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Pharmacological Purging of Syngeneic Bone Marrow *ex vivo*: Effect of Treatment with Doxorubicin and Lonidamine on Normal and Leukaemic Cells of DBA/2 Mice

Antonella Sandrelli, Paolo De Fabritiis, Alberta Capua, Franco Mandelli, Gabriella Zupi and Carlo Leonetti

The *in vivo* effect of *in vitro* treatment with doxorubicin plus lonidamine on normal and leukaemic cells was investigated in a mouse model of syngeneic bone marrow transplantation. Different numbers of L5178Y tumour cells or normal bone marrow cells alone, or mixtures of bone marrow and leukaemic cells were incubated with doxorubicin (0.25, 0.5, 0.75, 1 μg/ml) and/or lonidamine (50 μg/ml) and reinfused in DBA/2 mice. Lonidamine potentiated the cytotoxic effect of doxorubicin dependent on doxorubicin dosage and tumour cell concentration. Survival after injection of 10^4 *in vitro*-treated tumour cells was 42% for doxorubicin 0.75 μg/ml alone versus 100% for the combination with lonidamine and 50% for doxorubicin 1 μg/ml alone versus 100% combination. Reinfusion of normal bone marrow incubated with doxorubicin alone or in combination with lonidamine in lethally irradiated mice did not occur in 12–14% of mice injected, indicating that the repopulating ability of stem cells was spared. These data suggest the potential usefulness of lonidamine in *ex vivo* purging of bone marrow before autologous bone marrow transplants in haemopoietic malignancies.
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INTRODUCTION

SEVERAL REPORTS have recently suggested that optimal results with autologous bone marrow transplants in acute leukaemias require prior *in vitro* purging [1–4]. Despite encouraging results associated with purging for acute myelogenous leukaemia (AML), bone marrow transplants following *in vitro* treatment of acute lymphocytic leukaemia (ALL) in second or subsequent remission are characterised by a high relapse rate (nearly 80%). Until the origin of such relapse is clear, improved purging regimens may be necessary for the treatment of ALL and possibly AML. The usefulness of approaches to bone marrow purging that involve interleukin-2, lymphokine-activated killer cells and experimental chemical agents [5–8] awaits confirmation in preclinical and clinical trials.

We have recently tested the ability of lonidamine to enhance the *in vitro* cytotoxicity of several antineoplastic drugs[9]. Lonidamine, a derivative of indazole carboxylic acids, has antispermatogenic capacity and low cytotoxic effect on animal and human tumours, but enhances the effect of several antitumour agents. Lonidamine affects the energy metabolism of normal and neoplastic cells in a very peculiar way. It inhibits the oxygen consumption in both types of cells; it increases the aerobic lactate production in normal differentiated cells, but decreases that of the neoplastic ones by affecting the mitochondrially bound hexokinase, which is usually absent in normal cells. This agent is devoid of myelosuppression and is potentially non-cross-resistant with standard chemotherapeutic agents [10–12]. Several preclinical and clinical studies have indicated that lonidamine may be useful in potentiating the effectiveness of other anticancer strategies such as radiation, hyperthermia and chemical antitumour drugs [13–20]. We have also demonstrated that *in vitro* treatment of leukaemic cell lines and human AML cells with lonidamine enhances the antitumour effect of doxorubicin and mitoxantrone without affecting the repopulating ability of bone marrow cells [9]. In the present study we have evaluated the feasibility of *in vitro* purging with doxorubicin in combination with lonidamine in a mouse model of syngeneic bone marrow transplantation.

Correspondence to C. Leonetti.
G. Zupi and C. Leonetti are at the Laboratory of Experimental Preclinical Chemotherapy, Regina Elena Institute for Cancer Research, 291 V.le Regina Elena, 00161 Rome; A. Sandrelli, P. De Fabritiis, A. Capua and F. Mandelli are at Hematology, Department of Human Biopathology, University "La Sapienza" Rome, Italy.
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MATERIALS AND METHODS

Mice

Male 6–8 weeks old DBA/2 Cr (H-2^d) mice, weighing 20–22 g, were obtained from Charles River Labs. Each experimental and control group included 8–10 mice. Mice used to demonstrate bone marrow reconstitution after *in vitro* purging were subjected to lethal total body X-irradiation (950 R in a single dose) from a 6 MeV linear accelerator. Mice received neomycin (0.5 mg/ml), polymixin B (13 μg/ml), trimethoprimsulphamethoxazole (110 μg/ml) in acidified and sterilised drinking water 1 week prior to irradiation and engraftment and for 4 weeks post-transplantation. Sterilised cages, bedding and food were used for mice maintenance. Mice not irradiated were housed under conventional conditions and fed mouse chow and tap water *ad libitum*.

