Abstract. Background/Aim: Although acute myeloid leukemia (AML) has traditionally been considered an oncological emergency and initiation of therapy is believed to be crucial to minimizing disease-related morbidity and mortality, it has also been suggested that a certain delay in treatment has no negative consequences in terms of response, early mortality, or survival. We aimed to determine the effect of administration of sodium caseinate (SC), a salt of casein, the main milk protein, with cytarabine or with daunorubicin on survival in mice with well-established leukemia. Materials and Methods: To assay the time of establishment of leukemia in the bone marrow, BALB/c mice were inoculated with $2.5 \times 10^5$ WEHI-3 cells/mouse and after 3, 6 and 9 days were euthanized. Bone marrow mononuclear cells (BM-MNCs) of the femur were obtained and cultured for 120 h with or without rmIL-3 and cell proliferation was evaluated by the crystal violet technique. Then, the effect of administering SC-cytarabine or SC-daunorubicin on survival rates of mice with well-established leukemia was assayed. Another group of BALB/c mice was inoculated with WEHI-3 cell and after 10 days mice were treated with SC-cytarabine or SC-daunorubicin for 40 days. Survival rates were recorded daily and in surviving mice, the prevalence of bone marrow proliferation after treatment was assayed by the crystal violet technique. Results: The assay on the time of establishment of leukemia shows that in 9 days leukemia cells accumulate in the bone marrow in sufficient quantities to sustain an in vitro culture in the absence of growth factors, and we, thus, used this as a criterion of well-established leukemia. When mice with a burden of leukemic cells of more than 9 days were treated with SC-cytarabine or SC-daunorubicin, this resulted in 55% survival for both treatments, and the proliferation assays showed that the bone marrow retained its normal proliferation capacity. Conclusion: SC-cytarabine or SC-daunorubicin treatment prolonged the survival rate of BALB/c mice with a burden of well-established leukemia, and there was no negative impact on bone marrow functionality; however, SC-cytarabine or SC-daunorubicin combination options need to be sought to increase survival beyond 40 days.

Acute myeloid leukemia (AML) is an aggressive and heterogeneous hematological group of neoplasms characterized by increased proliferation of myeloid progenitor cells and reduced differentiation of the myeloid lineage (1) this results in catastrophic failure of the bone marrow (2). Although it can occur in any age group, AML is predominantly a disease with a mean age at diagnosis of 68 years (3). An overall survival of 5 years can be achieved in only 24% of adult patients who are selected for intensive therapy, 10-15% of older patients not eligible for intensive therapy (mostly by combinations of an anthracycline with high-dose cytosine arabinoside) survive up to five years, and almost...
80% of patients diagnosed at age 65 years die within one year. Thus, new treatment modalities are needed to improve the long-term perspectives for adult patients with AML.

New treatment regimens based on combinations of chemotherapy drugs at low doses and administration seem to be a viable option to enhance survival. In recent years, we have provided evidence of the hematopoietic regulation capacity of casein and sodium caseinate (SC) in inhibiting the proliferation of leukemic cell lines such as J774, P388, and WEHI-3 in vitro, enhancing the survival of mice in leukemia models and attenuating the manifestations of disease, but in healthy mice, SC promotes the production of hematopoietic regulators such as G-MCS and GM-CSF (4–7). We recently showed that in a WEHI-3 AML mouse model induced 24 h prior to the start of treatment, the combined administration of SC with daunorubicin or SC with cytarabine promotes the highest survival rate, without evidence of malignant cells in the bone marrow of survivors of more than 60 days and without damage to the BM-MNC (8).

It is highly recommended to start antineoplastic treatment as soon as AML is diagnosed due to the poor prognosis of untreated acute leukemia. Sekeres et al. found that in patients 60 years of age or older, the time from diagnosis to treatment (TDT) did not appear to affect complete remission or overall survival rates, response rate, or early death (9). Thus, it is reasonable to wait a short time for laboratory tests to better characterize leukemias and design therapeutic strategies adapted to diagnosis (10). With this in mind, we investigated whether in a mouse leukemia model with evidence of leukemia in progress by several days, combination treatment of SC with daunorubicin or SC with cytarabine promotes survival rate, without evidencing malignant cells in the bone marrow of survivors.

Materials and Methods

Animals. Female Balb/c mice (8-12 weeks old at the time of the experiments) were provided and housed by the Animal Facility of the Faculty of High Studies Zaragoza (FES-Zaragoza), National Autonomous University of Mexico (UNAM). All experimental protocols were approved by the Ethics Committee of FES-Zaragoza, UNAM (approval ID: FESZ/DEPI/CI/128/14), in accordance with the institutional guidelines. All animals were housed under standard conditions (12 h light/dark cycles at 25˚C) and a standard rodent diet, and all efforts were made to reduce the number of animals used and their suffering.

