IN VIVO AND *IN VITRO* RESEARCH ON THE BIOLOGICAL EFFECTS OF DEUTERIUM-DEPLETED WATER: 1. INFLUENCE OF DEUTERIUM-DEPLETED WATER ON CULTURED CELL GROWTH

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Abstract. Deuterium depleted-water (DDW) is a new available tool for decreasing deuterium concentration in the environment of cells in culture.

Several types of established cell lines, both normal and neoplastic were grown in culture media dissolved with DDW and compared with the same strains, in the same amounts, grown in media dissolved with normal distilled water.

Naive mice splenocytes were grown, under stimulation with proliferation triggers, like bacterial lipopolysaccharide (LPS) and Concanavalin A (ConA) in the same conditions. The growth and proliferation were estimated using the MTT assay.

Both established cell types and explanted splenocytes in the DDW-media had a significantly higher growth rate than cell cultured in normal media.

In an attempt to identify the membrane mechanisms involved in the growth stimulation by DDW, the membrane proton transporters Na^+/H^+ antiporter and H^+/K^+ATP-ase were inhibited with their selective blockers amiloride and respectively lansoprazole.

The results, however incomplete, point towards a lack of involvement of the Na^+/H^+ antiporter and a possible implication of the H^+/K^+ATP-ase.

Key words: deuterium depleted water, proliferation, proton pumps.

INTRODUCTION

The presence of deuterium as a stable non-radioactive isotope of hydrogen in sources of natural waters raised naturally the problem of its effects on living animals. Compared with the metabolic, structural and functional changes induced by the excess deuterium in water (Gross and Harding, 1961; Wang *et al.*, 1993; Kushner *et al.*, 1999), the biological consequences of the reduction of its normal concentration (145–150 ppm) are much less studied.

Classically, it is admitted that both the difference in the atomic mass of deuterium toward hydrogen and its concentrations, above 10 mM/l in body water give it qualities of independent chemical element, with its own physical, chemical and

biological properties. Most of the research concerning the effects of deuterium was made using deuterated (heavy) water in various concentrations. The increase of the D:H ratio over 1:6600 produces changes of density, diffusion and refraction index accompanied by the decrease in solubility and diffusion coefficient. These will induce on the metabolic plan alterations in the activity of several enzymes (Thomson, 1960) and the intensification of anaerobic glycolysis, which generates CO_2 and lactate (Litvinenco *et al.*, 1992). On the functional plane it has been shown that the excess deuterium in heavy water induces the decrease the membrane permeability (Potosin *et al.*, 1993).

Further experimental research showed that deuterium-depleted water influences the rhythm of cellular growth (Somlyai *et al.*, 1993) and vascular reactivity (Haulica *et al.*, 1998). Deuterium-depleted water inhibits the growth of fibroblasts and tumor cells (Somlyai *et al.*, 1997–1998). Thus, it has been precised that deuterium is involved in the regulation of cell growth and development and that deuterium-depleted water seems to have antitumoral effects.

Benefiting of the availability of a product which can decrease the deuterium content in the water used for solving the growth media – deuterium-depleted water (DDW) from the Institute of Criogeny and Isotopic Separations RM. Valcea, Romania, a series of experiments were made, intended to identify the effect of deuterium depletion in the water from the environment of the cells grown in culture. In order to have a wider range of effects and reduce variability in the results were used established cell lines, both neoplastic and normal and also explanted immune cells from naive animals.

In order to identify a research path concerning the mechanisms through which these effects of deuterium-depleted water takes place, a further step in the experiment was to use specific pharmacological blockers of the membrane transporters which are involved in the migration of protons through the membrane.

It is known that intracellular pH is one of the triggers of the cell division. A very important aspect of the Na^+/H^+ antiporter functionality is the detection of cytosolic pH. A common characteristic of several regulation systems is their sensibility to intracellular pH (Rich *et al.*, 2000).

Another membrane component that could be involved in transmembrane hydrogen transport could be K^+/H^+ ATP-ase. The knowledge on this membrane pump are less detailed that on other proton transporters, however there are specific inhibitors, omeprazole derivatives that induce, in a K^+ dependent manner, an intense intracellular alkalinization (Jaisser and Beggah, 1999).

While one of the hypothetical mechanisms through which a stimulating effect for the cell proliferation could arise was the involvement of the Na^+/H^+ antiporter, a complete blocking or at least a partial inhibition was tried, using selective inhibitors. For blocking the Na^+/H^+ antiporter was used Amiloride (Grinstein, 1994; Orlowski and Grinstein 1997). The blocking of the K^+/H^+ antiporter was tried with Lansoprazole (Jaisser and Beggah, 1999).