Mice were observed for mortality daily to 90 days after syngeneic bone marrow transplantation. Necropsy was performed on dead mice and selected tissue samples (spleen, liver,

lungs and bone marrow) were examined histologically to evaluate cause of death.

Maintenance and tumorigenicity of tumour cell line

The chemically induced leukaemia–lymphoma L5178Y (H-2^d) cell line derived from DBA/2 mice was maintained by weekly passages in mice injected intravenously with 0.25 ml of a suspension containing 10^5 viable tumour cells. The leukaemogenic capacity of L5178Y cells was evaluated based on survival of mice injected intravenously with 10^1–10^5 viable tumour cells.

Preparation of suspension of normal marrow and L5178Y cells

Marrow was collected from the long bones (femur, tibia) of healthy or leukaemic mice killed by cervical dislocation. Bones were immersed in calcium- and magnesium-free Hanks' balanced salt solution (HBSS), the marrow was flushed with HBSS under sterile conditions, filtered through nylon mesh and washed twice. Cells were resuspended and counted, and viability was assessed by Trypan Blue dye exclusion.

Drugs and in vitro purging

Lonidamine (Angelini SPA, Italy) was dissolved in dimethylsulphoxide at a concentration of 10 mg/ml and adjusted with isoton to a final concentration of 50 μg/ml. Doxorubicin (Farmitalia) was freshly dissolved in bidistilled water and diluted with HBSS to concentrations ranging from 0.25 to 1 μg/ml.

In experiments to evaluate either the antileukaemic efficacy of the two agents or bone marrow engraftment ability, 10^1–10^5 leukaemic cells per recipient and 2×10^6 bone marrow cells per recipient were utilised, respectively.

In experiments to evaluate purging of L5178Y cells in the presence of excess normal bone marrow cells, 10^4 leukaemic cells were mixed with 2×10^6 bone marrow cells (0.5% leukaemia cell contamination). Cells were incubated with doxorubicin (0.25, 0.5, 0.75, 1 μg/ml) for 60 min at 37°C in a shaking water bath, washed with HBSS and reincubated with lonidamine (50 μg/ml) or with an equivalent volume of RPMI-1640 for 60 min at 37°C. After reincubation, cells were maintained at 4°C for 10 min to block any further effect, washed twice with RPMI supplemented with 5% fetal calf serum and kept on ice until reinfusion.

To assess haematological reconstitution, peripheral blood from the tail caudal vein was evaluated weekly for white, red blood cell and platelet counts.

Statistical analysis

All data are from 2–3 independent experiments. Numbers of surviving mice in each group were compared by the χ^2-test. Mice still alive after 90 days were considered survivors.

RESULTS

Tumorigenic potential of L5178Y cell

All healthy mice injected intravenously with L5178Y tumour cells (10^1–10^5 per mouse) developed leukaemia; necropsy revealed leukaemic cells in bone marrow, spleen and liver, and in some cases diffuse peritoneal and pericardic infiltration. All mice died by 2 weeks postinjection and no statistically significant difference ($P > 0.05$) in the median survival time was observed among groups infused with different leukaemic cell concentrations. Mice receiving 10^1 and 10^5 tumour cells showed a median survival time of 11.5 and 8.5 days, respectively (Fig. 1).

Sensitivity of L5178Y cells to in vitro treatment with doxorubicin and lonidamine

To evaluate the antileukaemic efficacy of *in vitro* purging with doxorubicin and lonidamine, 10^2–10^5 L5178Y cells were treated with each drug alone or in combination and reinfused in healthy mice.

