The Journal of
Trace Elements in Experimental Medicine

WILEY-LISS

Enhanced Tolerance to High Cytostatic Doses by Means of Oligoelements Mn, Se, and Zn Plus *Lachesis muta* Venom: In Vivo and In Vitro Studies

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We evaluated, in vivo and in vitro, the increased tolerance to treatment with high doses of chemotherapeutic drugs produced by the administration of oligoelements (Zn, Se, Mn) plus *Lachesis muta* venom (O-LM). Cyclophosphamide, methotrexate, and 5-fluoruracil (CMF) were administrated to male rats treated daily with O-LM. The doses of CMF tested covered from 1- to 20-fold the dose used in human patients. Human neoplastic and normal cell lines were challenged in vitro with increasing doses of 5-fluoruracil (5-FU) in the presence and absence of O-LM. Results indicated a significantly higher global survival in O-LM-treated rats versus Control rats. LD_{50} in O-LM rats was 18.5-fold the basic CMF dose versus 14.1 in controls. Eighty percent of Control rats showed severe bone marrow aplasia, sepsis, and secondary leukemia, while only 20% of O-LM-treated rats showed these effects and no leukemia cases were observed. O-LM treatment produced a significant inhibition of cell proliferation of malignant cells, while in normal cells O-LM increased colony formation. In transformed cells, the cytostatic effect of 5-FU was significantly enhanced by O-LM pretreatment, in contrast with the protective effect exerted on normal cells. Toxicological studies clearly demonstrated that O-LM did not produce any toxic or undesirable effect when administered to normal animals. Whole body weight, water and food consumption, and laboratory parameters remained unchanged after O-LM administration, even with a dose 10-fold higher than that employed in the experimental work. Results obtained afford the possibility of applying this protective treatment to human patients undergoing high-dose chemotherapy. J. Trace Elem. Exp. Med. 16:39–53, 2003. © 2003 Wiley-Liss, Inc.

Key words: chemotherapy; selenium; zinc; manganese; *Lachesis muta*; trace elements

INTRODUCTION

The application of cytostatic drugs in human patients has enabled great progress in the treatment of malignant tumors, such as breast, colon, and lung carcinoma [1–3]. Progress has been achieved in the application of massive doses

with the help of hematopoietic stimulating factors, which allow bone marrow recovery following a high-dose of chemotherapy [4,5]. However, no more than 5,000 mg/m^2 of cyclophosphamide, equivalent to 6 or 7 times the usual dose, can be administered [4].

An important immunoregulatory and antioxidant performance of selenium (Se) was reported in human lymphocytes [6,7]. The ability of zinc to retard oxidative processes has also been recognized [8], and animal studies indicate that manganese is an essential co-factor for enzymes such as superoxide dismutase [9].

Lachesis muta venom (LM) has an important content of phospholipase A$_2$ (PLA$_2$). It has been demonstrated that PLA$_2$ acts as an effector system for certain enzymes such as cyclo-oxygenase and lipo-oxygenase, for cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6) [10], and it also produces histamine release from mast cells [11]. The histamine released may in part mediate the modulation of the immune response and the action on cell proliferation attributed to PLA$_2$. In recent years it has been extensively described with the role of histamine in proliferation of normal and cancer cells [12,13] and in the modulation of the immune response [14].

We have previously reported the therapeutic effects of a new combination (Se, Zn, Mn plus *L. muta* venom, O-LM) that was studied in cell cultures and in a mammary experimental tumor model [15,16]. This treatment was also applied to human patients with colon cancer with liver metastases [17], with breast cancer [18], or with pancreatic carcinoma [19], producing in all cases a significant increase in survival rates.

Cyclophosphamide 500 mg/m^2, methotrexate 40 mg/m^2, and 5-fluoruracil 600 mg/m^2 (CMF) is the most commonly used chemotherapeutic combination for treatment of human patients with breast cancer [1]. In the present paper we investigated, in vivo and in vitro, the enhanced tolerance to treatment with high doses of CMF produced by the O-LM combination.