While preliminary results of our research group emphasize possible radioprotective and immune stimulating properties of deuterium-depleted water (Bild *et al.,* 1999), a series of experiments involving the effects of DDW-media on naive immune cells were initiated, using splenocytes from mice which were not subjected to any immune stimulation. The growth was made under the stimulation of proliferation triggers Concanavalin A (Grinstein *et al.,* 1987) and bacterial Lipopolysaccharide (Mukaida *et al.,* 1996).

MATERIALS AND METHODS

1. GROWTH STUDIES WITH ESTABLISHED CELL LINES

The established cell lines used were: RAG – murine renal adenocarcinoma, ATCC catalogue number: CCL–142–RENCA, amoeboidal cell, with adherent growth, propagated in MEM with 10% FCS (Klebe *et al.*, 1970), TS/A – murine mammary adenocarcinoma, ATCC catalogue number CRL–2116, epithelioid aspect cells, with adherent growth, propagated in DMEM medium with 10% FCS (Chao and Chu, 1989) and NIH – murine embryonic fibroblasts, ATCC catalogue number IRR-3T3, with adherent growth, propagated in DMEM with 10% FCS. The aliquots of 10^5/ml were obtained from the cell bank of the immunology department of the „Max Delbruck" Molecular Medicine Center from Berlin, Germany.

The culture media were powdered media (RPMI 1640, Gibco BRL, SUA), reconstituted with bi-distillated and de-ionized water under magnetic stirring or with deuterium-depleted water from ICSI Râmnicu Vâlcea, Romania, in concentrations of 30, 60, 90 and 120 ppm at a pH of 7,35.

The cells were incubated at 37°C in air with 5% CO_2. The following of the cells development was made by direct observation through phase-reversed microscope. At confluence, the 3 flasks of primary culture were suspended in 5% trypsin and aliquots were harvested for reseeding. Cell counting was made by haemocytometer counting, after exclusion dying with trypan blue (which allows for the elimination of non-viable cells presenting membrane disruptions) (Cristofalo and Phillips, 1989).

Cells were reseeded in sterile conditions on 6, 12, 24 well culture plates (Coming C 25100), using RPMI 1640 as described above. For each type of cell were made 10 plates. Beginning after 48 hours from incubation, at each 24 hours a well from each plate was harvested. Culture media were removed, cells were suspended by incubation with trypsin 5% for 20 minutes at 37°C and than counted using a Neubauer haemocytometer.

2. STUDIES WITH INHIBITORS OF THE MEMBRANE PROTON PUMPS

For the studies with inhibitors of the membrane proton pumps a series of 3 × 24 well culture plates, beginning with 10^5 cells/ml for each cell type. In each culture media were added by trials, several dilutions of amiloride and lansoprazole, selective blockers for the H^+/Na^+ antiporter and the membrane H^+/K^+ ATP-ase respectively. The dilutions that were kept were of 1 mM for amiloride and 0,5 mM for lansoprazole.

Beginning from 48 hours of incubation, at each 24 hours a well from each plate was prelevated. The culture media was removed, cells were suspended by 5% trypsin incubation for 20 minutes at 37°C and then counted, after exclusion coloration with 0,4% trypan blue in PBS using a Neubauer haemocytometer.

Quantification of Cell Growth

The measuring of growth differences between the two types of cultures was made by calculating the population doubling level (number of generations) and the population doubling time. The calculations were made according to the formulae presented (Hayflick, 1973).

The concept of Population Doubling Level – PDL is based on the assumption that cells in culture have symmetrical sequential divisions, thus their population should increase exponentially (from 1 to 2, from 2 to 4, from 4 to 8, and so on). At the end of *n* generations a known inoculum should produce 2^n cells. Thus, the total number of cells at a certain moment after inoculation is $N_H=N_I2^n$, where N_H is the n umber of cells obtained at the end of the culture time and N_I is the number of inoculated cells. The number of generations is the number of population doublings, and can be expressed using base 10 logarithms, according to the following formula:

$$2^n = \frac{N_H}{N_I} \; or \; n \log 2 = \left(\frac{N_H}{N_I} \right) = \log N_H - \log N_I \qquad (1)$$

because log 2 = 0,301, 0,301 n = log N_H – log N_I, thus n = 3.32 (log N_H – log N_I).

