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Polyamine oxidase activity in rats treated with mitoguazone: Specific and permanent decrease in thymus

M. E. Ferioli and **A. Armanni**

Centro di Studio sulla Patologia Cellulare, C.N.R, c/o Istituto di Patologia Generale, Università di Milano, Milan, Italy

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Summary. To extend the knowledge on the role of polyamine oxidase in thymus physiology, we evaluated the in vivo effect of the polyamine biosynthetic pathway inhibitor mitoguazone. The drug markedly and permanently decreased the enzyme activity in the organ, in which the level of putrescine also decreased at the later times observed. A byproduct of the reaction catalyzed by polyamine oxidase is hydrogen peroxide, a well known inducer of apoptosis. The decrease in polyamine oxidase activity, with the consequent decrease in hydrogen peroxide production, is correlated with a positive effect on thymus physiology. Since mitoguazone has been successfully employed in patients with AIDS-related diseases, in which the reconstitution of the immune function is a favorable prognostic index, we hypothesized that mitoguazone may have the thymus as target organ, and that the decrease in polyamine oxidase activity may have a role in the positive effect of the drug.

Keywords: Polyamine oxidase – Spermidine/spermine N^1-acetyltransferase – Mitoguazone – Thymus – Rat

Introduction

There is increasing evidence that drugs can modulate the immune system via direct or indirect mechanisms. Alterations in immune functions due to exposure to xenobiotics may result in a change in an organism's ability to resist infectious disease or tumor cell growth, in hypersensitivity and autoimmune diseases, as well as in specific organ toxicity. The thymus gland is a central lymphoid organ in which bone marrow-derived T-cell precursors undergo a complex process of maturation, eventually leading to migration of positively selected thymocytes to the T-cell-dependent areas of peripheral lymphoid organs such as spleen and lymph nodes (reviewed by Savino and Dardenne, 2000).

Polyamines (putrescine, spermidine and spermine) are small molecular weight, positively charged compounds that are ubiquitous in all living cells and have many important roles in cellular physiology. Thus, compounds that interfere with polyamine metabolism have been used therapeutically for the treatment of cancer and infectious diseases. Polyamines have an intracellular salvage or catabolic pathway that leads from spermine to spermidine and then to putrescine. This pathway relies on the selective monoacetylation of the primary amino residues of spermine and spermidine followed by oxidation of the N-acetyl aminopropyl terminus by polyamine oxidase (EC 1.5.3.11, PAO).

Mammalian PAO transforms spermidine and spermine into putrescine and spermidine, respectively, and the polyamine oxidation by the enzyme results in the production of an aminoaldehyde and hydrogen peroxide (Seiler, 1995; Seiler and Atanassov, 1994). It was found that the role of polyamines is not restricted to growth-related processes. There are many reports confirming the importance of polyamine homeostasis for the normal functioning of the immune system (Seiler and Atanassov, 1994).

Additionally, polyamine metabolism has been demonstrated to be a chemotherapy target for Pneumocystis carinii, a common opportunistic pathogen associated with AIDS and other immunosuppressed conditions (Marton and Pegg, 1995).

Mitoguazone (MGBG) is an agent with a unique mechanism of action as an inhibitor of S-adenosylmethionine decarboxylase, a key enzyme in

polyamine biosynthetic pathway (Von Hoff, 1998). Clinical trials with MGBG as the antineoplastic drug were initiated in the early 1960s, but they were stopped because of the toxicity observed in a chronic (usually daily) schedule. After the demonstration of the extremely long plasma half-life of MGBG (Von Hoff, 1998), additional studies were carried out and clinical trials were conducted using weekly and every other week schedules of administration, thus reducing an accumulation of MGBG that could be responsible for the toxicity. More recently, MGBG has been successfully used in patients with AIDS-associated non-Hodgkin's lymphoma (Von Hoff, 1998), in which urinary polyamines were found to increase with progression and severity of the disease. Furthermore, polyamine levels are increased in cells infected with cytomegalovirus (Tyms and Williamson, 1982) and in lymphocytes from patients with HIV infection (Colombatto et al., 1989).

