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Original Research

Evaluation of metabolic reactivity in macrophages from mice with chronic sodium arsenite intake and experimental carcinogenesis

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Abstract: Arsenic is carcinogenic to human beings, and environmental exposure to arsenic is a public health issue that affects large populations around the world. Thus, studies are needed to determine the mode of action of arsenic and to prevent harmful effects that arise from arsenic intake. In particular, knowledge of the effects of arsenic exposure in individuals who are undergoing a carcinogenesis process is lacking. The present study was performed in mice to evaluate the effect of chronic As^{3+} administration on peritoneal and alveolar macrophages; the As^{3+} was administered in drinking water over 9 months and there was a two-stage carcinogenesis process. At the end of the experiment, the number of tumors stabilized to below the control values, but the tumors showed increased malignancy. Our objective was to evaluate the systemic effects of chronic As^{3+} ingestion in a population of macrophages that was derived from the peritoneal cavity and the broncho-alveolar trunk of cancerized mice since they are the first line of defense in the immune system. The results showed that the macrophages under all conditions retained their ability to self-regulate their metabolic reactivity. This feature was more evident in peritoneal macrophages than in alveolar macrophages. Furthermore, an increase in the number of macrophages from animals receiving higher doses of As^{3+} compared to untreated animals was observed. These findings indicate that certain parameters associated with two-stage skin carcinogenesis are modified by the presence of As^{3+} in drinking water.

Key words: As^{3+}; Arsenite; Alveolar macrophages; Peritoneal macrophages; Carcinogenesis; SenCar mice.

Introduction

Inorganic arsenic is typically found on the surface of rocks in the form of arsenic metal compounds. The exposure of living organisms to arsenic is unavoidable, varying according to local geochemical characteristics as well as the level of anthropogenic activity. Arsenic has different chemical forms in the environment, and As transport and distribution are complex due to its cycle as generated by water, soil and air (1).

The intake of drinking water is the primary route of entry for humans living in areas with high levels of inorganic arsenic (2). The correlation between the prolonged ingestion of arsenic and cutaneous and/or visceral damage has been documented in both humans and animals (3). Chronic exposure to arsenic in humans is associated with several cancers and many other pathological effects; these effects include clinical skin signs such as hyperhidrosis, hyperkeratosis and arsenical melanodermia (4), peripheral vascular disorders, dark abrasions on the feet (black foot disease) (5), alterations in the peripheral nervous system (6), diffuse pulmonary fibrosis and broncho-pulmonary lesions (7).

Sodium arsenite (As^{3+}) is the most extensively studied arsenic compound. Arsenite causes oxidative stress through the generation of reactive oxygen species (ROS) (8,9), which are associated with carcinogenesis (10, 11). Even at low concentrations, arsenite generates ROS, which could lead to the abnormal differentiation of monocytes (12) and alter the characteristics of human monocyte differentiation into macrophages, surface marker expression or phagocytic activity (13).

Macrophages are innate immune cells that play a broad role in host defense and in tissue homeostasis. These cells have pleiotropic activities and are characterized by a high phagocytic capacity and heterogeneity, which are consistent with their adaptation to different environments. A respiratory burst is triggered during phagocytosis, leading to a marked production of ROS linked to the activation of NADPH (nicotinamide adenine dinucleotide phosphate oxidase) (14, 15). Thus, during the respiratory burst, the release of several types of toxic oxygen products (15) can mediate the production of various inflammatory factors (16), resulting in a wide range of physiological responses (17). Experimentally, the respiratory burst can be triggered by soluble agents such as TPA.

Peters *et al.* reported that neutrophils have the potential to interact with one another (18). The inflammatory response is limited by auto-regulation when excess neutrophils are recruited to an area of tissue injury in an oral area with active inflammation. Similarly, Fernandez *et*

al. stimulated macrophages from the peritoneal exudate, and they described an auto-regulatory mechanism that could be linked to the protection of body tissues from the toxic effect of ROS, for which the number of reactive cells remained constant, irrespective of the total peritoneal cell density (19).

Alveolar macrophages (AM) are the most abundant antigen-presenting cells in the airways and alveolar spaces. A population of alveolar macrophages is used here as an example of a resident population. Their features could reflect the unusual environment in which they are found, which is characterized by high oxygen tension and high concentrations of surfactant proteins.