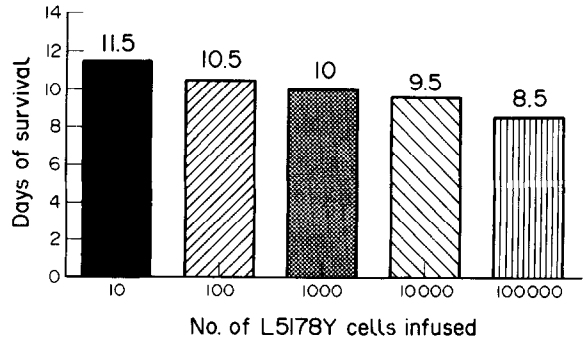

Fig. 1. Median survival time of mice infused with L5178Y cells.

In mice challenged with 10^5 tumour cells treated *in vitro* with doxorubicin at 0.25 or 0.5 μg/ml, no survivors were observed; after incubation with doxorubicin at 0.75 and 1 μg/ml, 15% and 25% survival was obtained, respectively. Addition of lonidamine produced a consistent but not statistically significant ($P > 0.05$) increase in survival when tumour cells were treated with doxorubicin at 0.5, 0.75 or 1 μg/ml (0% vs. 17%, 15% vs. 37%, 25% vs. 50% survival, respectively). However, no effect was observed when lonidamine was added to 0.25 μg/ml of doxorubicin. Mice that received untreated tumour cells all died using 10^2 and 10^5 tumour cells (median survival time 10.5 and 8.5 respectively, $P > 0.05$). No survivors were observed among mice infused with 10^4 tumour cells incubated with 0.25 μg/ml of doxorubicin alone or in combination with lonidamine. However, *in vitro* treatment with 0.5, 0.75 or 1 μg/ml of doxorubicin resulted in 33%, 42% and 50% survival, respectively, and incubation with both agents increased survival to 50%, 100% ($P < 0.01$) and 100% ($P < 0.01$), respectively. When lower numbers of treated leukaemic cells were used (10^3 and 10^2), doxorubicin at 0.5, 0.75and 1 μg/ml effectively eliminated tumour cells and produced 100% survival. However, 0% and 75% survival was observed after reinfusion of 10^3 and 10^2 cells, respectively treated with doxorubicin at 0.25 μg/ml. The addition of lonidamine increased survival significantly to 88% ($P < 0.001$) and 100% ($P < 0.001$) using 10^3 and 10^2 tumour cells, respectively as compared with survival obtained with doxorubicin alone (Fig. 2).

Effect of in vitro treatment with doxorubicin and lonidamine on bone marrow reconstitution

We have demonstrated previously [9] the low toxicity on CFU-GM (colony forming units gramulocyte–macrophage) *in vitro* treatment of human bone marrow of anthracyclinic drugs in association with lonidamine and a complete recovery of more immature precursors evaluated in long-term marrow cultures. To evaluate bone marrow reconstitution *in vivo* after *in vitro* treatment with doxorubicin and lonidamine normal bone marrow cells were incubated with the same concentration of drugs used on leukaemic cells and injected in lethally irradiated mice. In preliminary experiments 10^6 normal bone marrow cells, untreated or treated with doxorubicin at 1 μg/ml produced engraftment in lethally irradiated mice. About 2×10^6 marrow cells were treated *in vitro* and reinfused intravenously 24 h after total body irradiation. Median survival time of irradiated mice was 10.5 days (range 9–14); transplantation of 2×10^6 untreated bone-marrow-protected irradiated mice and less than 10% of