Drugs. The drugs used in the present study were purchased from the following manufacturers: sodium caseinate from Spectrum (New Brunswick, NJ, USA) and daunorubicin and cytarabine from Pfizer (New York, NY, USA). Drugs were administered in PBS at 2 g/kg, 3 mg/kg, or 0.5 mg/kg SC, cytarabine and daunorubicin, respectively. The antibiotics penicillin and streptomycin, crystal violet, glutaraldehyde and Histopack were purchased from Sigma–Aldrich (St. Louis, MO, USA). Iscove’s modified Dulbecco’s medium (IMDM), fetal bovine serum (FBS) and equine serum (ES) were purchased from Gibco-BRL (Carlsbad, CA, USA).

Cell line, and bone marrow mononuclear cell culture. Murine WEHI-3 leukemia cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The WEHI-3 cells were cultured in tissue culture flasks from Sarstedt AG & Co. (Numbrecht, Germany) in IMDM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and grown at 37°C under humidified 5% CO₂.

Healthy Balb/c mice were euthanized by cervical dislocation. The femurs were obtained under sterile conditions, and total bone marrow cells were obtained by flowing IMDM supplemented with 10% FBS. Subsequently, the bone marrow mononuclear cells (BM-MNCs) were separated by a density gradient with Histopack (density=1.077 g/ml), and 1x10⁵ cells were cultured in 96-well plates in IMDM with 15% FBS, 5% ES and recombinant mouse interleukin-3 (rmIL-3) and grown at 37°C under humidified 5% CO₂.

Advanced leukemia assay. We examined the burden of leukemic cells in the bone marrow of Balb/c mice inoculated with 2.5x10⁵ WEHI-3 cells/mouse or 200 μl PBS as vehicle after 3, 6 and 9 days. Three mice were euthanized, and at each time point, BM-MNCs were cultured for 120 h as above and without rmIL-3. Then, cell proliferation was assessed by the crystal violet technique as previously indicated (8). The optical density was evaluated at 570 nm in a plate spectrophotometer from SpectraTecan Image (Austria), and the data obtained were plotted. When autoproliferating cells were detected in the absence of rmIL-3, they were considered evidence of disease progression.

Treatment. For animal survival rate assays, mice were randomly divided into five groups (n=9). Group I mice were set as the control and given i.p. injection of PBS as a vehicle. The other four groups of mice were leukemia animals with individual i.p. of WEHI-3 cells (2.5x10⁵ cells/mouse), and 10 days after injection, antileukemic treatment was started as follows. Group II mice were set as the leukemia control and administered i.p. PBS was used as a placebo treatment, and groups III, IV and V were given a combination of SC and antineoplastic drugs, SC-cytarabine, or SC-daunorubicin by i.p. injection in PBS at 2 g/kg, 3 mg/kg, or 0.5 mg/kg SC, cytarabine and daunorubicin, respectively, every 2 days. A cytarabine-daunorubicin combination as antineoplastic standard treatment is ongoing. The animal studies were performed for 40 days for the evaluation of survival rates, and mice surviving at this time had recovered them. Their BM-MNCs were assayed for the prevalence of proliferation in bone marrow posttreatment as previously indicated.

Results

Leukemia burden in the bone marrow of Balb/c mice inoculated with WEHI-3 cells. It is well documented that i.p. injection of WEHI-3 cells in Balb/c mice can induce leukemia (11, 12). We previously showed that WEHI-3 cells reach the bone marrow as early as 24 h after being inoculated intraperitoneally (8); herein, we investigated the time at which leukemia cells accumulate in the bone marrow in sufficient quantity to sustain an in vitro culture in the absence of growth factors, which is a criterion of disease progression.
We showed that BM-MNCs recovered 3 days after inoculation of WEHI-3 cells do not proliferate in the absence of rmIL-3, and only those recovered 6 days after induction of leukemia reached a sufficient number to sustain cell proliferation in vitro in the absence of rmIL-3, thus proving the establishment of the disease in BM. While it is necessary to wait 9 days to recover from BM, a sufficient number of cells with autoproliferative capacity to reach 10% proliferation in the absence of rmIL-3 compared to the culture supplemented with rmIL-3 as growth factor is evidence of disease in progression (Figure 1). Representative images of these autoproliferative cultures show colonies in the absence of growth factor (Figure 2A) but with rmIL-3 stimulation for 120 h (Figure 2B). The culture progressed similarly to that of BM-MNCs from healthy mice in the presence of growth factor, with cells both growing in suspension as they grew adhered to the culture plate (Figure 2D), while there was no evidence of colonies or proliferation in BM-MNCs from healthy mice without rmIL-3 (Figure 2C).