However, the model for chemical carcinogenesis in skin has been valuable for the formulation of the basic principles underlying the initiation, promotion and progression of tumors (20, 21). Tumors do not develop immediately; rather, the mutated ras oncogene remains dormant in epidermal cells and requires additional events to progress to malignancy (22). Our group recently showed that following 7,12-dimethylbenz(a) anthracene (DMBA) /tetradecanoyl-phorbol-acetate (TPA) treatment in SenCar mice, the number of skin tumors increased in animals that had concomitantly received As^{3+} in the drinking water (23).

The aim of this study was to test whether the auto-regulation of oxidative stress in macrophages using mice drinking water with different concentrations of As^{3+} was maintained when they were concomitantly subjected to a two-stage carcinogenesis process.The analysis of the macrophage behavior in cancerized animals that were subjected to a microenvironment with sustained, potentially toxic levels of As^{3+} is a useful tool to elucidate the role they would play during a carcinogenic process and to explore the potential effect of arsenic on carcinogenesis.

Materials and Methods

Chemicals

Sodium arsenite (As^{3+}), 7,12-dimethylbenz(a) anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA), nitrobluetetrazolium (NBT), thioglycollate, paraformaldehyde and polyvinyl pyrrolidone (PVP) were purchased from Sigma Chemical Co. (St. Louis, MO USA).

Animals

Five lots containing 20 female 7-9-week-old SenCar mice each were used (24). The animals were bred at the facilities of the National Atomic Energy Commission of Argentina and housed under environmentally controlled temperature and humidity conditions with a 12 h light/dark cycle. The mice were provided with a standard pellet diet and water *ad libitum.* Institutional and national guidelines for the use and care of laboratory animals were strictly observed (25).

Mouse model for chemical tumorigenesis

Mouse skin tumors were induced by a chemical process involving an initiation-promotion regime (26). The animals were initiated with a single topical application of DMBA (25.65 µg/ml acetone; 200µl/mouse). The promotion stage started ten days later. The animals received biweekly dorsal topical applications of TPA (10 µg/ml acetone; 200 µl/application) over the course of 9 months.

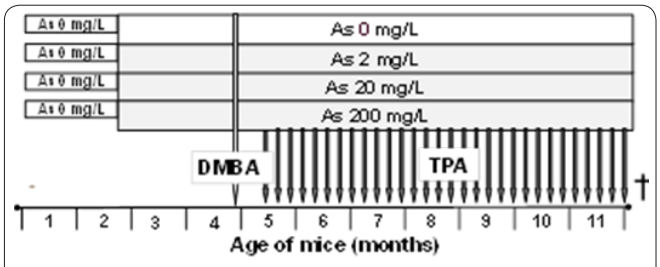

Figure 1. Summary of the experimental conditions.

As^{3+} in the drinking water

As^{3+} was delivered daily in the drinking water at different concentrations (0-200 mg/L) for two months before the beginning of the carcinogenesis protocol and during the 9-month promotion period.

Five groups of 20 SenCar mice were treated as indicated:

i) As0: 0 mg As^{3+}/L, control, ii) As2: 2 mg As^{3+}/L, iii) As20: 20 mg As^{3+}/L, iv) As200: 200 mg As^{3+}/L, and v) an additional age-matched untreated control (Co) (no carcinogenesis treatment or As^{3+} in the drinking water, same manipulation) was included.

Figure 1 summarizes the different experimental conditions.

At the end of this promotion period, the animals presented numerous skin tumors over their dorsal areas (23).

Macrophage recruitment

Half of the animals in each group were injected intraperitoneally with 2 ml of thioglycollate broth (8% in PBS), generating sterile inflammations. Four days later, the animals were euthanized and their activated macrophages were harvested by washing the peritoneal cavity with 3.5 ml of PBS.

The other half was used to obtain the resident alveolar macrophages by broncho-alveolar lavage as described elsewhere (27). In brief, after the animals were euthanized, their lungs were lavaged several times with cold PBS. The total volume of the lavage fluid was collected and centrifuged at 800g for 10 min at 4ºC. The cells were resuspended in 3.5 ml of the supernatant.

A 3-ml volume of cell suspension was used for the NBT test and the others to determine the cell number and cell viability.

The resulting viability was always higher than 95%, and over 98% of the harvested cells were macrophages.

These *ex vivo* studies were carefully performed to preserve the *in vivo* conditions as closely as possible.

The samples obtained from each mouse were analyzed independently due to the high variability in the resulting cell densities.

