Cancer Chemother Pharmacol (2005) 56: 659–665
DOI 10.1007/s00280-005-1014-3

ORIGINAL ARTICLE

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Methylene blue is more toxic to erythroleukemic cells than to normal peripheral blood mononuclear cells: a possible use in chemotherapy

Received: 5 November 2004 / Accepted: 22 January 2005 / Published online: 29 July 2005

Abstract Methylene blue (MB) is a phenothiazine with radio and photosensitizing properties and anti-tumoral activity. Our group has shown that MB was capable of inhibiting the in vitro growth of erythroleukemic cells with multidrug resistance (MDR). However, there are no studies comparing the cytotoxicity of this molecule for normal and tumoral cells. In this work, the cytotoxicity of MB was measured by MTT method in erythroleukemic and melanoma lineages, comparing it with that of normal cells:lymphocytes and melanocytes. MB was more cytotoxic for tumoral cells; however, there was no difference between erytroleukemic cells with or without MDR phenotype. Lymphocytes and erythroleukemic cells were much more sensitive to the effects of MB than melanoma cells and melanocytes. The proliferation of phytohemagglutinin-activated lymphocytes was inhibited when ^{3}H-thymidine incorporation to DNA was measured. We tried to analyze whether the cells were dying, via apoptosis or necrosis, using Anexin-V and propidium iodide. Despite higher levels of Anexin-V, it was not possible to distinguish necrosis from apoptosis, as the fluorescence of MB is in the same channel as propidium iodide. The production of hydrogen peroxide was measured by cytometry using dihydrorhodamine 123 (DHR). Despite the erythroleukemic cells and lymphocytes being capable of producing free radicals, there was no relation between the production and the sensitivity of various cells to MB. Our results suggest that MB should be used as a chemotherapeutic agent, because of its preferential cytotoxic effects over tumor cells, considering the fact that MDR cells are also sensitive, and due to its radio and photosensitizing activities.

Keywords Methylene blue · Peripheral blood lymphocytes · Reactive oxygen species · Multidrug resistance · Erythroleukemia

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Introduction

Treatment of multidrug resistant (MDR) tumors is one of the major concerns of oncologists because these tumors are refractory to conventional chemotherapy. The best understood mechanism of MDR is conferred by P-glycoprotein (Pgp), which pumps out of the cells a number of unrelated drugs. Much effort has been expended to circumvent this problem, and one of the most promising approaches is the use of Pgp modulators, including some phenothiazines [9].

Methylene blue (MB) is a phenothiazine with strong photodynamic properties, retained mainly in malignant tissues. It has been used in a variety of clinical approaches, such as staining of tumors for diagnosis and surgery [6, 14] and labeling of sentinel lymph node [11]. Its use in cancer chemotherapy has also been suggested, due to its photodynamic properties [7]. It was also shown that the photodynamic treatment with MB could modulate the multidrug resistance phenotype [15], substantiating its use in cancer chemotherapy.

We have previously shown that MB alone (without light) were cytotoxic to erythroleukemic cells and could also modulate the MDR phenotype in those cells [13]. Therefore, MB either associated or not with light, could be used to treat not only solid tumors, but also those originated from peripheral blood. Indeed, it was observed that MB inhibited the growth of leukemic cells in mice [4]. However, there is at present no study comparing its effects in tumor and normal cells. The purpose of the present work was to compare the cytoxicity of MB in two erythroleukemic cell lines and in normal

peripheral blood mononuclear cells (PBMC) activated or not with phytohemagglutinin (PHA).

Materials and methods

Cells

The human erythroleukemia cell line K562 and its MDR derivative K562-Lucena 1 [10] were cultured in RPMI 1640 medium (SIGMA, USA) with 10% fetal bovine serum (FBS, GIBCO, USA) in a concentration of 2×10^4 cells/ml.

PBMC were obtained by the separation of heparinized blood from healthy volunteers on Ficoll-Hystopaque (SIGMA, USA) density gradient centrifugation. After washing, PBL fraction was suspended in RPMI 1640 medium with 10% FBS, followed by incubation in culture dish for 1 h at 37°C in 5% CO_2 atmosphere for macrophage depletion. The cell number was then adjusted to 5×10^5 cells/ml.