**Survival of Balb/c mice with a 10-day leukemic cell burden in the bone marrow treated with combinations of SC-cytarabine or SC-daunorubicin.** We have used the WEHI-3 leukemia model in the past to determine the therapeutic potential of SC (5), and we recently analyzed the therapeutic effect of combined treatment of SC with antineoplastic drugs cytarabine or daunorubicin, administered as early as 48 h after induction of leukemia. We found that the combined treatment prolongs survival to a greater extent than individual treatments, both SC and antineoplastic drugs of regular use, even completely eliminating the autoproliferating cells of the BM (8). Herein, we asked ourselves whether this combination could provide the same synergistic effect in leukemic mice when the disease had progressed by 10 days before starting treatment, and the results indicated that leukemic mice treated with placebo PBS had a 0% survival at 33 days. However, at the same time, SC-cytarabine or SC-daunorubicin treatment resulted in both 55% survival, and antileukemic control treatment with cytarabine-daunorubicin resulted in only 11% survival (Figure 3), demonstrating that even if the disease had progressed for 10 days before starting treatment, the combination of SC and antineoplastic drugs prolonged survival and was even greater at the same time than the traditional combination of antineoplastic drugs.

**Leukemia cell burden in surviving Balb/c mice after 40 days of SC-daunorubicin treatment.** We allowed the study to progress for an additional 3 days, and the survival of leukemic mice in both SC antineoplastic treatments remained superior (44% and 33% with SC-cytarabine or SC-daunorubicin, respectively) to the 11% survival of leukemic mice treated with cytarabine-daunorubicin. Then, three days later (six days after that survival 0% in the placebo PBS treatment group), we recovered the BM-MNC from surviving mice with SC-daunorubicin and cytarabine-

![Figure 1. Proliferation of bone marrow mononuclear cells from Balb/c mice at 3, 6 and 9 days postinduction of leukemia by WEHI-3 cell inoculation. Cells were cultivated for 120 h in the presence or absence of recombinant mouse interleukin 3 ([+rmIL-3] or [-rmIL-3]) and evaluated by the crystal violet technique. Data shown are the mean±standard deviation (SD) of three independent experiments analyzed by using Tukey's test of analysis of variance. *Significantly different from healthy mice without rmIL-3 at p<0.05.](image-url)
Figure 2. Representative cultures of mononuclear cells from the bone marrow of leukemic mice (9 days postinduction of leukemia by WEHI-3 cell inoculation) after 120 h in culture in the absence (A) or presence (B) of recombinant mouse interleukin 3 (rmIL-3) and mononuclear cells from the bone marrow of healthy mice after 120 h in culture in the absence (C) or presence (D) of rmIL-3 by 120 h. Magnification 10×.

Figure 3. Survival rate of Balb/c leukemic mice (WEHI-3) treated with 1 ml of phosphate buffered saline (PBS) or 2 g/kg sodium caseinate plus 3 mg/kg cytarabine (SC-CYTA), 2 g/kg sodium caseinate plus 0.5 mg/kg daunorubicin (SC-DAUNO) or 3 mg/kg cytarabine plus 0.5 mg/kg daunorubicin (CYTA-DAUNO) every 48 h. Healthy mice treated with PBS (PBS-PBS) were included as a control. Kaplan–Meier curve, n=9 per group.
daunorubicin treatment to verify the proliferation capacity of the marrow, and as representative images show, cultures in the presence of rmIL-3 of BM-MNC from surviving leukemic mice with both types of treatment (Figure 4A and B, respectively) are similar to the culture with rmIL-3 of BM-MNC from healthy mice (Figure 4D), but culture of BM-MNC from both surviving leukemic mice can form colonies in the absence of rmIL-3 (Figure 4C and D).

Discussion

None of the currently used mouse AML models faithfully recapitulate the complex biology, cell-to-microenvironment interactions, and dynamic progression of AML. Nevertheless, they have been instrumental in deciphering the underlying pathology of the disease and advancing AML research. Historically, chemical, irradiation and viral models have set the field of AML modeling in mice and have been used to develop many AML drugs. Patient-derived cell xenotransplants in immunodeficient murine model systems provide an experimental platform to test the efficacy of novel therapeutic compounds against human AML cells, but these immunodeficient models are limited by their inability to address the interplay of leukemic blasts with different cells of the immune system (13). In this sense, the WEHI-3 murine cell graft model in immunocompetent Balb/c mice, although it induces a pathology of leukemia that could not fully phenocopy human AML, represents a model where leukemic cells over impose the immune system, establish themselves in the bone marrow and develop until they generate a disease pattern that, in the absence of treatment, invariably leads to death; thus, although it is not without limitations, this leukemia study system remains valid (12, 14-17).