The multiplication rate and the Population Doubling Time – PDT provide useful information on the characteristics of the logarithmic growing phase of a cell line. The multiplication rate (r) is the number of generations per time unit and is usually expressed as population doublings in each 24 hours. Population Doubling Time – PDT is the period, expressed in hours, for the cell number to double, and is the reverse of the multiplication rate (1/r). The formula is:

$$\text{Multiplication rate (r)} = 3.32 \, (\log N_H - \log N_I)/(t_2 - t_1) \qquad (2)$$

$$\mathbf{PDT = 1/r}$$

These parameters describe the growth characteristics of the cell population as a whole and do not *stricto sensu* characterize the division cycle of individual cells.

However, in practice, PDT is used for estimating the cell cycle and for determining the length of the phases of the cell cycle.

3. CELL GROWTH OF EXPLANTED MOUSE SPLENOCYTES

Six male Balb/C mice, weighing 15 grams, were sacrificed by cervical dislocation. The spleens were harvested and placed in Phosphate-Buffered Saline (PBS). Each organ was triturated and passed through a 25 μm sieve (Falcon) for eliminating the collagen fibers and the remains of splenic parenchyma. The triturate was washed with DMEM media with 10% Fetal Calf Serum (FCS) and the cells were harvested in 5 ml Eppendorf vials. These were centrifuged at 500 rpm for 10 minutes. The supernatant was removed and the cells were resuspended DMEM culture media with 10% FSC.

96-well plates were used for growth (Costar Inc., USA). In each well was set 100 μl culture media and 1×10^4 splenocytes were seeded. Also, in increasing amounts were put growth stimulators like LPS (Bacterial Lipopolysaccharide, Sigma Co.) or concanavalin A (ConA, Sigma Co.). Plates were wrapped in plastic sheet and left for 24 hours in incubator, at 37°C in 95% air with 5% CO_2.

After 24 hours of incubation the cell numbers were estimated using the takeover and quantitative extraction of MTT (3-[4,5-dimetiltiazol-2-il]-2,5-difenil-tetrazoliu-bromid). This, added to the culture media in a concentration of 5 mg/ml is taken up and converted to formazan by the mitochondrial dehydrogenases in the living cells. The protocol used is based on the method of Plumb *et al.* (1989), which eliminates the effect of pH on the results and offers a linear relationship between the cell number and formazan production both in high and in low cell densities.

The media from each well was removed and 100 μl of fresh media was added, containing 10 mM Hepes/l, at pH of 7.4. Immediately was added 50 μl of MTT solution (Sigma) in PBS. Plates were re-wrapped in plastic sheet and incubated 4 hours at 37°C. Than the media was removed and the MTT solution and immediately was added 200 μl of DMSO solution (Dimethylsulfoxyde, Sigma) in Sorensen buffer (0.1 M glycine, 0.1 M NaCl balanced at pH 10.5 with 0.1 M NaOH).

Using an Elisa reader, the plate extinction was read at 570 nm. The results from each set of wells, belonging to separate animals were then pooled and than subjected to statistical analysis using Student and ANOVA tests.

RESULTS

1. GROWTH STUDIES WITH ESTABLISHED CELL LINES

Following the use of the culture media with deuterium-depleted water, a stimulation of the cell proliferation was observed. This effect was seen in all types of cells, both neoplastic and normal (Figure 1), compared with cells grown in medias with usual water.

