} M concentration.

The finding that low doses of sodium arsenite(III), DMAs^{III} and MMAs^{III} stimulate PBMC proliferation confirms the data of other studies showing an enhancement of proliferation of human epidermal keratinocytes and PBMC induced by As(III) (11, 14). In particular, the stimulatory effect of DMAs^V on PBMC proliferation may be related to its capacity of being transformed into DMAs^{III} (8). On the other hand, stimulatory effect of low concentrations of As(III), DMAs^V and MMAs^{III} may be related with the capacity of As of acting as an essential element (15).

In conclusion, this study shows that not only inorganic As but also its organic intracellular metabolites exert marked inhibitory immune effects at high doses and stimulatory activities at low concentrations.

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