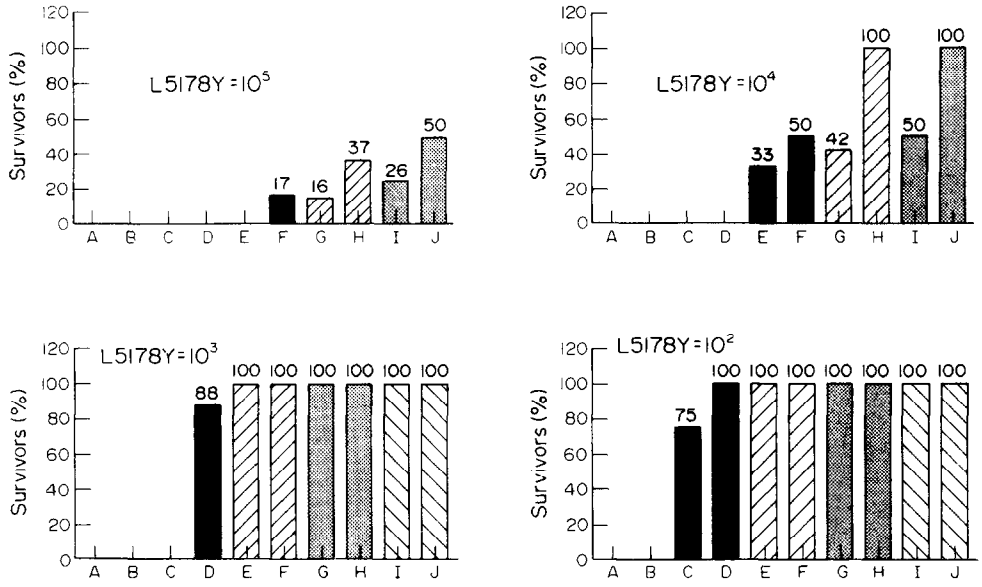

Fig. 2. Survival of mice reinfused with L5178Y cells treated *in vitro* with different concentrations of doxorubicin and with 50 μg/ml of lonidamine alone or in combination. A, untreated cells; B, lonidamine; C, doxorubicin (0.25 μg/ml) and D, + lonidamine; E, doxorubicin (0.5 μg/ml) and F, + lonidamine; G, doxorubicin (0.75 μg/ml) and H, + lonidamine; I, doxorubicin (1 μg/ml) and J, + lonidamine.

deaths in all experiments were transplant-related. Similar results were obtained in groups treated with lonidamine alone, confirming the absence of toxicity of this agent on bone marrow repopulating ability. All groups treated with doxorubicin alone or in combination with lonidamine showed a survival ranging from 88% to 100% 3 months after syngeneic bone marrow transplantation (Fig. 3). Necropsy of mice not reinfused revealed aplasia in bone marrow cytospins. Mice that died after reinfusion showed at necropsy death by haemorrhage or infections. In surviving mice blood cell count become normalised at 12–15 days after bone marrow infusion.

Survival of mice transplanted with mixtures of normal bone marrow and leukaemic cells in vitro *treated with doxorubicin and lonidamine*

Mixtures of 2×10^6 bone marrow cells and 10^4 L5178Y cells were incubated *in vitro* with doxorubicin and lonidamine at the concentrations described above and reinfused in lethally irradiated mice. Results were superimposable with those obtained when bone marrow and L5178Y cells were treated separately. In particular, all mice died within 3 weeks (range 9–21 days) when reinfused with cell mixtures treated with doxorubicin at 0.25 μg/ml alone or in combination with lonidamine. When the cell mixture was treated with doxorubicin at 0.5 μg/ml survival was not significantly increased by the addition of lonidamine (25% for doxorubicin alone vs. 37.5% for doxorubicin and lonidamine). As expected, lonidamine in combination with doxorubicin at higher concentrations resulted in a highly significant increase in survival (37.5% for doxorubicin alone vs. 100% for doxorubicin and lonidamine) both at 0.75 and 1 μg/ml of doxorubicin ($P < 0.001$).

DISCUSSION

Non-randomised clinical trials have shown a possible impact of purging on disease-free survival in AML; however, in

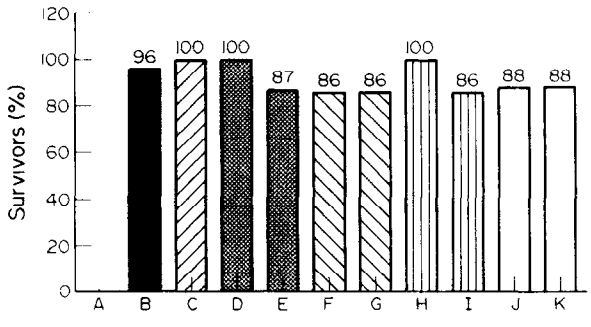

Fig. 3. Survival in lethally irradiated mice reinfused with 2×10^6 bone marrow cells treated *in vitro* with different dosages of doxorubicin and 50 μg/ml of lonidamine alone or in combination. A, no reinfused cells; B, cell reinfusion; C, lonidamine; D, doxorubicin (0.25 μg/ml) and E, + lonidamine; F, doxorubicin (0.5 μg/ml) and G, + lonidamine; H, doxorubicin (0.75 μg/ml) and I, + lonidamine; J, doxorubicin (1 μg/ml) and K, + lonidamine.