Treatment with PHA

PBL at the concentration of 5×10^5 cells/ml were incubated, either in 15 ml conical tubes or directly in a multiwell plate, with 5 μg/ml of PHA (SIGMA, USA) for different times at 37°C in 5% CO_2.

Proliferative assay using [^{3} H]-thymidine incorporation

Cells were incubated in a 96-well plate. After a total culture time of 72 h, measurements of cell proliferation were made by determining the incorporation of ^{3} H-thymidine (ICN Radiochemicals, USA), which was added to the culture 6 h before the end of the experiment. After that, the cells were harvested and radioactivity assessed with liquid scintillator.

Cellular viability

The viability of the cells was measured by thiazoyl blue colorimetric method (MTT). The cells were incubated in a 96-well plate with 20 μl of 5 mg/ml MTT 4 h before the end of the experiment. The plate was centrifuged and the pellet suspended in DMSO (SIGMA, USA). The optical density (OD) was measured in an ELISA reader, using the wavelength of 490 nm.

Treatment with MB

Various concentrations of MB (MERCK, Germany) were added to PBL before or after deactivation with PHA (5 μg/ml). *Before activation:* PBL were incubated with MB for 4 h. After that, the cells were washed and incubated in medium alone or in medium with PHA for the next 68 h. *After activation*: PBL were incubated with PHA for 48 h. After that, the cells were washed and incubated with medium alone or medium with MB for the next 24 h. After24 h, the viability of the cells was measured by MTT.

Measurement of MB uptake by flow cytometry

PBL (5×10^5 cells/ml) were incubated with 5 μg/ml PHA and various MB concentrations for different periods of time. The cells were then washed with PBS and the incorporation of MB into the cells was analyzed by flow cytometry in a Beckton–Dickinson cytometer (FACS Calibur).

Formation of hydrogen peroxide

The leuco probe dihydrorhodamine 123 (DHR) (Molecular Probes, USA), which preferentially reacts with H_2O_2 and is oxidized into the fluorescent dye rhodamine 123, was used to assess H_2O_2 production. Both the cell lines were pre-incubated for 15 min with 1 μM DHR and then treated with 1 mM H_2O_2 or MB for various times at 37°C. Cells were then washed twice in PBS and kept on ice. Flow cytometry analyses were performed, as described earlier.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), considering the treatments as factors. The

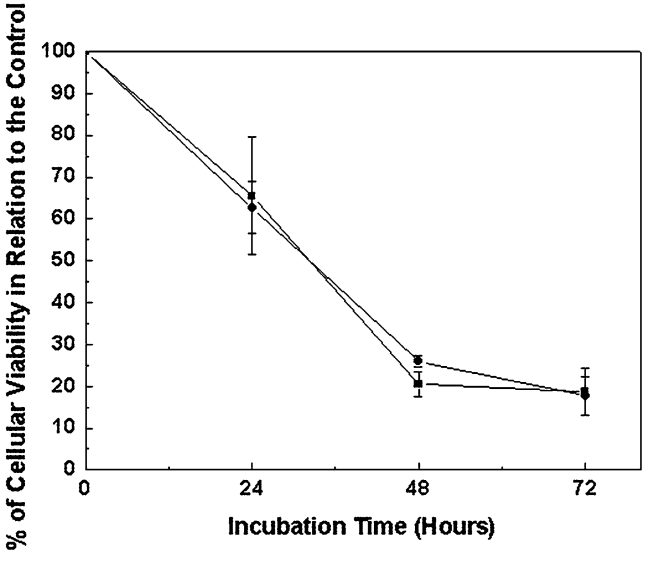

Fig. 1 Time-course of viability of leukemic cells following exposure to MB (2 μg/ml). Results are expressed as percentage of MB treated cells in relation to untreated cells. (●) Lucena cells and (■) K562 cells

significance of differences was verified by post-test Newman–Keuls multiple comparison test. Each mean was obtained from at least three different experiments.