MATERIALS AND METHODS

In Vivo Studies

Animals

One hundred twenty-eight (128) 4-month-old male Sprague-Dawley rats inbred in our laboratory and weighing 380–420 g were used. Animals were kept in groups of 5 per cage with water and food ad libitum, temperature at 22–23°C, humidity at roughly 56%, and a 12-h light cycle. Body weight was monitored every other day. In all cases, the animals were in the conditions recommended by the Guide for the Care and Use of Laboratory Animals, National Research Council [20].

Treatments

Chemotherapeutic drug injection. CMF was given as a single IP dose. The lowest CMF dose given, indicated as 2-fold, was calculated by extrapolating the double value of that one used in human patients: cyclophosphamide 500 mg/m^2, methotrexate 40 mg/m^2, and 5-fluoruracil 600 mg/m^2 [1]. The dose ranges em-

ployed were multiples of the latter and were indicated as 4-, 10-, 12-, 14-, 16-, 18-, and 20-fold, respectively.

Protocol of CMF and O-LM administration. The 128 animals were divided into 16 batches of 8 rats each. Groups denominated A, B, C, D, E, F, G, and H (64 rats) received one IP dose of 2-, 4-, 10-, 12-, 14-, 16-, 18-, or 20-fold CMF, respectively, and a daily 0.5 SC injection of vehicle (saline) from 10 days before CMF and during 70 days (Control groups). Groups A', B', C', D', E', F', and H' (64 rats) received respectively one IP dose of CMF and a daily SC injection of O-LM (O-LM groups). The O-LM combination was administered as a 0.5-mL SC injection of the oligoelements Mn, Se, and Zn (1.5 µg/kg BW each) plus *L. muta* (0.1 ng/kg BW), beginning 10 days before the CMF injection and continuing during 70 days.

All survival animals were sacrificed 60 days after CMF injection, on day 70.

Histopathological studies. Autopsies were performed on all the animals either at the time of spontaneous death or at the end of the experiments. Viscera and the sixth dorsal vertebrae were removed and immediately fixed in 10% formaldehyde buffer. After macroscopic study, specimens of lung, liver, heart, kidney, spleen, genitalia, and bone marrow were harvested for histological studies. Tissues were embedded in paraffin and stained with hematoxylin-eosin, Giemsa, periodic acid-Schiff, or other special techniques.

Parameters recorded. (a) Body weight was monitored every other day; (b) observation of collateral macroscopic effects including changes in fur coloration, hair loss, and nasal and gut hemorrhage; (c) LD_{50} determination; (d) necropsy to determine the cause of death; (e) histopathological studies of organs and tissues.

Toxicological studies. Another set of experiments was performed to investigate the possible side effects of O-LM combination. Twenty-four female and 24 male adult rats were divided in three groups ($n = 8$ each). The first group received a daily dose of O-LM identical to the one employed in the protocol with CMF. The second group received a 10-fold dose, and the third one received vehicle alone (saline). These toxicological studies were followed up during 180 days.

The parameters analyzed were (a) body weight: the weight of animals was measured with a special scale once a week. (b) Water and food daily consumption: food and water consumption was determined per animal and per body weight (BW) using metabolic cages. (c) Clinical laboratory parameters: hematology (erythrocytes, hematocrit, hemoglobin, neutrophiles, lymphocytes, monocytes, eosinophiles, basophiles, platelets), chemistry (glucose, urea, creatinine), and enzymes (glutamic oxaloacetyl transaminase [GOT] and glutamic

pyruvic transaminase [GPT]) were determined employing conventional bio-chemical methods recommended by the International Federation of Clinical Chemistry. (d) Histopathological studies of different organs: liver, kidney, lung, brain, and testis.

In Vitro Studies

Cell cultures

Cell lines derived from different human neoplasias as SOJ-6, PANC-1 from pancreatic carcinoma, MCF-7 from breast carcinoma, WM35 and WM983 from melanoma, and HBL-100 from normal mammary epithelium were employed. Cells were cultured in RPMI medium, 10% FCS, and incubated at 37°C in a 5% CO_2 humidified atmosphere.