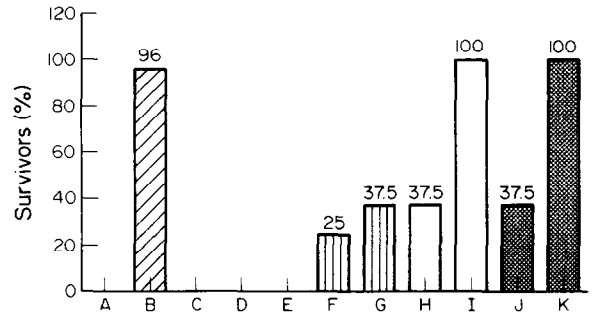

Fig. 4. Survival in lethally irradiated mice reinfused with mixtures of 2×10^6 normal bone marrow and 10^4 leukaemic cells treated *in vitro* with different dosages of doxorubicin and 50 μg/ml of lonidamine alone or in combination. A, no reinfused cells; B, untreated bone marrow cells reinfusion; C, untreated mixture reinfusion; D, doxorubicin (0.25 μg/ml) and E, + lonidamine; F, doxorubicin (0.5 μg/ml) and G, + lonidamine; H, doxorubicin (0.75 μg/ml) and I, + lonidamine; J, doxorubicin (1 μg/ml) and K, + lonidamine. L5178Y untreated cells = 0% survival. Lonidamine-treated cells = 0% survival.

advanced ALL the majority of patients relapse after autologous bone marrow transplantation, and *in vitro* treatment of bone marrow from ALL in remission has not proved of clinical benefit [1–4].

The present study demonstrates the feasibility and the efficacy of pharmacological purging of bone marrow *in vitro* in a mouse model of syngeneic bone marrow transplantation. L5178Y tumour cells at all four tested concentrations killed 100% of intravenously injected mice within 2 weeks. When a relatively high number of tumour cells (10^4) was treated *in vitro* only, the addition of lonidamine to the higher concentrations of doxorubicin was able to produce an optimal bone marrow purging.

A more marked effect was shown by the two agents when lower numbers of tumour cells were treated *in vitro*. Doxorubicin alone produced 100% survival at 0.5, 0.75 and 1 μg/ml. However, at 0.25 μg/ml doxorubicin produced no survivors when 10^3 tumour cells were treated, whereas in combination with lonidamine, 88% animals survived beyond 3 months after syngeneic bone marrow transplantation ($P < 0.001$).

Very few studies have addressed the possibility of using anthracyclines *in vitro* to purge remission marrow from residual leukaemic cells, because of their potential stem cell toxicity. In our experiments to evaluate the effect of doxorubicin and lonidamine treatment on bone marrow haematological reconstitution, 2×10^6 cells per recipient were incubated with doxorubicin and/or lonidamine at the same dosages used to treat leukaemic cells and reinfused in lethally irradiated mice; 86–100% survival was obtained in the different groups, consistent with our previous findings [9] of a lack of toxicity *in vitro* of bone marrow precursors evaluated in long-term marrow cultures. Similar experiments using mixtures of leukaemic cells and normal bone marrow cells (ratio 0.5:100) to resemble remission marrow *in vivo* confirmed the absence of toxicity of the 2 drugs on bone marrow cells and a significant antitumour effect even in the presence of a 200-fold excess of bone marrow cells. The lack of lonidamine toxicity on normal stem cells is demonstrated by the near 100% survival of irradiated mice reinfused with stem cells treated *in vitro* with doxorubicin and lonidamine. This is confirmed at the necropsy by the presence of bone marrow reconstitution in mice which died after reinfusion of stem cells.

The use of L5178Y tumour cells as a target to test the efficacy of an *in vitro* purging regimen may or may not reflect the human situation of an acute leukaemia of lymphoid origin. It is possible that a high number of proliferating cells (or other mechanisms) in this cell line leads to over- or underestimates of the efficacy of doxorubicin in eliminating tumour cells. Only appropriate clinical trials will define the role of bone marrow purging in leukaemias and the efficacy of lonidamine in enhancing the *in vitro* tumour cell elimination by anthracyclines.

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