The absence of a functional thymus negatively affects clinical outcome of HIV infection (Haynes et al., 1999). Disease-induced immunosuppression is attenuated when thymic function was at least partially restored after antiretroviral regimens (Viganò et al., 2000). Accordingly, we hypothesize that MGBG may target the thymus, and that the drug may modify the activity of the catabolic enzyme PAO, which plays a role in thymus physiology. In our previous studies, we demonstrated that hormones, such as glucocorticoids and prolactin, which differently affect thymus physiology, in the same way exert a different effect on thymic PAO activity. In fact, the negative effect exerted by glucocorticoids was correlated with an induction of PAO activity (Ferioli et al., 1999a), whereas the positive action of prolactin in thymus was correlated with a decrease in enzyme activity (Ferioli et al., 2000). One could suppose that the treatment-related response of PAO activity may represent an important event specific for the thymus, because this enzyme seems to have a role in apoptosis (reviewed by Schipper et al., 2000). In addition, it has been suggested that PAO may play an important role in the regulation of interleukin-2 (IL-2) production (Seiler and Atanassov, 1994), and that the enzyme may have a general function in cellular immune defense mechanisms (Seiler, 1995; Seiler and Atanassov, 1994).

Based on these data, we initiated studies to examine the in vivo effect of MGBG administration to rats on PAO activity in the thymus with the notion that decreased PAO activity may explain the positive effect exerted by this drug in AIDS-associated diseases (Von Hoff, 1998). We also determined the activity of another polyamine catabolic enzyme, spermidine/spermine N^1-acetyltransferase (SAT), and polyamine contents. At the same time, we investigated the effect of MGBG administration on PAO activity in the spleen.

Materials and methods

Reagents

[acetyl-1-^{14}C]Acetylcoenzyme A (58 mCi/mmol) was purchased from Amersham International. All other chemicals were analytical-grade products and were purchased from Sigma, Merck or Boehringer-Mannheim.

Animals and treatments

All experiments were conducted in accordance with guidelines described in the NIH Guide for the Care and Use of Laboratory Animals. Male Wistar rats (35–38 days old, 150–170 g) (Charles River, Calco, Italy) were used in all experiments. They were housed under 12 h light/12 h dark controlled lighting conditions with free access to water and a standard rat chow diet. The animals were given a single ip injection of MGBG (80 mg/kg in saline solution) between 9:30 and 10:00 a.m. The dosage of the drug was chosen on the basis of previous reports showing an effect on SAT activity in rats (Pegg et al., 1985). We chose to administer the drug by a single ip injection because it has been shown that MGBG has an extremely long half-life (more than 5 days) and with this schedule the agent has minimal toxicity (Von Hoff, 1998). The rats were killed at different times (from 4 to 144 h) after drug or saline administration, and thymus and spleen were rapidly excised, frozen with solid CO_2 and stored at $-80°C$ until enzyme or polyamine assays were performed. Some control rats were killed at the end of the experimental period to avoid differences due to the body weight and the organ-to-body weight ratios. At all observation times, mean body weight and absolute and relative thymus and spleen weights were not significantly different in MGBG-treated rats and the corresponding vehicle controls killed at the same times.

Assay of PAO activity

PAO activity in homogenates was assayed by a previously reported method (Ferioli et al., 1999b), which measures the H_2O_2 formed due to the oxidation of N^1-monoacetyl-spermine by converting homovanillic acid into a highly fluorescent compound in the presence of horseradish peroxidase. Each enzyme determination was performed in duplicate, and assay blanks were carried out without substrate. The incubations were carried out in the presence of pargyline and aminoguanidine to inhibit monoamine oxidases and diamine oxidases present in the homogenate. The reaction was corrected for quenching by the addition of 1–2 nmol of H_2O_2 in place of the substrate. Fluorescence was measured at 323 nm excitation and 426 nm emission.

Assay of SAT activity

SAT activity was performed as previously described (Ferioli et al., 1999b). In brief, the cytosolic extract resulting from 1 h

centrifugation at 100,000 × g was used as a source of enzyme, and the activity was measured at 30°C with spermidine as a substrate. The conditions were such that the activity was proportional to the amount of added protein and the time of incubation. Each enzyme determination was performed in triplicate, and the blanks were incubates in the absence of spermidine. Enzyme activity was expressed as units per mg of added protein, where 1 units is equal to the production of 1 pmol/min of acetylated spermidine. Our results are based on measurements of total acetylating activity.