Proliferation was assayed by colony formation technique as described previously [21]. Cells were seeded in 6-well plastic dishes (2×10^3 cell per well) in complete medium and were allowed to grow for 8–10 days. Then cells were fixed in buffered formalin, stained with toluidine blue, and evaluated under OM by counting the numbers of colonies containing more than 50 cells. The effect of O-LM was tested by adding 20 μL per well of the combination to the culture medium in order to obtain a final concentration of Zn, Se, and Mn of 10^{-9} g/L and for LM 10^{-12} g/L.

Experimental Protocol

Stock monolayer cultures were trypsinized, and cells resuspended in complete medium were seeded in 6-well plastic dishes. The plates were incubated for 24 h; afterward, a proportion of the cultures was treated with freshly thawed 5-flu-oruracil (5-FU) in final concentrations ranging from 1 to 100 μM and further incubated for another 24 h. The overlayer medium was then removed, cells were washed, and fresh complete medium was added. After 1 week cells were fixed in buffered formalin, stained with toluidine blue, and evaluated by counting the colonies containing more than 50 cells. Cell survival was calculated as the relation between the number of colonies in 5-FU-treated cultures and the colonies formed in control plates.

In identical parallel experiments, cell cultures were grown in complete medium containing Zn, Se, and Mn 10^{-9} g/L plus LM 10^{-12} g/L during the whole experimental period. The treatment with 5-FU was performed under the same experimental conditions and in the above mentioned concentrations. Control cultures were grown in complete medium alone. All experimental points were performed in quadruplicate.

Statistical Analysis

Two-way ANOVA and Bonferroni post-test were used to compare the body weight of O-LM groups versus the Control group. Survival curves were fitted by

the Kaplan–Meier method and compared by log-rank test. LD_{50} doses were estimated by the Litchfield–Wilcoxon method [22]. In dose–response studies non-linear regression analyses were performed by using the Graph Pad Prism 3.0 software.

Reagents

Zinc chloride, manganese chloride, sodium selenite, and *L. muta* venom were obtained from providers of pharmaceutical drugs according to USPXX Pharmacopoeia requirements. Methotrexate was from Lederle Parenterals (USA), cyclophosphamide was from Labinca (Buenos Aires, Argentina), and 5-fluoruracil was from Roche (Basel, Switzerland). Cell lines derived from human pancreatic carcinoma (SOJ-6, PANC-1) were obtained from Dr. A. Mazo (University of Barcelona, Spain), and cell lines WM35 and WM983 were from Dr. Andras Falus (Semmelweis University of Medicine, Budapest, Hungary). The normal mammary epithelium cell line HBL-100 and MCF-7 line were obtained from Dr. A. Baldi (Institute of Medicine and Experimental Biology, Buenos Aires, Argentina). Cell culture reagents were from BRL Gibco (Gaithersburg, MD).

RESULTS
In Vivo Experiments

All parameters studied indicated an enhanced tolerance to systemic CMF treatment in O-LM versus Control rats.

A significantly higher survival was observed in animals receiving O-LM compared to Control groups: while 84.3% of O-LM rats remained alive 60 days after CMF injection, only 59% of Control ones did so (Fig. 1, $P < 0.004$, log-rank test). The highest percentage of deaths occurred between the 7th and 12th days post-CMF injection; in Control groups 8% of rats died between days 13 and 25 post-CMF.

The groups injected with 14-, 16-, and 18-fold CMF doses showed the most significant difference in survival between the O-LM-treated and the corresponding Control group ($P < 0.006$, $P < 0.005$, and $P < 0.002$, log-rank test, Fig. 1b–d, respectively).

In rats daily injected with O-LM, the LD_{50} was significantly higher than the value determined in Control rats: 18.5-fold versus 14.1-fold the basic CMF dose ($P < 0.01$ Litchfield–Wilcoxon, Fig. 2).

As it is observed in the Fig. 3, rats treated with O-LM and receiving 2- to 10-fold of the basic CMF dose showed a significant increase in whole body weight when compared to Control groups ($P < 0.05$, two-way ANOVA and Bonferroni post-test).