Quantitative measurement of superoxide anions (NBT assay)

The intracellular generation of superoxide anions (O_2^-) produced by phagocytes during the respiratory burst was evaluated as previously described by Segal (28). The active oxygen species was evidenced by the amount of blue formazan precipitate in the cells fol-

lowing NBT reduction. This assay was performed immediately after peritoneal and bronco-alveolar lavage, and the respiratory burst was triggered by TPA.

The cell suspensions were divided into three fractions of one ml each as follows: the *basal fraction* (PBS-acetone), *non-stimulated fraction* (NBT-acetone) and *stimulated fraction* (NBT-TPA). One ml of PBS or NBT (0.1% in PBS) was added for 15 min at 37ºC followed by the addition of 5 µl of acetone or TPA (100µg/ml acetone) for 45 min at 37ºC under mild agitation. Then, paraformaldehyde (4% in PBS) was added to stop the NBT reaction and fix the cells. Later, the fractions were centrifuged and resuspended in PVP (10% in PBS). Smears were prepared for each fraction to perform a cell digital image analysis.

Digital image analysis

Image analysis of the macrophages was performed in accordance with previous studies (29). In brief, the cells were observed under a MPM 800 Carl Zeiss microscope (Jena, Germany) using an interference filter (570 nm) and a 40x objective coupled to a camera. The image intensity was digitized and analyzed with an image analysis system (IBAS-Kontron, Jena, Germany). The chosen software allows for the scanning and evaluation of single cells. It affords data on the total optical density (TOD) of the whole reactive cell.

A TOD of 200 cells was evaluated for each smear. A percentage histogram with a TOD range of 0-300, with arbitrarily selected intervals of 50 units (reaction levels), was performed to calculate the number of cells at each interval.

Statistical analysis

The results were expressed as the means ± SE. A statistical evaluation of the data was performed using an analysis of variance (ANOVA), followed by Tukey's *post hoc* test. A probability value of <0.05 was considered the minimum level of statistical significance.

Results

Microscopic observations revealed that some macrophages in the stimulated fraction were reactive (blue formazan granules in the cytoplasm), while others exhibited no reaction (Figure 2).

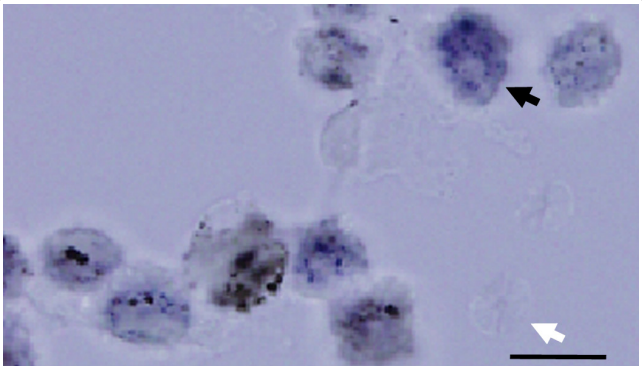

Figure 2. Microphotograph of the stimulated fraction of alveolar macrophages. White arrows indicate non-reactive macrophages. Black arrows indicate macrophages with reduced NBT. Scale bar = 10 µm. Note that some macrophages show dark non-specific particles that are usually present in alveolar macrophages.

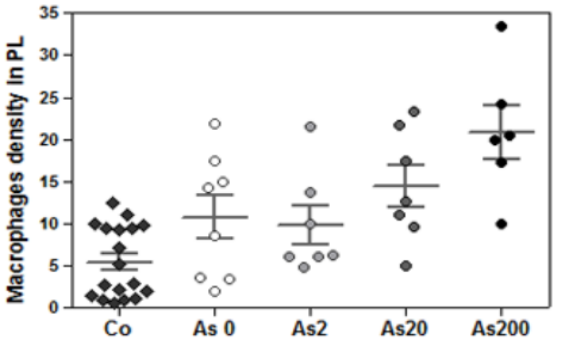

Figure 3. Macrophage density in the peritoneal lavage (PL) (10^6 cells/ml) as a function of increasing As^{3+}. Each point represents one animal and the lines represent the means ± standard error.

Peritoneal macrophages

In the animals whose drinking water was supplemented with A20 and A200 (Figure 3), the cell density in the peritoneal exudate showed higher values than that of the controls (As 0 and Co).