Polyamine analysis

Tissue samples were homogenized in 0.2 N perchloric acid and deproteinized. Aliquots of the supernatant were used for the determination of polyamines by HPLC with fluorescence detection as previously descried (Ferioli et al., 1999b). Polyamine levels were then calculated based on external standard curves run within 24 h of the sample chromatograms.

Cytofluorimetric analysis

Control and MGBG-treated thymuses were mechanically disaggregated, and thymocytes resuspended and prepared for cytofluorimetric analysis according to Pellicciari et al. (1996). 10^6 cells/ml were stained for DNA with propidium iodide (50 μg/ml in water containing 0.002% Nonidet NP40 and 100 units/ml of RNase type A, for 30 min). Measurements were taken with a FACStar flow cytometer (Beckton Dickinson, San José, CA) equipped with an argon laser excitation (power 200 mW) at 488 nm; red fluorescence signals were detected using a 610 nm longpass filter. When present, apoptotic thymocytes give rise to the typical sub-G1 peak in DNA histograms. In each experiment, at least 20,000 events were measured in the gated regions used for calculation.

Statistical analysis

One-way analysis of variance (ANOVA) was employed to determine differences between groups. A value of $P < 0.05$ was considered statistically significant.

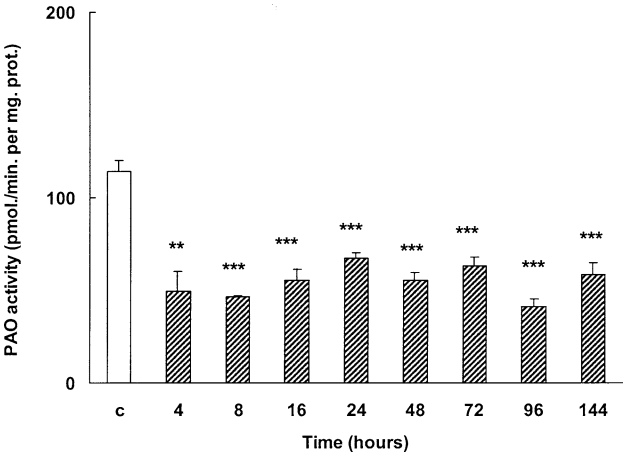

Fig. 1. Time course of the effect of MGBG on PAO activity in rat thymus. Data are the mean ± SE of 6–7 rats per group. All determinations were carried out in duplicate. ** $P < 0.005$; *** $P < 0.001$ compared with control (*c*) (saline-treated) group

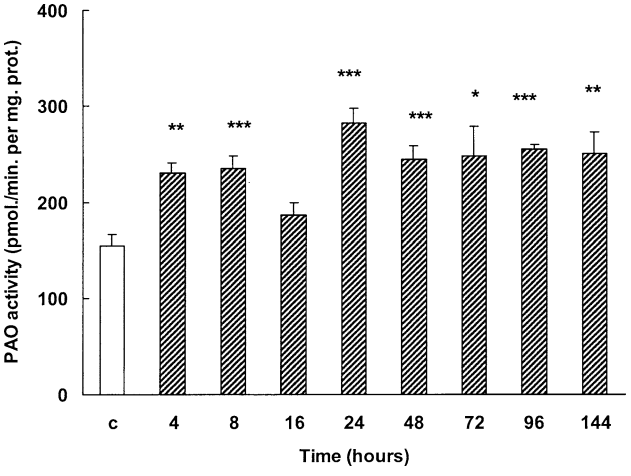

Fig. 2. Time course of the effect of MGBG on PAO activity in rat spleen. Data are the mean ± SE of 6–7 rats per group. All determinations were carried out in duplicate. * $P < 0.01$; ** $P < 0.005$; *** $P < 0.001$ compared with control (*c*) (saline-treated) group

Results

The time course of the effect of MGBG administration on PAO activity was examined in lymphoid organs of the rats. Figure 1 shows the results of the treatment in the thymus, in which the enzyme activity was dramatically decreased by MGBG at all observation times after administration. The decrease was permanent until the end of the observation period and could have generated an immuno-stimulating effect, as has been found with PAO inhibitors (Seiler and Atanassov, 1994).