Collateral macroscopic effects, such as nasal and gut hemorrhage, fur discoloration, and hair loss, were invariably milder in O-LM groups compared to
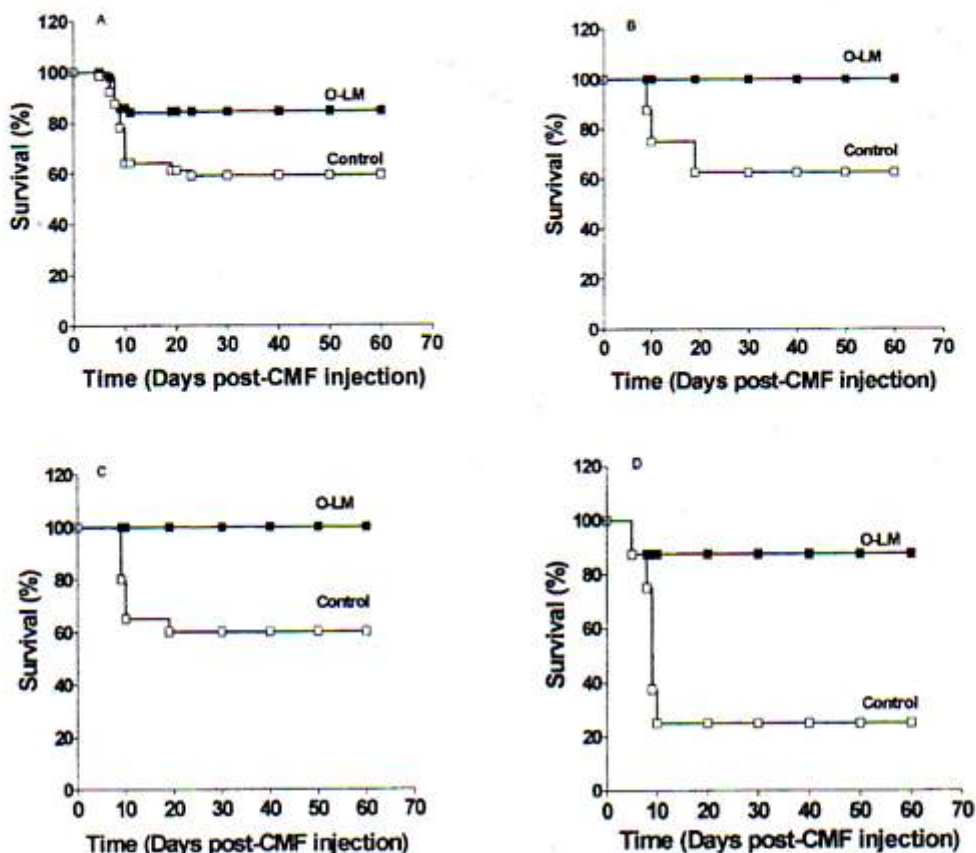
Fig. 1.   Survival curves of animals treated with a single IP dose of 2- to 20-fold CMF (cyclophosphamide 500 mg/m², methotrexate 40 mg/m², and 5-fluoruracil 600 mg/m²). For all the CMF doses employed rats protected with a daily injection of O-LM (■) exhibited higher survival than Control rats (□). (A) Global survival ($P < 0.004$). (B) Survival curves of rats injected with a 14-fold CMF dose ($P < 0.005$). (C) Survival curves of rats injected with a 16-fold CMF dose ($P < 0.005$). (D) Survival curves of rats injected with an 18-fold CMF dose ($P < 0.002$). Kaplan–Meier method and log-rank test used.)

Control animals; for the 16-fold dose, 50% of Control rats showed severe nasal and gut hemorrhage while only 25% of O-LM group did so; for the 18-fold dose, 75% of Control and 50% of O-LM rats, respectively, showed severe collateral effects; finally, for the 20-fold dose, 100% of Control rats showed severe hemorrhage while O-LM rats exhibited similar but less severe lesions.

