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Brief Communication

The Immune Response and the Therapeutic Effect of Metronomic Chemotherapy With Cyclophosphamide

Viviana R. Rozados,*[1] Leandro E. Mainetti,*[1] María J. Rico,* Mariano F. Zacarías Fluck,* Pablo Matar,*† and O. Graciela Scharovsky*‡

*Instituto de Genética Experimental, Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Rosario, Argentina
†Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rosario, Argentina
‡Consejo de Investigaciones, UNR, Rosario, Argentina

Metronomic chemotherapy (MCT) is a novel therapeutic strategy for cancer treatment endowed with an antiangiogenic effect. It refers to regular administration of low doses of cytotoxic drugs, with minimal or no drug-free breaks. Previously, we demonstrated the immunomodulating activity of a single low-dose of cyclophosphamide (Cy) and the antitumor effect of MCT with Cy on established rat lymphomas and sarcomas. Here, we examined whether the immune response is responsible for the antitumor effect of MCT with Cy on L-TACB lymphoma. Inbred *e* rats and nude mice were subcutaneously challenged with L-TACB. After 7 days, they were distributed into two experimental groups: 1) treated animals, which were injected IP with Cy (10 mg/kg body weight) three times per week, and 2) control animals, which received IP saline injections. Exponential growth and decay and tumor doubling time were calculated. Also, serum IL-10 levels were measured. One hundred percent of treated rats showed tumor regression versus 0% of control rats. The increase of tumor-induced IL-10 levels was reverted by the treatment with Cy. On the other hand, there were no tumor regressions, in treated or control nude mice. However, the tumor doubling times of treated nude mice were significantly higher than those of control mice, implying that other antitumor mechanism(s), independent of the adaptive immune response, might be taking place. Our present results indicate that modulation of the immune response would be involved in the antitumor effect of MCT with Cy, because the absence of the specific immune response impairs, at least in part, its therapeutic effect in a lymphoma tumor model.

Key words: Metronomic chemotherapy; Immune response; Lymphoma; Cyclophosphamide

INTRODUCTION

Metronomic chemotherapy (MCT) is a novel therapeutic strategy for cancer treatment. It refers to regular administration of comparatively low doses of cytotoxic drugs, with minimal or no drug-free breaks, over prolonged periods of time (1). We have previously demonstrated that a single low dose of cyclophosphamide (Cy), a treatment completely devoid of toxicity, inhibits the growth of spontaneous and experimental metastasis of a rat lymphoma, while it does not affect primary tumor growth (2). Such an effect would be mainly due to modulation of the immune response (3–6). We also demonstrated that administration of Cy at low doses on a thrice weekly schedule, with no rest periods, to established rat lymphomas and sarcomas is a successful antitumor therapy (100% and 83% complete tumor regression, respectively), that does not cause loss of weight and is devoid of hematological, cardiac, hepatic, and renal toxicity (7). Preclinical and clinical experiments have shown that the therapeutic effect of MCT is achieved through inhibition of angiogenesis (1,8,9). Our objective here was to investigate whether the immune response is also responsible for the antitumor effect obtained with metronomic administration of Cy in a rat lymphoma model.

MATERIALS AND METHODS

Animals

Inbred adult female IIM *e*/Fm rats (10) from the Facultad de Ciencias Médicas, Universidad Nacional de Rosario breeding facilities and N:NIH (S)-nu nude mice

[1]These authors provided equal contribution.
Address correspondence to O. Graciela Scharovsky, Ph.D., Santa Fe 3100 (S2002KTR), Rosario, Argentina. Fax: 54-341-4804569; E-mail: graciela.scharovsky@gmail.com

from Universidad Nacional de La Plata breeding facilities were used for these study. The animals were fed with commercial chow and water ad libitum, and were maintained in a 12-h light/dark cycle. All the experiments were done during the first half of the light cycle and in accordance with animal care standards of the institution, which complies with the guidelines issued by the Canadian Council on Animal Care.

Cyclophosphamide

Cyclophosphamide (Cy) was obtained from Laboratorio Filaxis, SA, Argentina. It was dissolved in sterile distilled water to a concentration of 20 mg/ml.

Tumor

Lymphoma L-TACB is a poorly differentiated B-cell lymphoma, which arose spontaneously in an inbred *e* rat (11) and it is maintained by in vivo periodic challenge in syngeneic rats.