The effect of MGBG on PAO activity in the thymus seemed to be specific, because in the other lymphoid organs, i.e., the spleen, the drug had an opposite effect on enzyme activity, with an increase at all the observation times (see Fig. 2). Moreover, determination of the time course revealed that the decrease in thymic PAO activity and increase in splenic PAO activity were parallel processes.

Since previous studies have reported an induction of SAT activity in response to a number of toxic insults (Casero and Pegg, 1993), and particularly after MGBG in some tissues (Karvonen and Pösö, 1984; Pegg et al., 1985), SAT activity was measured in the same rats. As can be seen in Fig. 3 (Panel A), SAT activity levels in the thymus of treated rats were not significantly different from the control throughout all the observed period. In the spleen, in accord with

previous data showing that MGBG only marginally increased the enzyme activity in the organ (Pegg et al., 1985), we found a small increase in SAT activity, which was statistically significant only at 24, 48 and 96 h after MGBG administration (see Fig. 3, Panel B).

To study the correlation between enzyme activities and the content of polyamines, polyamine determinations were carried out on the same tissues used for enzyme assays. As shown in Figure 4, administration of MGBG increased the thymic level of putrescine at 8 and 16 h after administration. At later times (96 and 144 h), the drug significantly decreased putrescine level in the thymus. However, in the same organ neither spermidine nor spermine concentrations showed significant alterations (see Fig. 4). Thus, we suppose that the increase in putrescine level observed at early time points after MGBG administration (see Fig. 4) could have been primarily due to the decrease in terminal oxidative deamination, as a result of inhibition of diamine oxidase (DAO) activity by the drug (Höltta et al., 1973), as well as to ornithine decarboxylase (ODC) induction (Karvonen and Pösö, 1984). The decrease in putrescine level at later times after MGBG administration was in accord with data showing the therapeutic potentials of polyamine biosynthesis inhibitors in some immune-related diseases (Seiler and Atanassov, 1994).

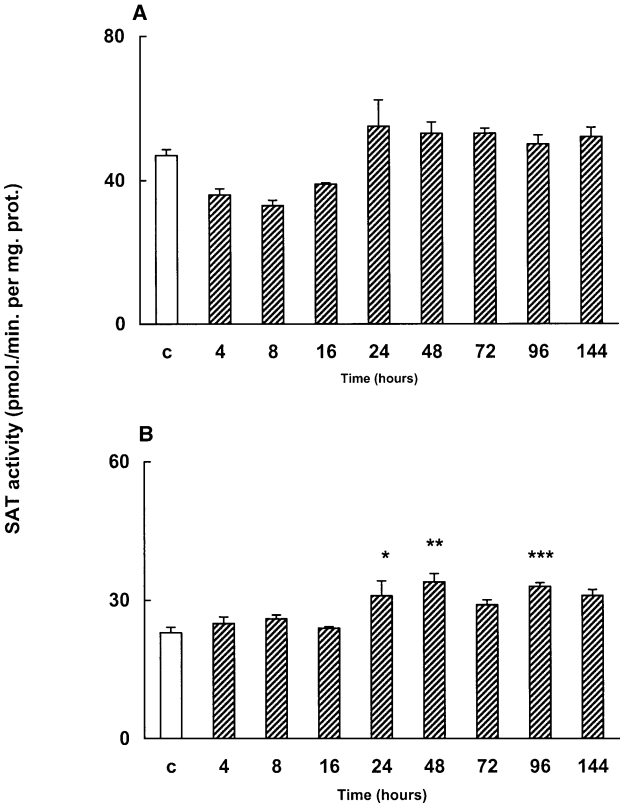

Fig. 3. Time course of the effects of MGBG on SAT activity in rat thymus (Panel **A**) and spleen (Panel **B**). Data are the mean ± SE of 4–5 rats per group. All determinations were carried out in triplicate. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$ compared with control (*c*) (saline-treated) group

As can be seen in Fig. 5, polyamine levels in spleen of MGBG-treated rats were correlated with enzyme activities, especially PAO activity. In fact, in the first 24 h after treatment, MGBG decreased spermidine and increased putrescine content as a result of the marked increase in PAO activity at these times.