Histopathological Results

The histopathological studies indicated that all deaths were related to the cytotoxicity of the drugs employed. During the first 2 weeks, severe bone marrow aplasia, grade II or III (Fig. 4), was observed. Acute sepsis with bacterial colonies
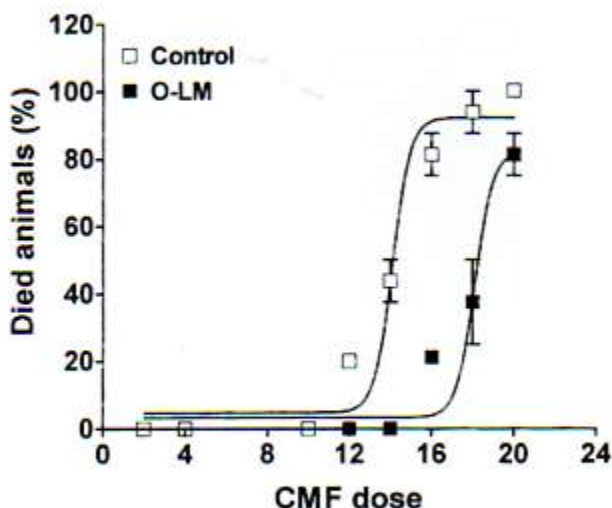
Fig. 2. Percentage of mortality versus dose of CMF (expressed as fold of the basic dose) for rats receiving CMF alone (□) or for animals simultaneously treated with O-LM (■). The LD_{50} value determined resulted significantly lower for Control rats: 14.1-fold versus 18.5-fold the basic CMF dose; $P < 0.01$, Litchfield–Wilcoxon test. Data represent $X \pm$ SEM from two independent experiments ($n = 4$ each).

were evident in spleen, liver, kidney, and other studied organs (80% of cases) of Control rats (Fig. 5); acute renal tubular necrosis was found in an important proportion of cases (60%), and centrilobular hepatic lesions were also evident in 40% of the cases. In the rats that died during the 3rd and 4th weeks, histopathological observations indicated kidney lesions or sepsis. The groups treated with O-LM showed different alterations according the applied chemotherapeutic
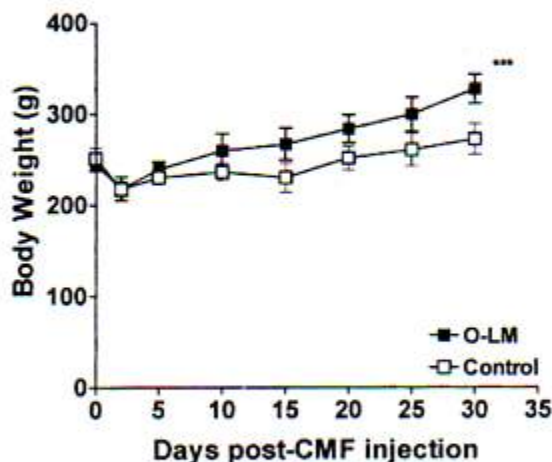