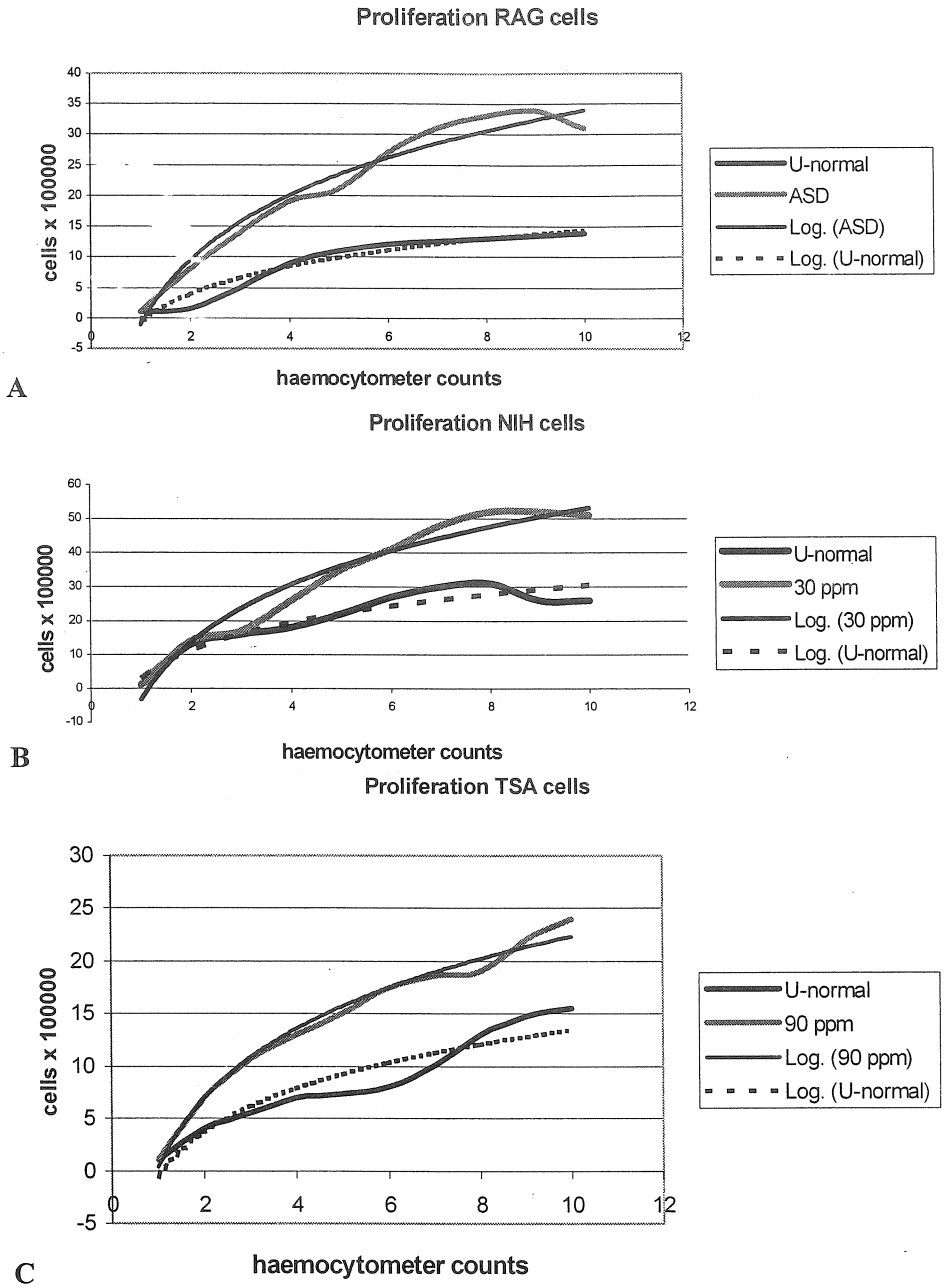


Fig. 1. – Cell growth curves, together with the log-plots of the data series using haemocytometer counts and exclusion dying with trypan blue:
A – murine renal adenocarcinoma (RAG)
B – normal murine fibroblasts (NIH)
C – murine mammary adenocarcinoma (TSA)

Cell growth, quantified both by PDT (Population Doubling Time), and by PDL (Population Doubling Level) is significantly stimulated by the presence in the growth environment of the cells of DDW, compared to control groups, reseeded in media with normal water.

This effect has been observed both for normal cells (mouse fibroblasts) (Figure 2), and for neoplasic cells (mouse renal and mammary adenocarcinomas).

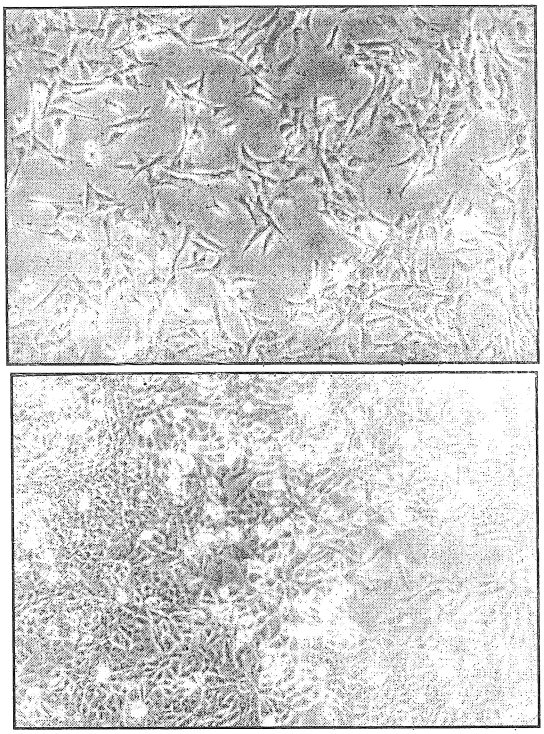


Fig. 2. – Microscopic view of the cell layers of mice fibroblasts (NIH) in the 3rd day from seeding, RPMI 1640 culture media, reconstituted with distilled water (A) or with DDW (B) (reversed microscope, 40 ×)

Following the calculations, the following values were obtained for the PDL's and PDT's of the three cell types (Tables 1 and 2)(Figure 3).