Concerning the quantitative assessment of superoxide generation (NBT assay), the stimulated fraction showed that 35-45% of the cells were reactive, with different reaction levels (TOD >50). Figure 4 shows the number of cells per ml, as sorted by level of reaction, as a function of the whole macrophage density. The number of reactive cells never exceeded values of approximately $7.5x10^6$cells/ml regard less of the number of macrophages obtained in the peritoneal lavage and after the different As^{3+} concentrations were assayed. Thus, the subpopulation of reactive cells is independent of the total number of peritoneal macrophages that were recruited, whereas the number of non-reactive macrophages and total cell densities are linearly related (R 0.8). In the non-stimulated fraction, only 2% was reactive.

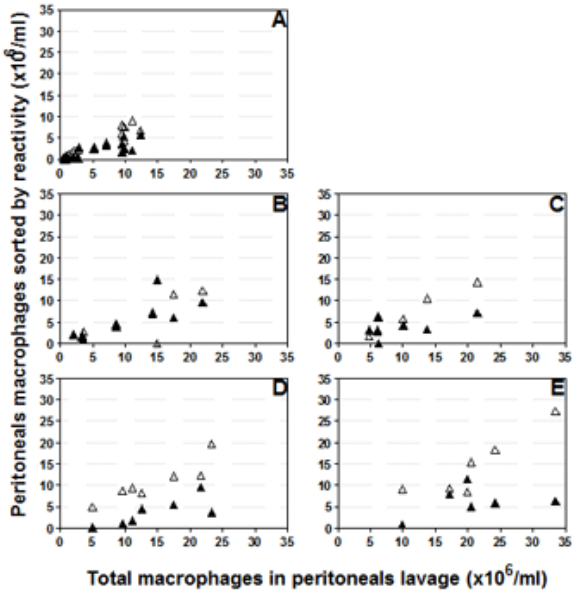

Figure 4. Macrophage reactivity (TOD △ 0-50, ▲ >50) in the stimulated fraction as a function of the whole macrophage population collected from the peritoneal cavity (partial density *vs.* total density). Each animal is represented by a pair of points. A: Control, B: As 0, C: As 2, D: As 20, and E: As 200.

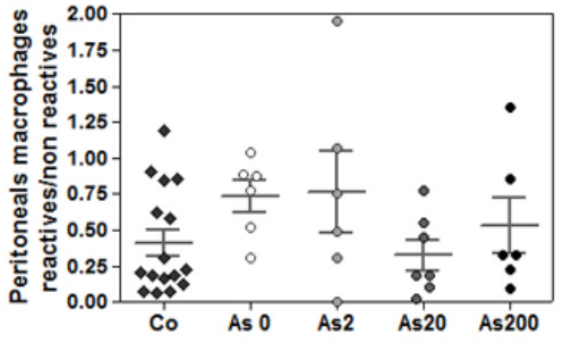

Figure 5. Peritoneal Macrophage Reactivity: The reactive cells (TOD>50)/non-reactive cells (TOD 0-50) for the stimulated fraction (each point represents one animal and the lines indicate the mean ratios ± standard errors).

In Figure 5, the reactive capacity in the stimulated fraction (the ratio of reactive cells *vs.* non-reactive cells) is depicted. This parameter was calculated for each animal. Increasing As^{3+} concentrations are associated with a reduction in this ratio, indicating less reactive capacity.

Alveolar macrophages

The range of macrophage densities in the broncho-alveolar tree of animals that were subjected to an increasing concentration of As^{3+} in drinking water is smaller than that found in the peritoneal cavity (Figure 6).

Regarding the quantitative assessment of superoxide generation (NBT assay) in the stimulated fraction, 40-50% of the cell population was reactive (TOD> 50). Figure 7 indicates the number of cells per ml as sorted by reaction level as a function of the macrophage density obtained in the lavage. The number of reactive cells was never larger than $8x10^5$ regardless of the total number of recruited cells. It is also apparent that the higher the number of recruited cells, the higher the number of non-reactive cells, with both variables being linearly related (R = 0.9). In the non-stimulated fraction, 18% were reactive and in the basal fraction, 8% of the cell population showed intracellular deposits of dark particulate traces coming from the microenvironment to which they are exposed (data not shown).

The reactive capacity of cells for each animal in the stimulated fraction is shown in Figure 8. The values for the increasing As^{3+} concentrations were below that of the control (As 0).