Fig. 3. Evolution of body weight after a single IP injection of 2-fold to 10-fold CMF doses in Control (□) and in O-LM-treated rats (■). Data represent $X \pm$ SEM. Rats receiving O-LM showed a significant increase in whole body weight. ***$P < 0.001$ two-way ANOVA and Bonferroni post-test.
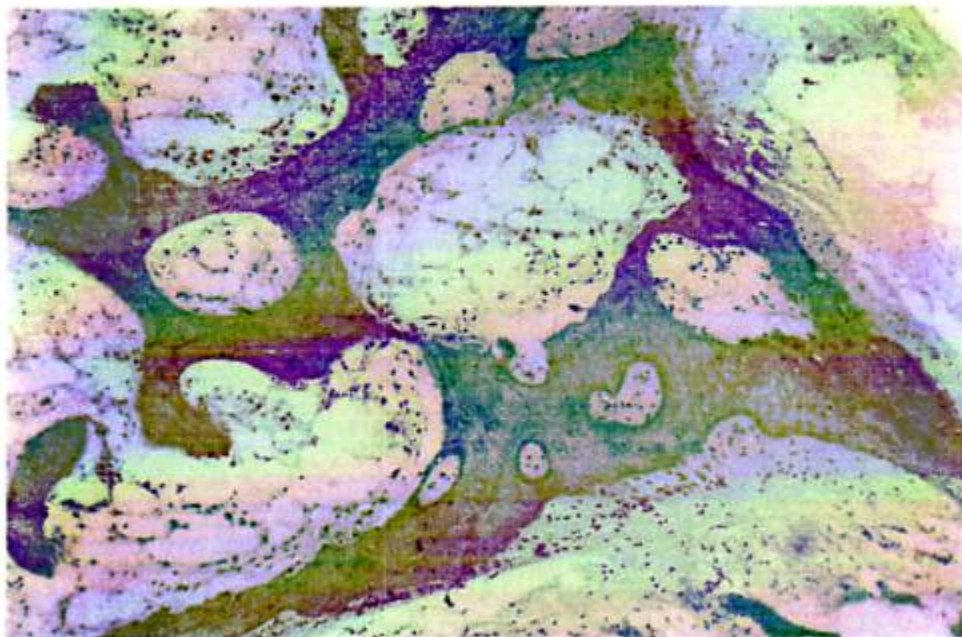
Fig. 4. Bone marrow with loss of granulocytic and erythroblastic components and persistence of lymphocytes, histiocytes, and stroma cells in a Control rat that died on day 8 after receiving 14-fold CMF. Aplasia grade III (HE, original magnification 400×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

dose: whereas at 16-fold the CMF dose, 80% of Control rats died with septic histopathological lesions, only 20% of O-LM treated rats showed signs of sepsis; at the same dose, 80% of Control animals exhibited bone marrow aplasia whereas only 20% of O-LM rats showed this lesion at the time of death.

On the other hand, studies performed on surviving rats that were sacrificed 60 days post-CMF injection, showed chronic myeloid leukemia in bone marrow in all Control batches (Fig. 6). In contrast, none of the O-LM-treated rats presented those abnormalities, showing normocellular or slightly hypercellular bone marrow population.

Toxicological Studies

Body weight. No significant difference was observed in whole body weight of rats treated with the low dose or the 10-fold dose of O-LM in comparison with non-treated rats.

Water and food daily consumption. Table I shows the water and food consumption of Control and treated rats with two different O-LM doses. No significant difference was observed in treated versus non-treated rats.
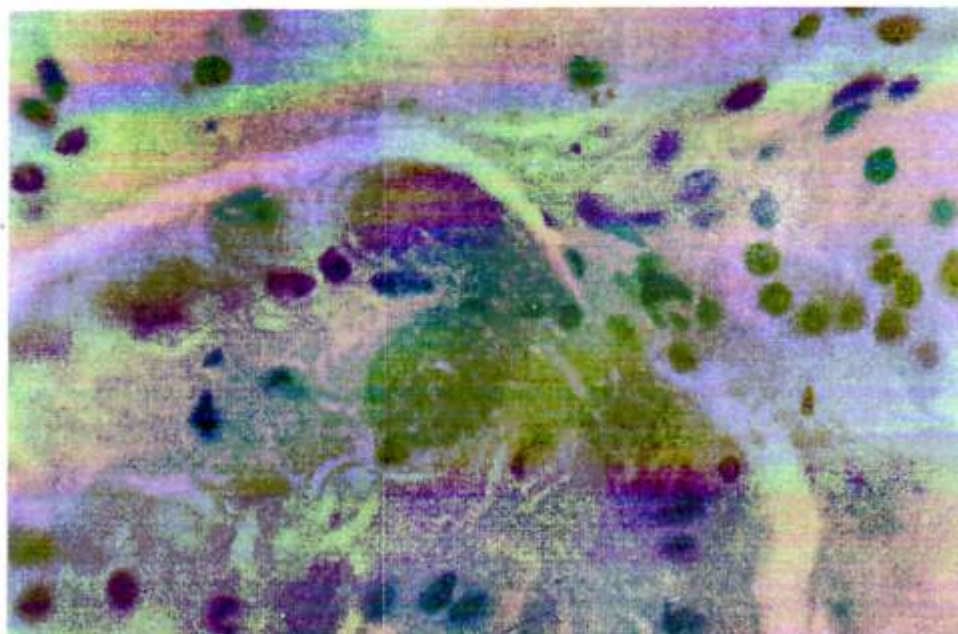
Fig. 5. Bacterial colonies in myocardium of a Control rat that died on day 11 after receiving 10-fold CMF (HE, original magnification 630×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Clinical laboratory parameters. As presented in Table II the value of biochemical parameters from Control and treated rats showed no significant differences.