Table 1

Population doubling levels for the three types of cells

	Normal water medium (145 ppm)	DDW media (30 ppm)
TSA	3.95	4.58
RAG	3.78	5.07
NIH	4.95	5.68

Table 2

Population doubling time (in hours) for the three types of cells

	Normal water media (145 ppm)	DDW media (30 ppm)
TSA	150	126
RAG	63.4	42.1
NIH	39.3	33

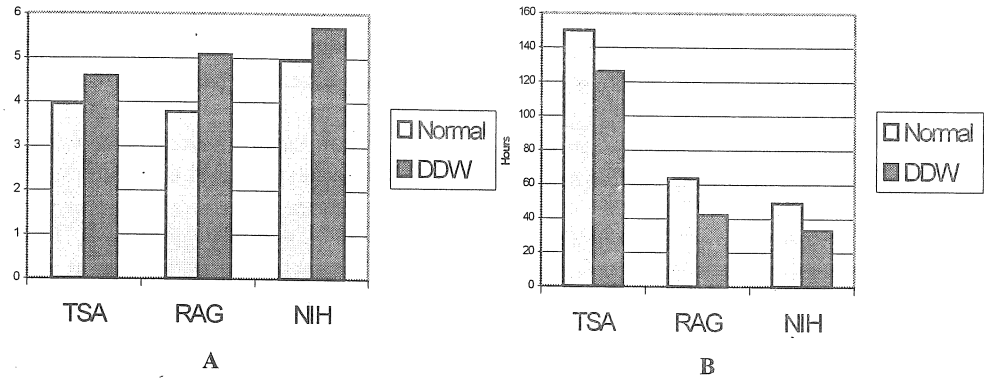


Fig. 3. – A. Population Doubling Levels (number of generations) between the normal medium and DDW-medium cultures.
B. Population Doubling Times – comparison between cultures with normal media and DDW-media.

2. STUDIES WITH INHIBITORS OF THE MEMBRANE PROTON PUMPS

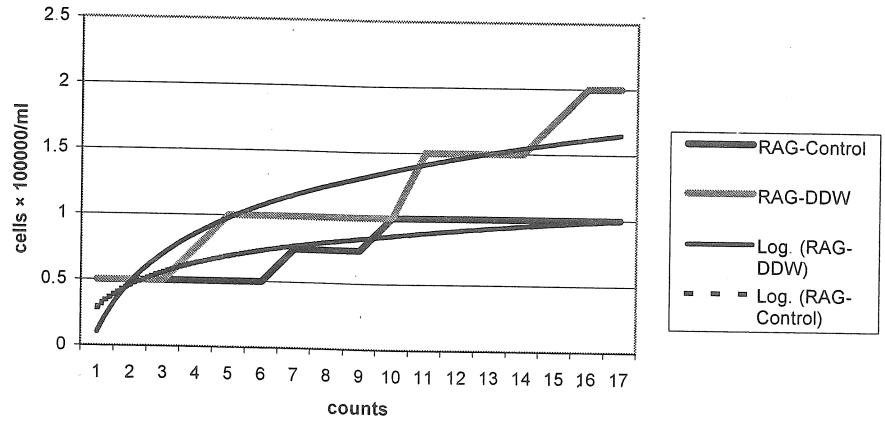
Following the administration of amiloride in the growth media there were no significant changes in the growth rates of the cell types taken into study. The fibroblasts (NIH) and the renal carcinomatous cells (RAG) had significantly different rates of growth, as can be seen in Figure 4, A and B.

Only one cell type, the murine mammary adenocarcinoma (TS/A) had a similar rate of proliferation in both types of media, the differences between the cell growth parameters being statistically non-significant.