To confirm our hypothesis about a correlation between PAO activity and apoptosis in the thymus, we performed DNA analysis. The formation of DNA ladders, an indicator of apoptotic cell death, was not observed in thymuses of MGBG-treated rats (results not shown). Moreover, in order to be sure that there was no apoptosis in thymuses of treated rats, we performed a cytofluorimetric analysis of our samples. The results (reported in Fig. 6) confirmed that MGBG did not induce cell death in the thymus.

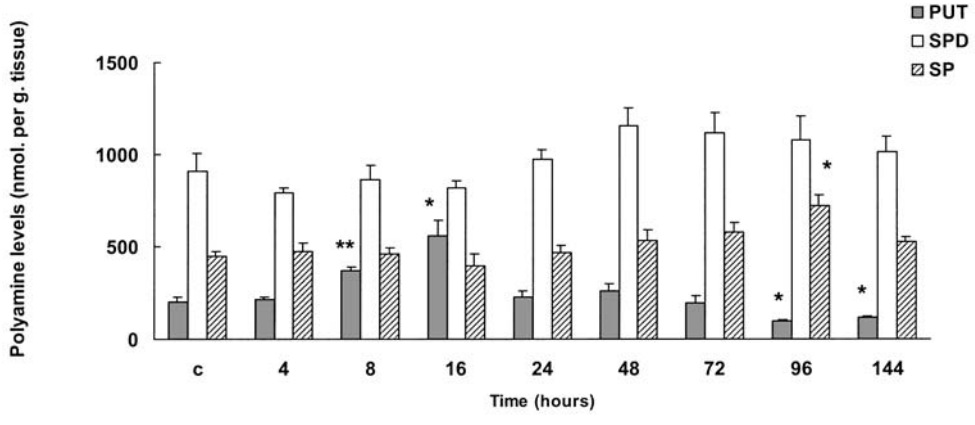

Fig. 4. Time course of the effects of MGBG on polyamine levels in rat thymus. Data represent the means ± SE of values from 4–6 rats per group. All determinations were carried out in triplicate. * $P < 0.05$; ** $P < 0.005$ compared with control (*c*) (saline-treated) group

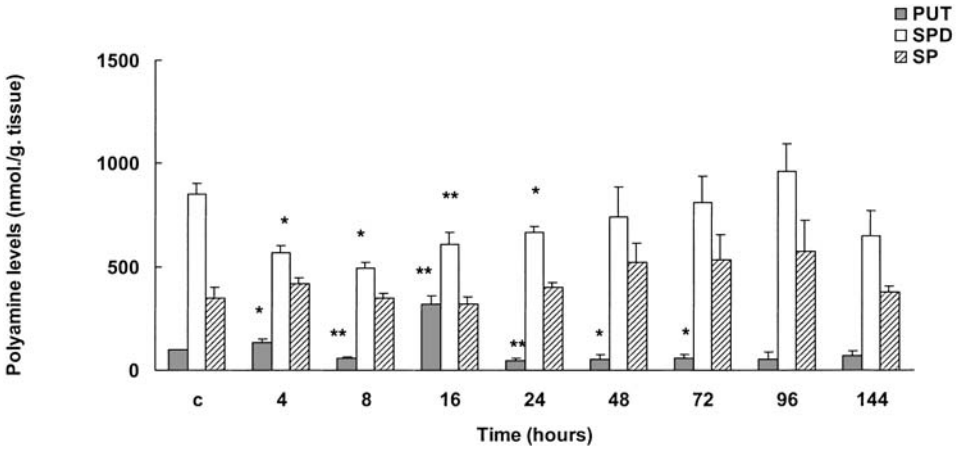

Fig. 5. Time course of the effects of MGBG on polyamine levels in rat spleen. Data represent the means ± SE of values from 4–6 rats per group. All determinations were carried out in triplicate. * $P < 0.05$; ** $P < 0.005$ compared with control (*c*) (saline-treated) group