In a recent study, we showed that mice inoculated with WEHI-3 cells do not survive more than 34 days after being treated with PBS as a placebo, but if they are treated with SC-cytarabine or SC-daunorubicin as soon as 48 h after induced leukemia, survival rates are achieved at 40 days of 40% and 35% and at 70 days of 10% and 20%, respectively (8). Herein, we showed that if the start of treatment is delayed 10 days after leukemia induction, there are no obvious manifestations of disease (lethargy, bristling hair, splenomegaly, abdominal distension, solid tumor, etc.) However, when survival is 0% with PBS (Day 33), 55% remain alive in both SC-cytarabine

Figure 4. Cultures of mononuclear cells from bone marrow in the presence of rmIL-3 for 120 h (A and B) or PBS (C and D) from leukemic mice after 30 days or treatment every 48 h with 2 g/kg sodium caseinate plus 0.5 mg/kg daunorubicin (A and C) or 3 mg/kg cytarabine plus 0.5 mg/kg daunorubicin (B and D). Culture of mononuclear cells from the bone marrow of healthy mice (4E) after 120 h in the presence of rmIL-3 was included as a control of normal proliferation. Magnification 10×.
and SC-daunorubicin treatment, while more than 75% survive when SC-cytarabine treatment is started as early as 48 h after leukemia induction (8). Although this represents a reduction in survival compared to treatment administered when the disease takes a few days of induction, it is encouraging that even in conditions of progressing disease, the combination of SC-cytarabine or SC-daunorubicin prolongs survival by a proportion even superior to the short-term survival of 41.5% obtained with CPX-351 (a liposomal formulation that encapsulates cytarabine and daunorubicin) (18). Unfortunately, while there were only 40 days of survival in early treatment almost 40% survived with SC-cytarabine and more than 70% with SC-daunorubicin, in mice with advanced disease, 39 days, 11% and 22% survived with SC-cytarabine and SC-daunorubicin, respectively (data not shown).

Traditionally, the initiation of oncologic therapy is thought to be crucial to minimize disease-related morbidity and mortality. Bertolli et al. analyzed the effect of TDT in newly diagnosed AML patients treated by induction chemotherapy between 2000 and 2009 and did not find any harmful signs concerning the effect of TDT on overall survival, early death, or response rate in younger and older patients with newly diagnosed AML treated by intensive chemotherapy (10). This is consistent with a recent meta-analysis that highlights that in patients with clinically stable AML and no signs of organ dysfunction, TDT is not related to survival (19); instead, the patient condition is decisive, and if the disease is de novo, it is in a relapse after a CR or whether it is a difficult-to-achieve CR1 or a second CR (20).

Although the negative impact of the cytarabine-daunorubicin regimen on normal bone marrow functionality is known and estimated 5-year survival rates of only 10%-15% are obtained in the group of patients with the highest incidence (age >60 years), it remains the accepted standard of care (21). We previously showed that early initiation of combined SC-daunorubicin or SC-cytarabine treatment prolongs survival without negatively compromising bone marrow proliferative capacity, as does conventional cytarabine-daunorubicin (8); therefore, we inquired if the bone marrow of the surviving mice retained its proliferative capacity, and as before, SC-daunorubicin treatment does not compromise the functionality of the bone marrow. Interestingly, cytarabine-daunorubicin treatment did not occur; however, in both cases, we observed the presence of colonies in cultures without rmIL-3.

In conclusion, delaying the initiation of SC-cytarabine or SC-daunorubicin treatment still prolongs the short-term survival rate, and there is no negative impact on bone marrow functionality; however, leukemia is not eradicated. It is necessary to search for combination options of SC-cytarabine or SC-daunorubicin in advanced disease conditions to increase survival and eradicate leukemia both short- and long-term.

Conflicts of Interest

The Authors declare no competing financial interests.

Authors’ Contributions

Itzen Aguñiga Sanchez, Edgar Ledesma Martínez and Edelmira Santiago Osorio designed and performed the research, analyzed data, and wrote the manuscript; Frida Melendez Ibarra, Benny Weiss Steider, Isabel Soto Cruz, Guadalupe Fajardo Orduña analyzed the data and helped write the manuscript; Jose Luis Lara Castañeda designed and performed the research and analyzed data.

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