Experimental Model

Adult *e* rats and nude mice were subcutaneously challenged with L-TACB by trocar (day 0). When tumors were detectable (day 7), animals were distributed as follows: treated rats—*e* rats ($n = 6$) were injected IP with Cy (10 mg/kg body weight) three times per week, from day 7; control rats—*e* rats ($n = 6$) were injected IP with saline three times per week, from day 7; treated mice—nude mice ($n = 7$) treated the same as treated rats; control mice—nude mice ($n = 7$) treated the same as control rats. Tumors were measured twice weekly and tumor volumes were calculated as follows: $V = 0.4\ (ab^2)$, where V = volume (mm^3), a = largest diameter (mm), and b = smallest diameter (mm). The experiments ended on day 19 (rats) or day 17 (nude mice), or when animals reached the maximum permitted tumor volume. Blood samples, taken from the tail vein of *e* rats, were collected on day 0 (basal level, before tumor inoculation) and at the end of the experiments, and were stored at −20°C until used. IL-10 serum levels were measured by a sandwich ELISA assay (OptEIA™, BD Biosciences Pharmingen, San Diego, CA, USA), in duplicate.

Statistics

Exponential growth and decay were calculated applying an exponential regression curve and one-phase exponential decay curve, respectively. For tumor volume doubling time (TvDT), a nonlinear estimation procedure was used to fit the experimental individual tumor volume–time data, set with an exponential model: $V(t) = \text{Start.exp}\ (k.t)$, where $V(t)$ = tumor volume at time t, Start = tumor volume at time 0, k = constant, t = time (days), and $0.69/k$ = doubling time. Goodness of fit was quantified by the coefficient of determination (R^2), and also judged by the convergent on a solution and the significance of a runs test performed to verify the random distribution of the residuals. For statistical analysis, doubling time was treated as a new random variable and differences between groups were analyzed with the nonparametric Mann-Whitney *U*-test.

All the statistical tests were done with the GraphPad Prism® version 3.0 (GraphPad Software, San Diego, CA, USA). Differences were considered to be statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The antiangiogenic mechanism of action of MCT, be it normalization of tumor vasculature, inhibition of repopulation of tumor cells, or amplification of the antivascular effects of chemotherapy (12), is nowadays widely accepted. Nevertheless, other mechanisms may well contribute to the therapeutic effect of MCT, mainly depending on the chemotherapeutic agent administered.

Cyclophosphamide is the alkylating agent most commonly used in cancer chemotherapy and one of the first cytotoxic drugs employed in metronomic chemotherapy (13). While high doses of Cy [i.e. maximum tolerated dose (MTD)] cause immunosuppression, a single low dose has an immunomodulatory effect (4,5,14,15). Thus, our question was if the regular administration of low doses of Cy would cause stimulation of the antitumor immune response like a single low dose does, or would induce its suppression, like Cy when it is administered in the MTD. Hence, we examined whether the therapeutic effect obtained when low doses of Cy are administered with no significant breaks in tumor-bearing animals involves the adaptive immune response.

Cross-species comparisons have been consistently used to evaluate the efficacy of different antitumor treatments in an environment devoid of specific immune response. Therefore, we developed syngeneic and xenogeneic models in which euthymic rats and athymic nude mice were challenged with L-TACB. The treated groups received low dose Cy in a metronomic schedule, beginning the treatment on day 7, when tumors reached a mean volume of 520 and 460 mm^3 in rats and nude mice, respectively. After a short period of tumor growth that lasted until day 12, 100% of treated rats showed sustained tumor regression (Fig. 1A, inset) versus 0% of control rats (Fig. 1B). We analyzed the exponential growth and decay of the treated (Fig. 1A, left and right, respectively) and control rat tumors (Fig. 1B). We found that during the growing phase, TvDT (tumor volume doubling time) was higher (slower growth rate) in treated rats than in control rats and the difference was