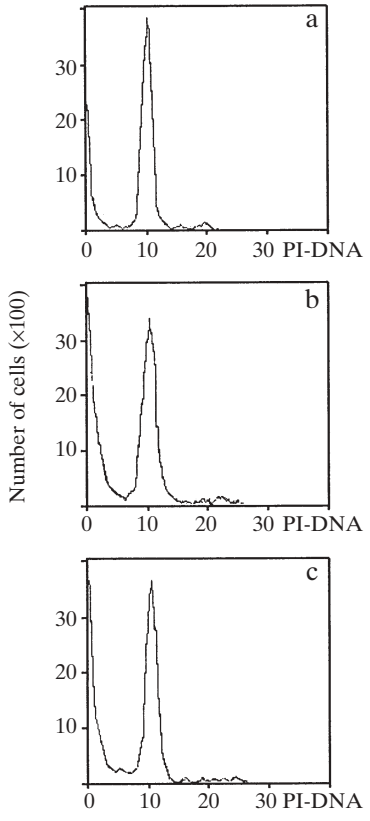

Fig. 6. DNA-histograms after propidium iodide (PI) staining in control (saline-treated) (**a**), in 24-h MGBG-treated (**b**), and in 144-h MGBG-treated (**c**) thymocytes. The fraction of dead apoptotic cells (at the left of the GI peak) did not increase in treated samples, compared with the controls. Similar results have been obtained in three independent experiments

Discussion

Many reports have confirmed the importance of polyamine homeostasis for normal functioning of the immune system, although some data are conflicting (reviewed by Seiler and Atanassov, 1994). Putrescine prevents the inhibitory effect of difluoromethylornithine (DFMO, a well-known inhibitor of ODC activity) on the immune response (Seiler and Atanassov, 1994). However, inhibitors of ODC can produce immunostimulating or immunosuppressive effects (Seiler and Atanassov, 1994).

Substances that interfere with polyamine biosynthesis or function have considerable potential for use as anti-cancer drugs (Marton and Pegg, 1995; Seiler et al., 1998). Since some anti-cancer drugs work in combination with a reduction of polyamine levels in malignant tissues, the endogenous polyamine concentration should be taken into consideration to achieve the best therapeutic outcome. Mice grafted with the Lewis lung carcinoma exhibited immune suppression, which was reversed by a polyamine-deficient diet and inhibitors of ODC and PAO (Seiler et al., 1998). In particular, inhibition of PAO activity has been recently proposed as a potential therapeutic approach against tumor cells (Seiler et al., 2000).

The thymus and spleen both fulfill central functions in the immune system of higher animals. These organs are highly sensitive to damage by xenobiotics and are thus examined routinely in connection with immunotoxicological evaluations. The thymus represents the major site of the production and generation of T cells expressing $\alpha\beta$-type T-cell antigen receptors. Moreover, the effects of thymic hormones are not restricted within the immune system, but are rather pleiotropic and potentiate the efficacy of antineoplastic drugs (reviewed by Savino and Dardenne, 2000).

During HIV infection there is a profound loss of $CD4^+$ cells due to direct and indirect cell death and to a failure to replace $CD4^+$ cells. Progressive destruction

of the thymus and other secondary lymphoid organs in HIV infection can have severe long-term consequences, since thymopoiesis plays a central role in T-cell homeostasis in HIV-infected individuals (reviewed by Evans et al., 2000). Thus, in cases of immune depletion due to HIV infection, the ability of the thymus to regenerate a complete T-cell receptor repertoire is important for full immune system recovery (Evans et al., 2000). Accordingly, after antiviral therapy there is a rapid and sustained increase in thymic output, and the reconstitution of immune function is a favorable prognostic index in HIV disease (Viganò et al., 2000).

The present study was addressed to the oxidative catabolism of polyamines via PAO using an in vivo model, since modifications of this enzyme activity could have a role in regulation of the apoptotic process (Ferioli et al., 1999a, 2000; Schipper et al., 2000) and of IL-2 production (Seiler and Atanassov, 1994). Deregulation of apoptosis has been associated with diseases such as cancer, autoimmunity and AIDS. Therefore, the identification of modulators of apoptosis has several therapeutic implications (Schmitz et al., 2000), since apoptosis may represent a mechanism which possibly arrests the immune response. Addition of MGBG to cultured thymocytes has been proved to induce apoptosis, and the process was reversed by spermine (Brüne et al., 1991). However, removing thymocytes from their microenvironment makes the cells more susceptible to some stimuli (Moore et al., 1992). As regards polyamine metabolism, in isolated thymocytes there is a complete disappearance of the high levels of ODC characteristic of the thymus of young rats (Stefanelli et al., 1992). Thus, we chose to use an in vivo system as a model to study the effect of MGBG on PAO activity in the thymus in order not to alter the physiological status of the organ.