Results

Sensitivity of erythroleukemic cells and normal PBMC to MB

We have previously shown that only about 30% of K562 and Lucena cells were viable after treatment with MB for 48 h [13]. Figure 1 shows that the toxicity of MB is already seen after incubation for 24 h, but it reaches a maximum after 48 h. Thus, this time was used in the next experiments.

To verify whether there were differences in sensitivity to MB between normal and tumor cells, PBMC obtained from healthy volunteers, as well as the erythroleukemic K562 and Lucena cells, were incubated with various MB concentrations during 48 h and the cellular viability was measured by MTT. K562 and Lucena cells were much more sensitive to the effects of MB than PBMC (Fig. 2). To analyze data mathematically, a curve fitting was performed. Data were best fitted by a first order exponential decay: $y = y_0 + A_1\ e^{-\ x/t}$. The LD50 was 0.078 μg/ml for K562 cells, 0.180 μg/ml for Lucena cells and 3.530 μg/ml for PBMC. Moreover, the rate of decay was greater for K562 cells (values of t were 0.083 for K562, 0.197 for Lucena, and 0.545 for PBMC), suggesting that Lucena cells are in fact more resistant to MB-cytotoxicity than K562 cells, for MB concentrations below 0.5 μg/ml ($p < .05$). However, for concentrations equal to or above 0.5 μg/ml, the differences in the viability of K562 and Lucena cells were not statistically significant, corroborating the data obtained previously by Trindade et al. [13].

Free radical production

It has been shown that MB can induce the formation of reactive oxygen species (ROS) [3], and some studies related ROS production to MB-induced cell death [5, 2]. Thus, it was tested whether MB-induced cell death observed in the present study was due to free radical production. In order to do this, the cells were incubated with the non-fluorescent probe DHR, which is oxidized to rhodamine 123 in the presence of reactive oxygen species. In Fig. 3, it can be seen that 2 μg/ml MB increased ROS in all cells studied, which is time-dependent. However, there were striking differences in the cellular behavior regarding ROS production. The greater increase was seen in K562 cells. Interestingly, ROS production by Lucena cells apparently reached saturation after 5 min of incubation with MB. This may be due to the fact that this cell line over-expresses P-glycoprotein, which is able to export rhodamine 123 out of the cells [10], and this may be masking the measurement of ROS production. On the other hand, we have previously shown that Lucena cells have about 4× more catalase activity than K562 cells [12]. Thus, the apparently saturation of ROS production by Lucena cells may be due to either a smaller intracellular retention of Rho123 or increased catalase activity. However, despite the possible difficulties in actually measuring ROS production by Lucena cells, it is clear

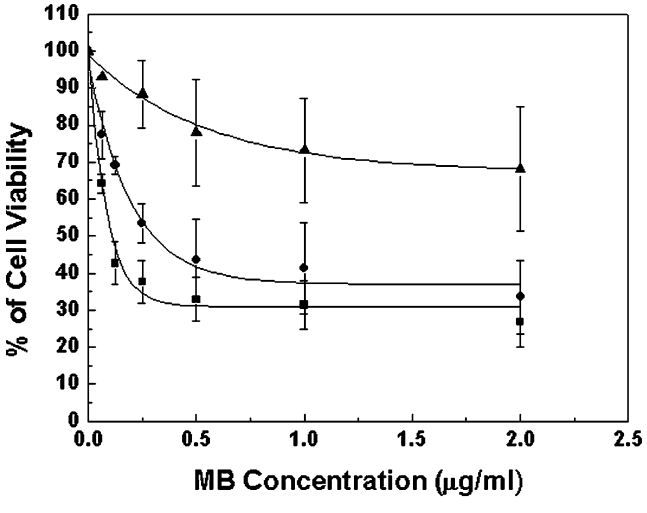

Fig. 2 Effect of MB on tumor cells and normal PBMC. Cells were exposed to different concentrations of MB for 48 h. Results are expressed as % of viable cells in relation to control (untreated) cells and represent mean ± SEM of three independent experiments. (▲) PBMC, (●) Lucena cells, and (■) K562 cells. $p < .05$ between PBMC and Lucena and also between PBMC and K562, analyzed with one-way ANOVA and with post-test Newman–Keuls multiple comparison test