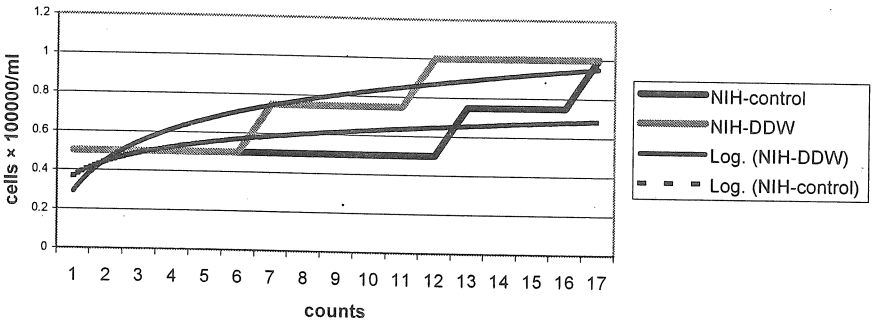
Due to the ubiquitous presence of the H^+/Na^+ antiporter, the dosage of amiloride in the culture media was extremely difficult. Doses higher of 1 mM induced the blocking or the inhibition of the cell growth and proliferation, becoming toxic. A decrease in the amiloride dosage below 1 mM did not induce any changes in the cell proliferation, situation which can lead to the hypothesis that either the H^+/Na^+ antiporter is not affected by the changes in the isotopic concentration of deuterium in the growth media either the approach using a pump blocker from the amiloride family a is not the correct one.

Following the counts made in the wells with culture media with DDW or with regular distilled water and in the presence of lansoprazole 0,5 mM it has been observed that the cell proliferation rates were similar between the two types of cultures for all kinds of cells used for this study (Figure 5).

These results suggest an involvement of the H^+/K^+ ATP-ase in the mechanisms that stimulate the proliferation of cells induced by the reduction of the environmental deuterium in the culture media using DDW, both for neoplastic and for normal cells.



A



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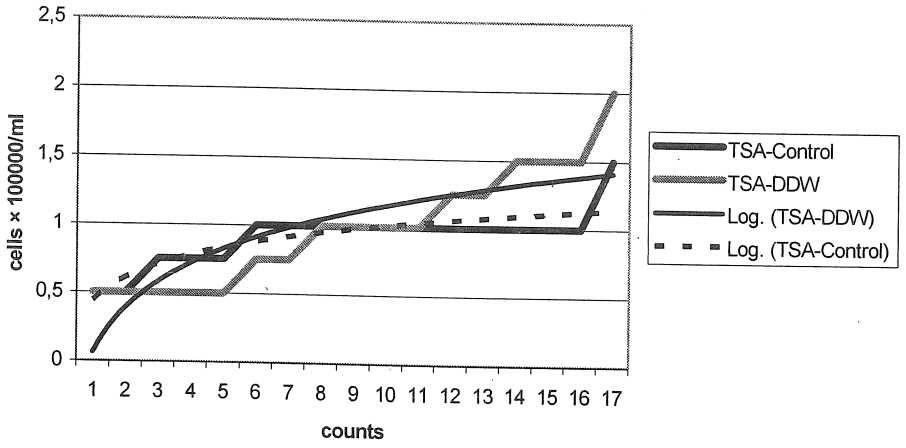
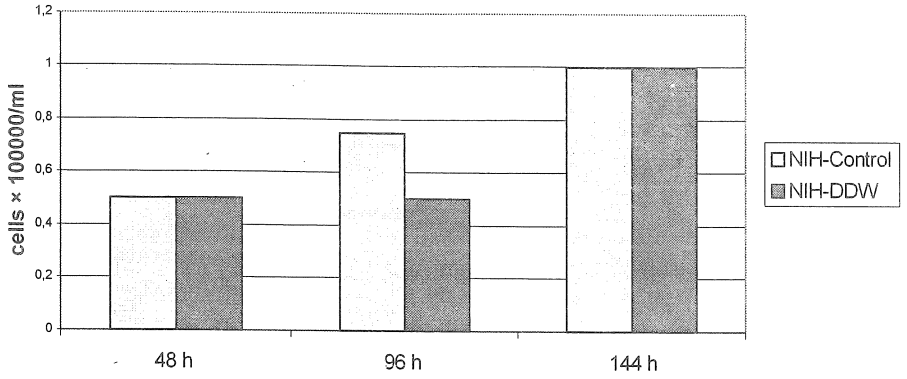
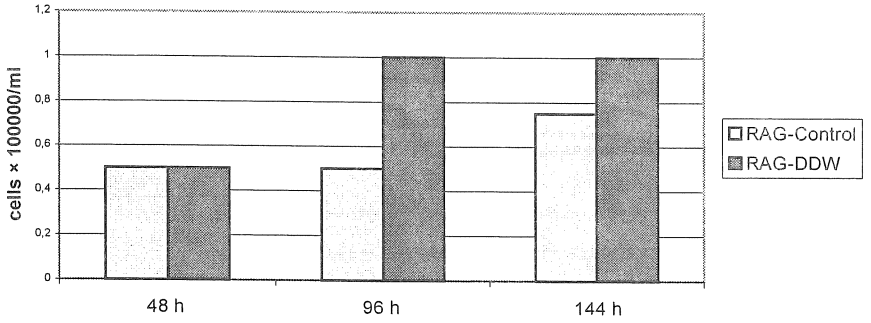