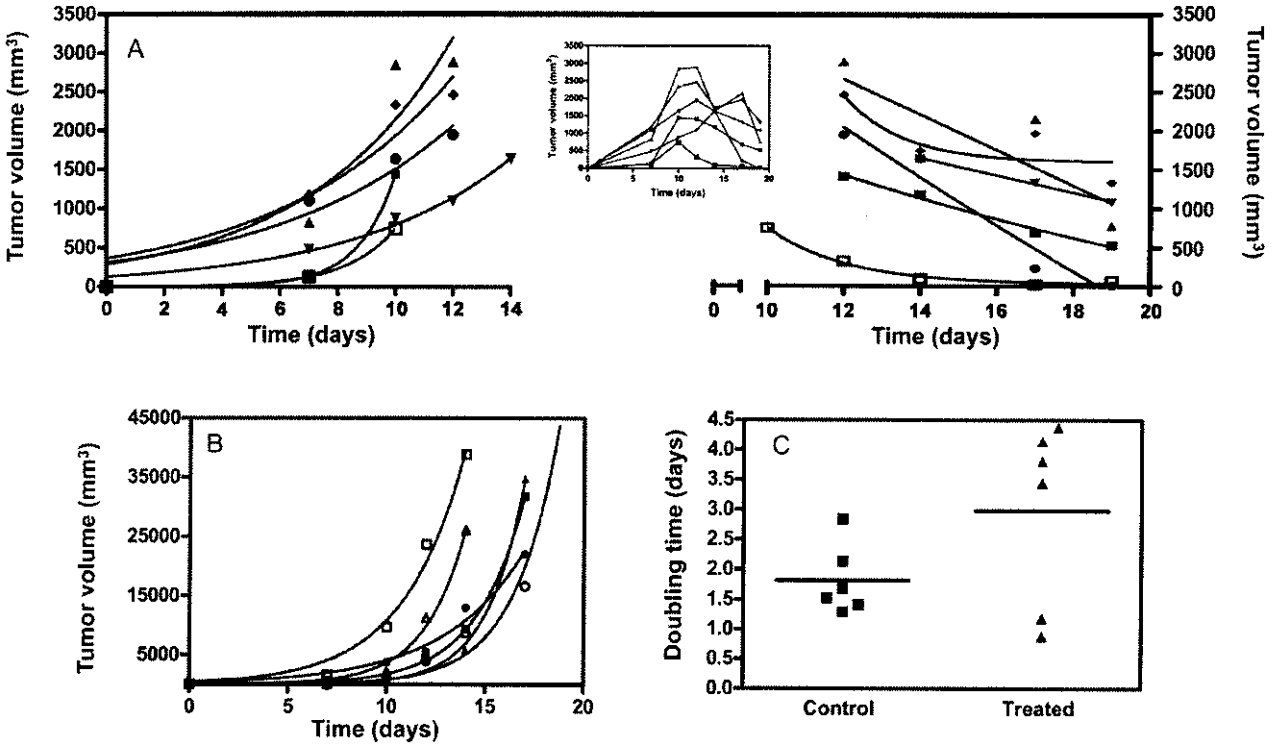

Figure 1. Effect of MCT with Cy in euthymic rats challenged with L-TACB lymphoma. (A, left panel) Exponential growth curves of individual treated rats; (A, right panel) exponential decay curves of individual treated rats. Inset: Individual tumor growth curves of treated rats. (B) Exponential growth curves of individual control rats. (C) Median tumor volume doubling times (TvDT) of control and treated rats. Control versus treated rats: $p = 0.057$, Mann-Whitney's *U*-test.

marginally significant ($p = 0.057$) (Fig. 1C). This outcome emphasized the early tumor response to metronomic chemotherapy.

Interestingly, neither treated (Fig. 2A) nor control nude mice (Fig. 2B) showed signs of tumor regression. The contrasting response in treated rats and mice suggests the involvement of the adaptive immune response in the antitumor effect of MCT with Cy in the L-TACB tumor model. We have previously demonstrated in the same model that the shift from immunosuppression to immunopotentiation induced by treatment of tumor-bearing rats with a single low-dose Cy is mainly mediated by a reduction in T-cell-derived IL-10 production (5). Accordingly, we measured IL-10 serum levels in L-TACB-bearing rats treated with Cy in an MCT schedule. As shown in Figure 3, IL-10 levels in control rats increased significantly with tumor progression (ANOVA, $p = 0.0124$; basal versus control: Tukey-Kramer, $p < 0.01$). However, the levels of IL-10 in MTC-treated tumor-bearing rats did not differ from basal levels.