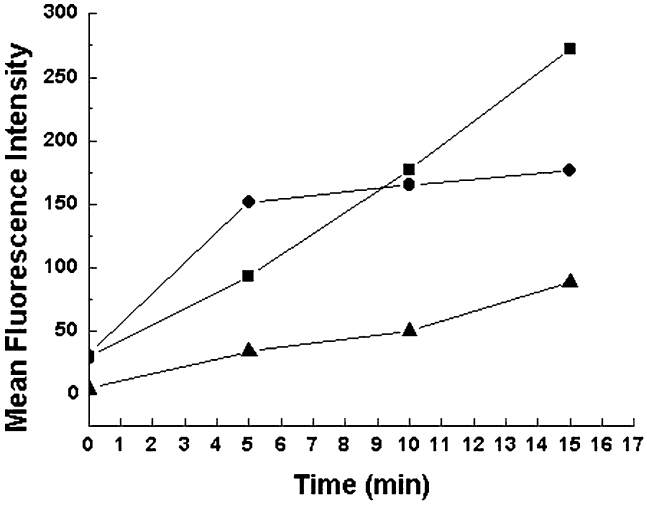

Fig. 3 Free radical formation. All the cells were adjusted to 5×10^5 cells/ml in PBS and pre-incubated with 1 μM dihydrorhodamine for 30 min. Methylene Blue (2 μg/ml) was added and the measurements were performed at the times indicated in the figure. (▲) PBMC, (●) Lucena cells, and (■) K562 cells

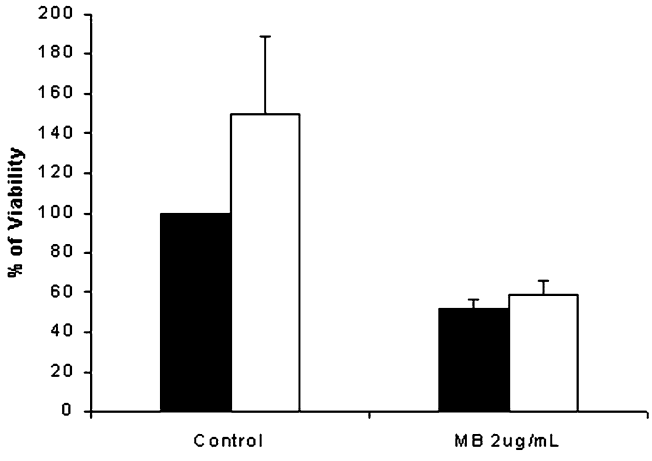

Fig. 4 Effect of MB on the viability of activated lymphocytes. PBMC were stimulated or not with 5 μg/ml of PHA for 48 h in the presence of various concentrations of MB. Results are expressed as % of viable cells in relation to untreated cells and represent mean ±SEM of three independent experiments. *Black columns*: resting PBMC; *white columns*: PBMC stimulated with PHA

that they produced much more ROS than PBMC, suggesting that ROS is indeed related to MB-induced cell death.

MB-sensitivity of activated lymphocytes

Proliferating cells are usually more sensitive to physical and chemical agents, and this could be the reason why K562 and Lucena cells are more sensitive to MB than resting PBMC. Therefore, the MB-sensitivity of resting and PHA-activated PBMC were compared. In Fig. 4, it is shown that, although PHA stimulated PBMC growth, there is virtually no difference in MB-sensitivity of PBMC activated or not with PHA. This result suggests that normal PBMC are indeed more resistant to MB than the erythroleukemic cells.