C

Fig. 4. – Growth curves and log-plots for the three cell types in media with amiloride 1 mM
A – Mouse embryonic fibroblasts (NIH)
B – Murine renal adenocarcinoma (RAG)
C – Murine mammary adenocarcinoma (TS/A)



A



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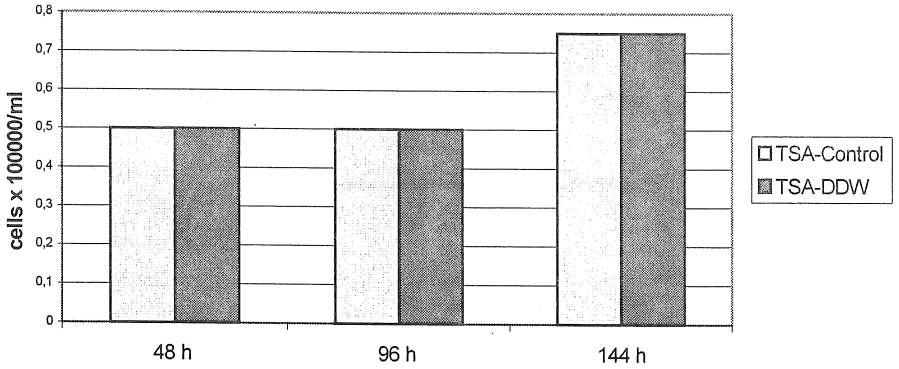

C

Fig. 5. – Evolution of cell numbers in media with distilled water (control) and DDW (30 ppm) in presence of lansoprazole 0,5 mM
A – Mouse embryonic fibroblasts (NIH)
B – Murine renal adenocarcinoma (RAG)
C – Murine mammary adenocarcinoma (TS/A).

3. CELL GROWTH OF EXPLANTED MOUSE SPLENOCYTES

A statistically significant increase of absorbance at 570 nm for the well in which the cells grew in medium with DDW, compared to the control wells, where the cells grew in media with normal water ($t = 1.20841$, $p = 0.02516$, at the level 0.05 the two averages were significantly different) (Figure 6).

Table 3

Average values of extinction at 570 nm for splenocytes in explant

	no stim	0,0625 µg/ml	0,125 µg/ml	0,25 µg/ml	0,5 µg/ml	1 µg/ml	2 µg/ml	
ConA-ASD	0.008	0.023	0.026	0.054	0.092	0.1	0.148	I
ConA-Control	0.014	0.029	0.05	0.054	0.043	0.056	0.09	II
LPS-Control	0	0.001	0.003	0.015	0.022	0.02	0.028	III
LPS-DDW	0	0.017	0.014	0.015	0.027	0.027	0.053	IV

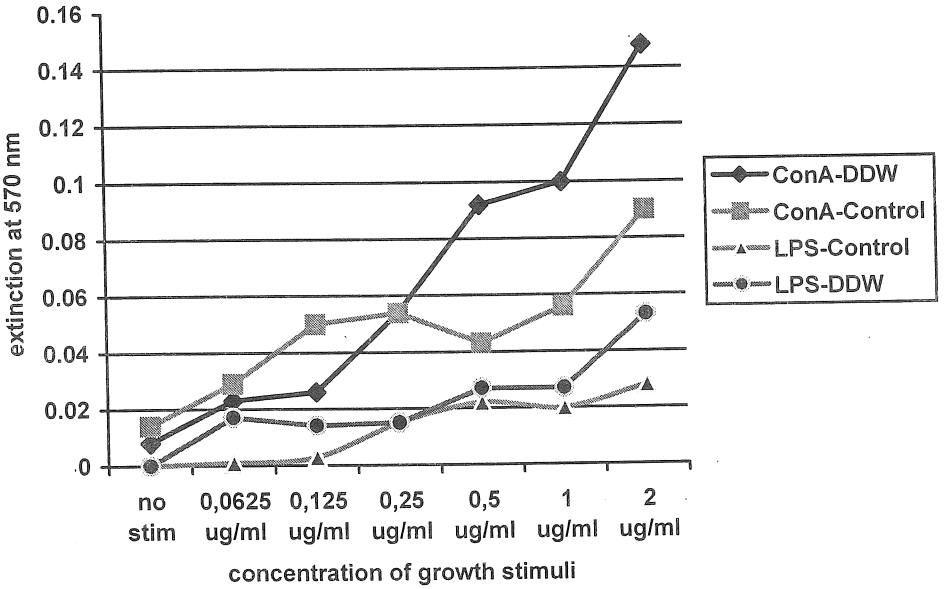

Fig. 6. – Proliferation of naive splenocytes under the influence of LPS and ConA, in environments with deionized water and deuterium-depleted water.