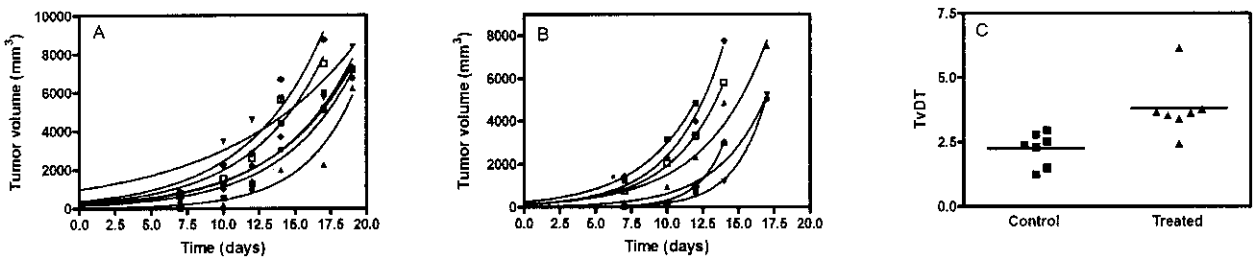

Figure 2. Effect of MCT with Cy in nude mice challenged with L-TACB lymphoma. (A) Exponential growth curves of individual treated mice. (B) Exponential growth curves of individual control mice. (C) Median tumor volume doubling times (TvDT) of control and treated mice. Control versus treated mice: $p = 0.002$, Mann-Whitney test.

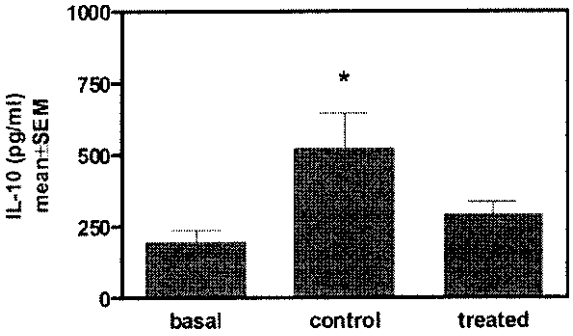

Figure 3. Measurement of serum IL-10 concentration in e rats before L-TACB inoculation (basal), and in L-TACB-bearing rats treated with saline (control) or MCT with Cy (treated), at the end of the experiment (day 19 or when animals reached the maximum permitted tumor volume). Data are expressed as mean (bars, SEM) serum concentration. ANOVA, $p = 0.0124$; *basal versus control: $p < 0.01$, Tukey-Kramer multiple comparison test.

Also, we found that treated and control nude mice showed a different response to MCT with Cy. Specifically, TvDT of tumors from treated nude mice was significantly higher ($p = 0.002$) than that of control mice (Fig. 2C). Because nude mice have an impaired immune response, other antitumor mechanism(s) distinct to immune rejection would explain the delay in tumor growth observed after Cy treatment.

The results herein obtained indicate that absence of the adaptive immune response impairs, at least in part, the therapeutic antitumor effect of MCT with Cy in the L-TACB lymphoma model. Moreover, and as expected, we observed that even in an environment unable to develop an adaptive immune response, metronomic administration of Cy in L-TACB tumor-bearing nude mice decreases the tumor growth rate. The antiangiogenic effect previously observed when administering MCT with Cy (16) may account for the observed delay in tumor growth.

According to our previous results, a unique low dose of Cy is able not only to reduce Th2 and to increase Th1 cytokine production by spleen cells of L-TACB-bearing rats (6) but also to diminish the number of Treg cells (17). The immunomodulatory properties of a single low dose of Cy have been thoroughly studied. Nevertheless, there were no such studies for Cy when administered with a regular low-dose schedule. Recently, it was demonstrated that metronomic administration of Cy in end stage cancer patients depleted Treg cells and restored immune functions (18).

The present results and those obtained by Ghiringhelli et al. (18) call attention about the fact that certain chemotherapeutic drugs, like Cy, may contribute to the therapeutic effect through stimulation of the immune response when administered in a metronomic fashion. This mechanism could be exploited therapeutically in order to increase the efficacy of the treatment. The clinical efficacy obtained with metronomic low-dose cyclophosphamide therapy in a patient with an advanced mucosa-associated lymphoid tissue lymphoma (19) underlines the importance of improving this therapeutic modality. Also it suggests the necessity of studying the relationship between other drugs usually employed in metronomic schedules and the immune system.

In summary, MCT with cyclophosphamide, a therapeutic modality that was initially thought as mainly antiangiogenic exerts, in addition, an immune system-mediated effect in a B-cell lymphoma tumor model. This knowledge will possibly lead to the proposal of novel combinations of antiangiogenic protocols and immunotherapy that may collaborate in the fight against lymphoma.

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