MB-uptake by subpopulations of PBMC

From Fig. 4, it seems that there is a subpopulation resistant to MB cytotoxicity, which is independent of PHA-activation. We observed that cells incubated with MB became light or dark blue. Therefore, to observe whether the incorporation of MB is related to cell death, PBMC were incubated with 2 μg/ml MB and the cellular fluorescence was measured by flow cytometry. In Fig. 5, it is clear that the percentage of cells incorporating MB

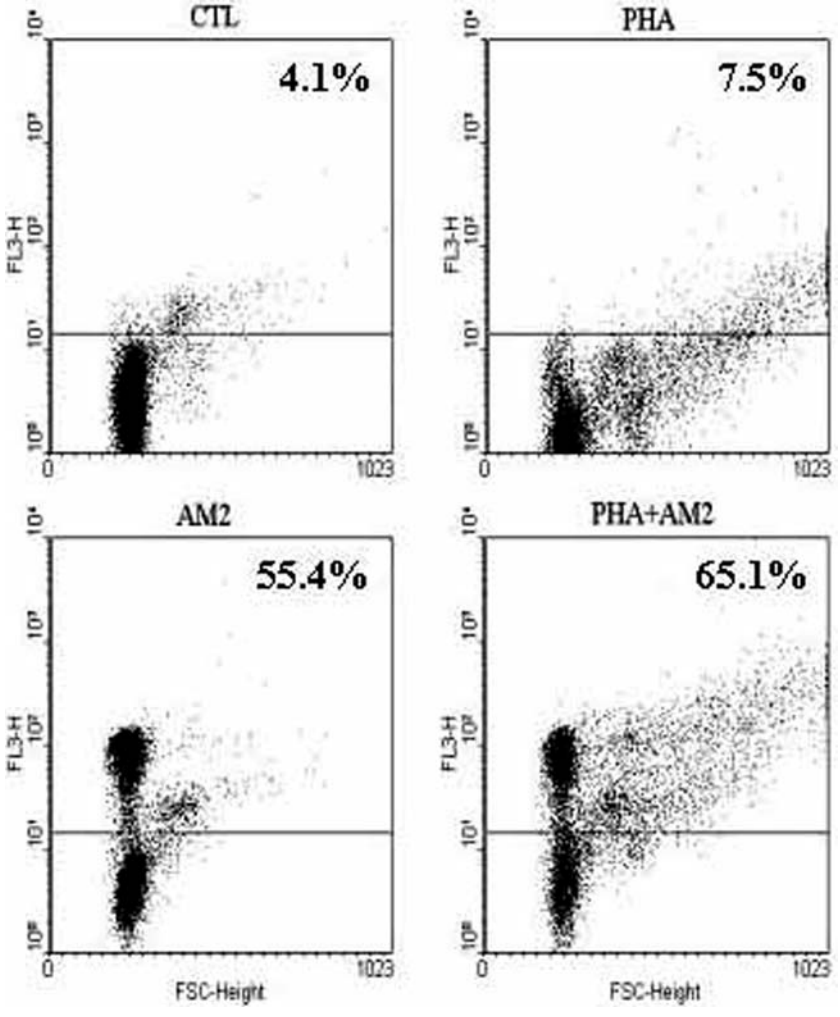

Fig. 5 Measurement of MB uptake by the cells. PBMC were incubated with 2 μg/ml MB for 4 h and the fluorescence intensity was measured by flow cytometry. Experiment representative of three different experiments. Numbers refer to the percentage of cells fluorescing