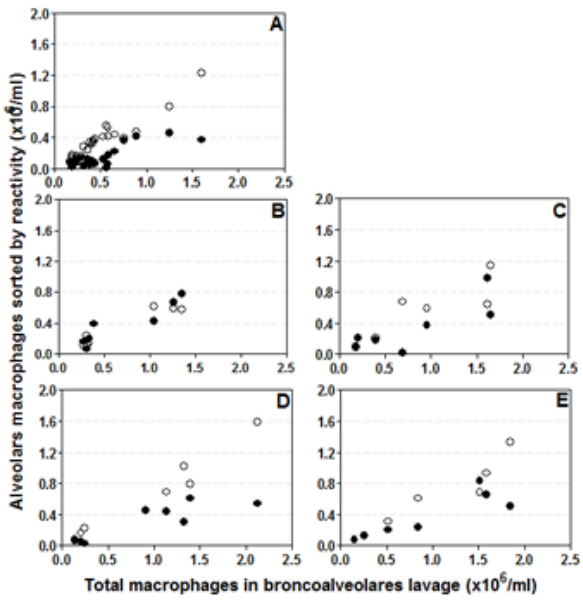

Figure 7. Macrophage Reactivity (TOD ○ 0-50, ● >50) in the stimulated fraction as a function of the whole macrophage population that was collected from the broncho-alveolar trunk (partial density *vs.* total density). Each animal is represented by a pair of points. A: Control, B: As 0, C: As 2, D: As 20, and E: As 200.

Discussion

Exposure to arsenic is an environmental risk for the human population in general and particularly for subpopulations living in areas with high levels of arsenic in the drinking water. Several investigations have been performed around the world in recent years, and they relate the toxicity of this agent to many human pathologies arising in target organs or systems since arsenic has been recognized as a human carcinogen in international epidemiological records (2). However, some compounds that contain arsenic have effective actions as therapeutic agents for treating certain types of neoplasia (30, 31), especially those of hematological origin (32).

In vivo experiments have shown that inorganic arsenic can alter macrophage functions (12). These effects were associated with a marked reduction in the adhesion properties, chemotactic migration and phagocytic activity of splenic macrophages (33). Macrophages are

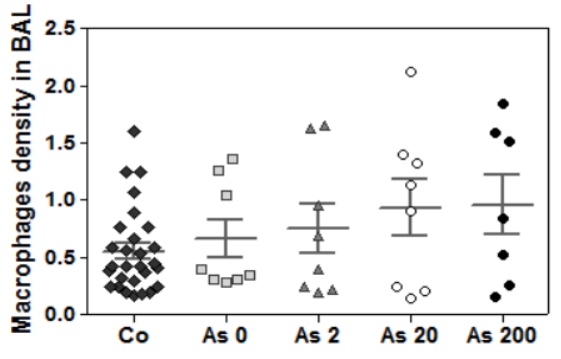

Figure 6. Macrophage density in broncho-alveolar lavages (BAL) (10^6 cells/ml) as a function of the increasing As^{3+} concentration. Each point represents one animal, and the lines indicate the means ± standard errors.

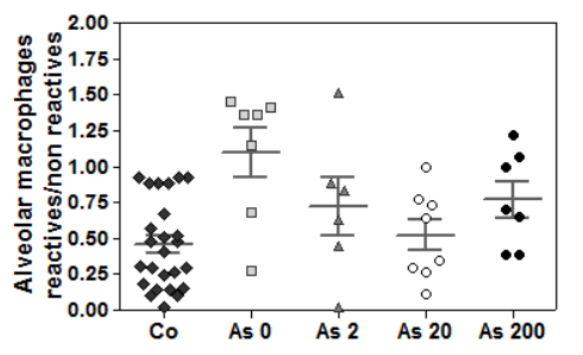

Figure 8. Alveolar Macrophages: The reactive cells (TOD>50)/non-reactive cells (TOD 0-50) in each animal for the stimulated fraction (each point represents one animal and the lines reflect the mean ratios ± standard errors).

sensitive targets of inorganic arsenic, contributing to the metabolic alterations produced by this environmental contaminant. The high degree of heterogeneity in macrophages is consistent with their capacity to adapt to different tissue environments, conditioning them to develop a high degree of specialization (34).

In a previous study, we reported the effect of chronic As^{3+} administration through drinking water as being superimposed along with a 2-stage carcinogenicity protocol for 9 months. The study focused on the progressive development of skin tumors, and the results showed a time-dependent pattern in two stages (23). In the present study, we focused on characterizing macrophages at the completion of the second stage. In taking advantage of this experimental model, the behavior of peritoneal and alveolar macrophages were used as an end-point to evaluate the consequences that chronic arsenic ingestion might have on people who are affected by some types of neoplasia.