The splenocyte growth and metabolism were stimulated by the presence of DDW in the culture media in both groups, but in the case of the LPS stimulation the growth differences were not great enough to be statistically significant. However, when using concanavalin A as proliferation stimulus for native splenocytes, a significant growth stimulation in the DDW – group was observed, mainly for larger doses.

The results obtained using this quantification method are in agreement with those obtained through traditional methods of direct counting of cells in the culture media.

DISCUSSION

As the results presented above show, the presence of the growth media with DDW has stimulated the growth and replication, both for established lines of cells and for naive cells obtained from explants.

Given that deuterium in excess arrests mitosis (Schroeter *et al.*, 1992) through various mechanisms, including derangement of microtubule arrays in interphase (Lamprecht *et al.*, 1991) or inhibition of the transport of cations across the membrane (Vereninov *et al.*, 1985) and even cytotoxicity at the level of the immune cells (Joenje, 1983), it seems probable that a reduction in the environmental ratio of D^+/H^+, realized by the introduction of DDW in the culture media, might have reversing effects, like a stimulation of mitosis.

While in normal water the concentration of deuterium is around 140–150 parts per million atoms of hydrogen, in the DDW this concentration was reduced to 30 ppm deuterium/protons. Experiments of whole cell recording and patch-clamp (DeCoursey and Cherny, 1997) using heavy water demonstrated that deuterium is able to pass through proton channels only in a very reduced proportion. The deuterium conductance was even lower than the presumed one, obtained through calculation of mass solvent effects, situation that suggests that deuterium specifically interacts with channels and pumps involved in proton transport.

This situation might lead to an increased efficiency in the functioning of the proton exchangers, which would further produce an alkalinization of the cytosol, which might trigger the mitosis, situation objectivated by the above results.

These effects seem to be responsible for the radioprotective and immune stimulation presented elsewhere (Bild *et al.*, 1999) through a possible effect of enhancing the recovery of fast-proliferating cells belonging to the immune and hematopoetic systems, which are mainly affected by ionizing radiation or chemical toxicity. These effects seem to be due to the stimulation of non-specific proliferation triggers, mainly connected to the intracellular pH changes.

The effects of the inhibition of the membrane proton exchangers point toward the support of this working hypothesis. The involvement of the ubiquitous Na^+/H^+ exchanger does not seem probable from the above-mentioned results. However, more investigations are needed, involving studies of membrane dynamics of this pump, because the use of amiloride is extremely sensitive, the slightest increase in dosage leading to toxic effects and a complete replication block, followed by the death of the cell, while a decrease in the concentration of the pharmacological blocker reestablishes the proliferation curves to the normal aspect. On the other hand, an eventual predominance of the amiloride-resistant NHE_3 antiporter (Orlowski and Grinstein, 1997) in the cell lines taken in study might render useless the experimental approach using amiloride.

The involvement of the K^+/H^+ ATP-ase seems to be more clearly established, D_2O acting on it as a modifier of its ionic sensitivity (Lobyshev *et al.*, 1982), its

suppression with lansoprazole eliminating the differences in the proliferation rates between the test and control groups. However, the distribution of this K^+/H^+ ATP-ase, even considered almost ubiquitous (Kraut *et al.*, 2001), is not so clearly known for the membranes of the cells taken in study, so further studies, with cell lines where the K^+/H^+ ATP-ase is much clearer characterized, are necessary.

In what concerns the growth and division of immune cells obtained from live animals, it respects a similar aspect of stimulation, with minor differences regarding the proliferation stimuli.

It is known that ConA induces a rapid, amiloride-sensitive, sodium-dependent increase in the cytosolic pH, of 0.13 pH units, indicative of the stimulation of the Na^+/H^+ exchanger, which has as effect a triggering of cell division (Grinstein *et al.*, 1987). It is thus probable that such a stimulation of the antiporter is also produced by an increase in the cytosolic pH.

In the same time, the mechanisms responsible for the proliferation induced by the LPS are due to a completely other line of signaling, involving the activation of several types of transcription factors, particularly nuclear factor-kappaB (NF-kappaB) (Mukaida *et al*, 1996). This might account for the seemingly lack of response of the cells stimulated with LPS to the variations in the deuterium content induced by the use of DDW in the formation of the culture media.

Further studies are necessary for investigating the differences between the mechanisms through which LPS and ConA stimulate cell division and the involvement of hydrogen pumps or exchangers are necessary.

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