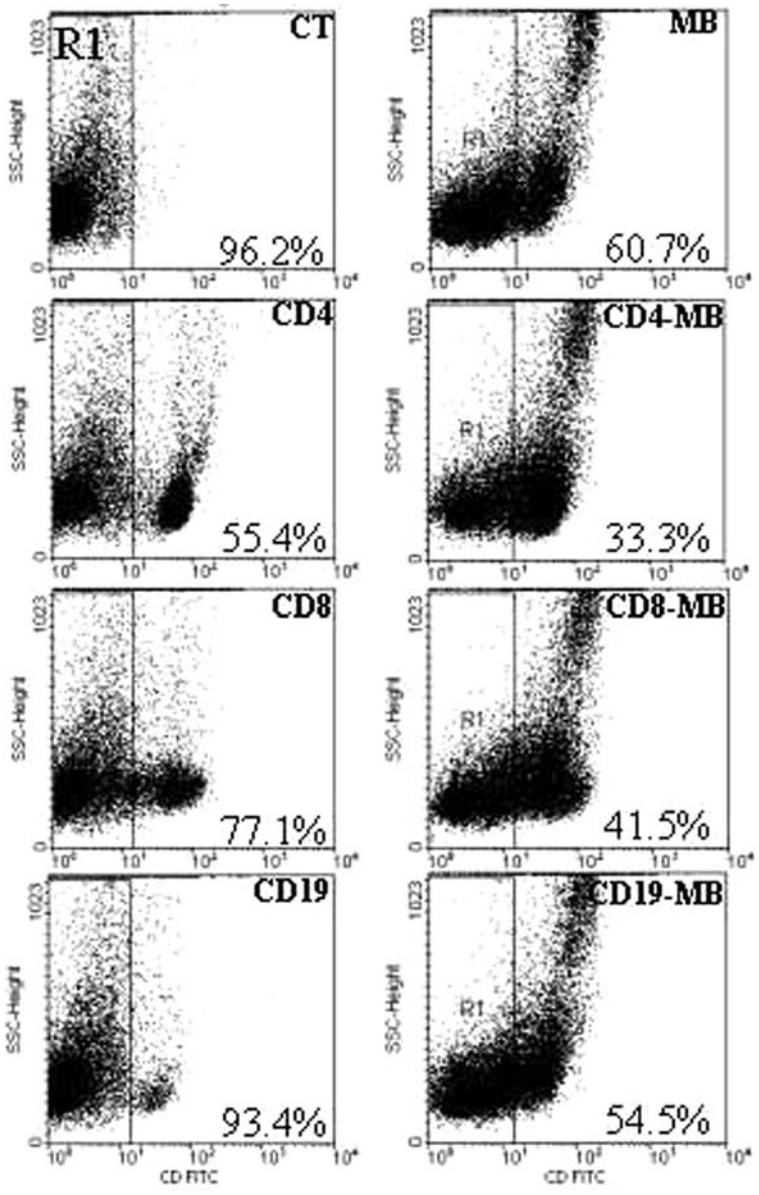

Fig. 6 Uptake of MB by lymphocyte subpopulations. PBMC were incubated with 2 μg/ml MB for 4 h and subsequently labeled with antibodies against CD4, CD8, and CD19. Experiment representative of three different experiments. *Left panel*: cells incubated only with antibodies. *Right panel*: cells incubated with MB and subsequently incubated with antibodies. Numbers refer to the percentage of cells remaining in region R1

is very close to that of cells being inactivated by MB (Fig. 4), and there is virtually no difference between activated and non-activated PBMC.

To observe whether there is a subpopulation of lymphocyte retaining more MB, cells were incubated with MB, subsequently incubated with antibodies against CD4, CD8, and CD19 and analyzed by flow cytometry (Fig. 6). As MB fluorescence interferes with antibody labeling, the analysis was performed using the population of cells that did not label with MB or with any antibody (region R1 in Fig. 6). It can be seen that approximately 55% of cells were $CD4^-$, 77% were $CD8^-$, and 93% were $CD19^-$. After incubation with MB, roughly 22% of the $CD4^-$, 35% of $CD8^-$, and 39% of $CD19^-$ cells incorporated MB, suggesting that there is no significant difference in MB-sensitivity in the subpopulations studied.

Inhibition of lymphocyte proliferation by MB

The results presented earlier suggest that MB is capable of inhibiting PHA-activation or, alternatively, MB prevents proliferation of PHA-activated lymphocytes. In order to study this issue further, PBMC were incubated with 2 μg/ml MB in two distinct situations: before PHA-activation and 4 h after PHA activation. The results are presented in Fig. 7a, b. In Fig. 7a, it is clear that pre-incubation with MB for 4 h is sufficient to completely block PHA-activation. However, Fig. 7b suggests that MB is also able to impair proliferation of lymphocytes previously activated with PHA. To confirm this, the [^{3}H]-thymidine assay was used to measure inhibition of DNA synthesis by MB. In Fig. 8, it is shown that, as expected, 2 μg/ml MB completely inhibited the DNA synthesis in PHA-activated lymphocytes.