The NBT assay was used to evaluate the production of superoxide anions during the respiratory burst (29, 35) in peritoneal and alveolar macrophages of cancerized mice that were drinking arsenical water. Using digital image analysis, a quantitative estimation of cell-to-cell variations produced during the respiratory burst at the cellular level was performed as previously described by Fernandez *et al.*(19). They described a mechanism of macrophage auto-regulation to explain the response of a population after stimulation. They reported that the macrophage reaction to a stimulus is self-regulated and is associated with variations in the cell density within the peritoneal cavity of mice. When an excess of macrophages was recruited in the peritoneal cavity, only a portion shows a reaction. This is the real modulating factor because this fraction of reactive cells is kept almost constant, guaranteeing a physiologically adequate number of reacting cells that are not harmful to the host tissues.

Under the experimental conditions assessed herein, the peritoneal macrophages that originated in cancerized mice were subjected to a long period of drinking water containing As^{3+}. Even so, the macrophages kept their physiological self-regulation properties because, irrespective of the cell density obtained in the peritoneal cavity, the reactive cell numbers were maintained within a defined range. However, the total number of peritoneal macrophages recruited to the peritoneal cavity tends to rise with increasing As^{3+} concentrations.

Concerning alveolar macrophages, the increase in density is conditioned by the limited bronchial space in which these cells are found. Therefore, the self-regulation mechanism is not as evident as it is in the peritoneal cavity. However, a trend towards higher cell densities was observed in mice that were subjected to the cancerization process with or without As^{3+} (5-17 x 10^5 cells/ml). These results are consistent with those of Maus *et al.*(36), who reported similar cell densities in a mice lung inflammation model induced by bacterial endotoxin.

Lemaire *et al.* (13) showed that low concentrations of As_2O_3 altered the cytoskeletal reorganization of F-actin in macrophages and can help to reduce the pool of functional macrophages by inhibiting the macrophage differentiation of blood monocytes or altering the endocytosis and phagocytosis processes. Furthermore, higher As_2O_3 concentrations can induce apoptosis in differentiated macrophages. An increase in the macrophage population could be expected to overcome these physiological deficiencies.

Thus, the continuous intake of As^{3+} and a sustained carcinogenesis process might produce the increase in the number of macrophages in the peritoneal cavity as well as in the broncho-alveolar tree.

It is noteworthy that a constitutive reactive macrophage fraction is observed in the broncho-alveolar lavage, and it is probably associated with the presence of airborne particulate matter in the bronchial tree.

The auto-regulation of macrophage metabolic reactions is directly associated with variations in cellular density and is considered as an intrinsic mechanism of phagocytic cells. This mechanism is probably related to the protection of normal host tissue from the toxic effects of superoxide anions and reduction products.

Cancerized SenCar mice that drink arsenical water show higher ratios of reactive/non-reactive cells compared with control mice. This effect might be associated with their sensitivity to the development of experimental skin tumors (37). Clutton *et al.* also reported that an increase in O_2^- generation is correlated with the development of carcinogenesis processes (38). However, in cancerized animals, when drinking different doses of As^{3+}, a decrease in the proportion of reactive/non-reactive cells is produced, revealing that the presence of chronic As^{3+}intake through drinking water altered this proportion. The number of non-reactive cells notably increased in the peritoneal cavity, while the number of reactive cells remained within a defined range, irrespective of the As^{3+} concentration. This behavior can be attributed to the functional phenomen on of self-regulation in which the presence of a controlled number of reactive cells ensures protection, preventing the harmful effect of an unlimited number of reactive cells.

The present data reveal that the modulation of the macrophage response is not affected by the presence of As^{3+}. Thus, when excess macrophages are recruited into the peritoneal cavity or to the broncho-alveolar tree, macrophages with no reaction would be one of the primary modulators of the population response. The fraction of reactive cells is kept virtually constant, guaranteeing a physiologically adequate number of reacting cells that is not harmful to the host tissues.

Based on the results described above, it is important to consider that the biological effect of arsenite would appear to be detrimental to the macrophages because, in spite of their increased density, the malignancy index of the tumor formations is increased (23). These characteristics should be taken into account, especially when there is a risk of overlapping chronic arsenite intake and the development of a carcinogenic process and possible adverse clinical effects.

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