One can suppose that the positive effect exerted by MGBG in patients with AIDS-related diseases (Von Hoff, 1998) may also be mediated through its effect on polyamine catabolism. Pentamidine, a substance which is frequently used in patients with AIDS to treat their Pneumocystis carinii pneumonia, inhibits the same enzymes that MGBG inhibits (Balana-Fouce et al., 1986; Libby and Porter, 1992). Accordingly, our data showed a marked decreased in thymic PAO activity after MGBG administration, thereby suggesting that the positive effect of the drug in HIV-associated diseases (Von Hoff, 1998) could be exerted, at least in part, through its effect on the enzyme.

Since it has been reported that hydrogen peroxide production by PAO had a role in polyamine analogue-induced apoptosis (reviewed by Schipper et al., 2000), the significant decrease in thymic PAO activity, with a possible consequent decrease in hydrogen peroxide formation, may contribute to the maintenance of an appropriate thymic function and to counteract involution of the organ due to HIV infection (Viganò et al., 2000). MGBG also inhibits thymic DAO activity (Höltta et al., 1973), thus further decreasing hydrogen peroxide production. Low glucocorticoid concentrations, which exert stimulatory effect on immune response (Wilkens, 1995) and antagonize T-cell receptor-mediated apoptosis in thymocytes (Savino and Dardenne, 2000), also decrease PAO activity (Ferioli et al., 1999a). Moreover, inhibition of polyamine oxidation by N,N′-bis(2,3-butadienyl)-1,4-butanediamine (MDL-72,527, an inactivator of tissue PAO) generates an immuno-stimulating effect in T cells (Seiler and Atanassov, 1994). In addition, hydrogen peroxide formation by PAO decreased the production of IL-2, and inhibition of oxidation prevented the down-regulation of IL-2 formation by polyamines (Seiler and Atanassov, 1994). The central role of IL-2 within the immune system emphasizes the importance of the regulation of T-cell functions by polyamines (Seiler and Atanassov, 1994).

Previous data demonstrated that the therapeutic effect of DFMO was much better for female than for male mice (Ask et al., 1992). We recently showed a sexual dimorphism in PAO, with lower activity in female than in male rats (Ferioli et al., 1999b). Moreover, although male rats had higher enzyme activity than females, in the thymus PAO level was lower than in other tissues, e.g., in the spleen (Ferioli et al., 1999b), thus suggesting that the decrease in thymic PAO activity may have a role in the positive effects of MGBG in HIV-related diseases. It is well known that in tumors PAO activity is lower than in equivalent normal tissues, and this low activity may contribute to the low level of apoptosis seen in tumor cells (Schipper et al., 2000).

To the best of our knowledge, this study provides the first proof that, when administered in vivo, MGBG exerts a positive effect on the thymus, in contrast to the apoptotic effect exerted in cultured thymocytes (Brüne et al., 1991). This result is consistent with recent data showing a protective effect of MGBG against ethanol-induced damage of the gastric mucosa (Al-Shabanah et al., 2000) and suggests that the drug

may have positive effects on different organs. Future studies may be addressed to determine whether animal data can be directly applied to and correlated with positive health effects in humans.

Another interesting finding from the study was that MGBG differently affected the activity of PAO in the thymus and in the spleen, with a decrease in the former and an increase in the latter organ (see Figs. 1 and 2). The difference once again confirms that the regulation of enzyme activity is organ specific (Ferioli et al., 1999a, 2000, and this study). Moreover, the inhibition of PAO activity by MGBG in the thymus represents another step in which this drug may influence polyamine metabolism in animals treated with the drug.

Finally, the recent cloning and characterization of human PAO (Wang et al., 2001) will provide a useful tool to clarify the role of the enzyme in regulation of many important metabolic pathways.

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Authors' address: Dr. Maria Elena Ferioli, Centro di Studio sulla Patologia Cellulare, C.N.R, c/o Istituto di Patologia Generale, Via Mangiagalli 31, I-20133 Milano, Italy, Fax +39-02-50315338; E-mail: MariaElena.Ferioli@unimi.it