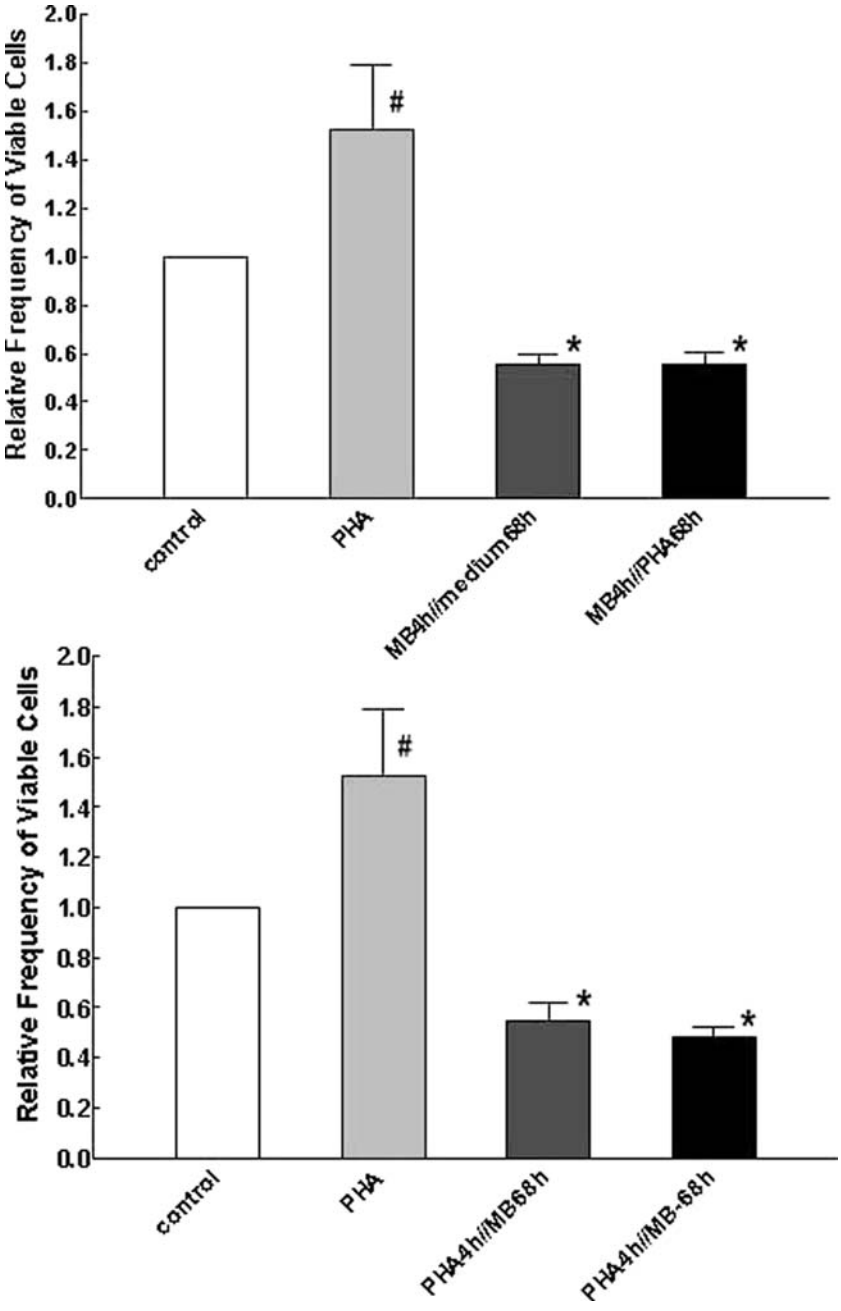

Fig. 7 Effect of Methylene Blue before and after activation with PHA. *Upper panel*: PBMC were incubated with 2 μg/ml of MB for 4 h, washed and incubated or not with 5 μg/ml of PHA for 68 h. *Lower panel*: PBMC were stimulated with 5 μg/ml of PHA for 4 h, washed and incubated with MB (2 μg/ml) for 68 h. Results are expressed as % of viable cells in relation to untreated cells and represent mean ± SEM of three independent experiments.# $p < .05$ when compared to control and * $p < .01$ when compared to PHA, analyzed with one-way ANOVA and Newman–Keuls multiple comparison test

Discussion

As other phenotiazines, such as trifluoperazine, have been shown to inhibit lymphocyte proliferation [1, 8], it is necessary to study the effects of MB in normal cells from peripheral blood.

In a previous study, we showed that MB alone (without light) was equally cytotoxic for two human erythroleukemic cell lines, K562, and the MDR K562-Lucena 1 [13]. Those results suggested the possible use of MB as a chemotherapic, besides its use as a photodynamic agent, in the treatment of tumors, including MDR leukemia. However, the toxicity of MB to normal lymphocytes was not known. Because there are evidences that another phenothiazine, trifluoperazine, is toxic to lymphocytes and inhibit many of their functions [1, 8], it was necessary to evaluate the toxicity of MB to normal lymphocytes.

In the present study, we showed that PBMC from normal volunteers are much more resistant than erythroleukemic cells to the toxic effects of MB. Furthermore, MB-treated erythroleukemic cells produced greater amounts of ROS, compared to lymphocytes, in agreement with other authors [3]. This suggests that ROS production is related to MB-induced cytotoxicity in both erythroleukemic and normal PBMC.

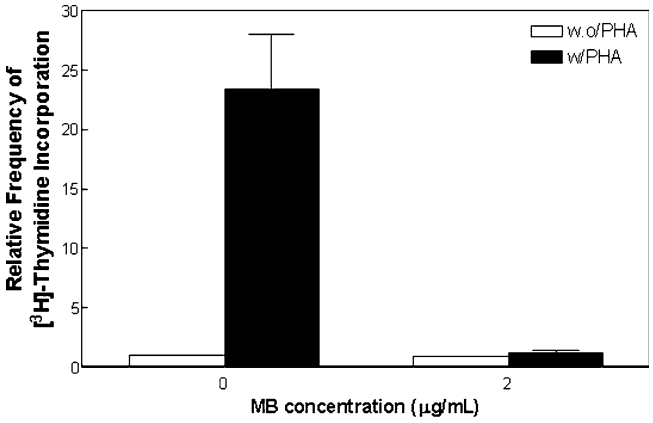

Fig. 8 Effect of MB on lymphocyte proliferation. PBMC were stimulated or not with 5 μg/ml of PHA for 48 h in the presence of MB 2 μg/ml. Results are given as the relative frequency of [^{3} H]-thymidine incorporation and represent the mean ± SEM of at least six independent experiments

We could not observe significant difference in MB sensitivity between resting and PHA-activated lymphocytes. This was in accordance to the fact that no difference was seen in MB uptake by those cells and that we also could not observe any difference in ROS production between resting and activated lymphocytes (data not shown). Moreover, our results suggest that there is no difference in resistance to MB cytotoxicity between the subpopulations of lymphocytes. However, the induction of lymphocyte proliferation by PHA was completely inhibited by MB-treatment, which was able to block DNA synthesis in PHA-activated lymphocytes.

MB has been used in the staining of tumors for diagnosis and surgery [6, 14], and labeling of sentinel lymph node [11], due to its property of being retained mainly in malignant tissues. Our results suggest that, although MB could be used in the treatment of malignant and MDR tumors of peripheral blood, care should be taken in the management of patients, due to a possible impairment of immune response during the treatment.

Acknowledgments The present work was supported by Howard Hughes Medical Institute (departmental sharing of Grant# 55003669), and by grants from Conselho Nacional de Pesquisa (CNPq), Capacitação de Pessoal de Ensino Superior (CAPES), and Programa de Apoio aos Núcleos de Excelência (PRONEX).

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