Histopathological studies. Macroscopic appearance and weight of the organs studies were normal and comparable to healthy rats. No alterations of shape, color, or general appearance were observed. Histological preparations of the autopsied animals showed normal structures in all cases. Neither damage to cellular structures nor inflammatory changes were found. Only physiological peribronchial mononuclear infiltrates were evident in lung samples and were similar to those observed in Control rats.

In Vitro Experiments

As shown in Table III, treatment with O-LM significantly inhibited proliferation of the different neoplastic human cell lines studied. Remarkably, normal epithelial cells (HBL-100) grown in the presence of O-LM showed a non-significant *increase in proliferation.*

In vitro experiments indicated that the cytostatic effect of 5-FU was significantly enhanced by O-LM pretreatment in transformed cells while in normal
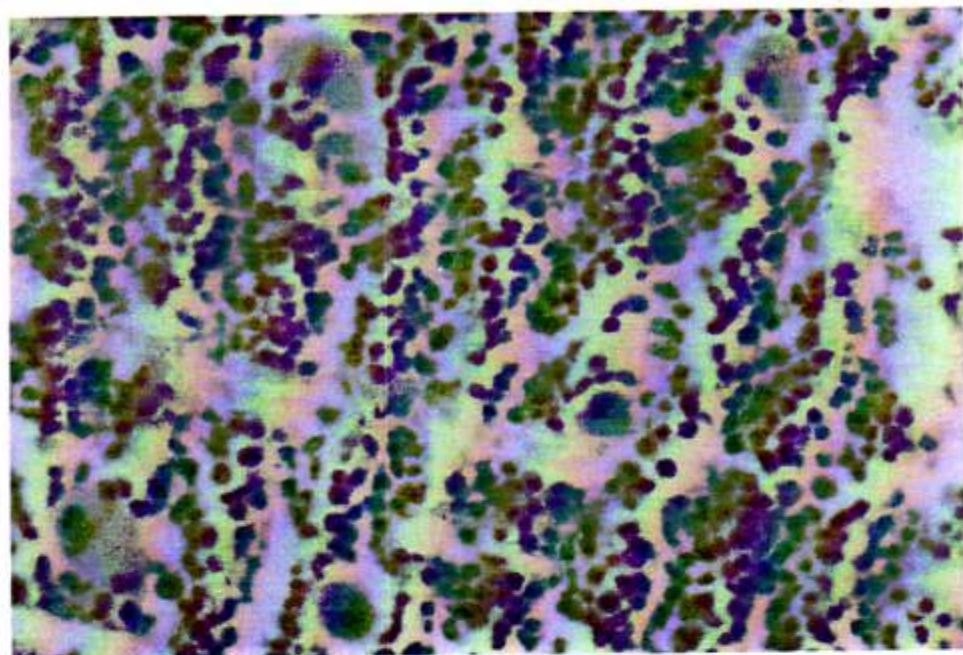
Fig. 6. Bone marrow with chronic myeloid leukemia infiltrate and disappearance of normal adipose tissue in a Control rat receiving 16-fold CMF and sacrificed after 60 days (HE, original magnification 400×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells O-LM exerted a protective effect with a marked decrease of toxicity. When transformed and normal cells were exposed to different doses of 5-FU, in both cases dose-dependent reductions of cell survival were observed, with identical EC_{50} values of 6 ± 2 μM. After 24 h of treatment with 5-FU 100 μM, no viable cells were observed. In Fig. 7a and b are shown the dose–response curves obtained with pancreatic carcinoma cells SOJ-6 and with the normal HBL-100 epithelial cell line, respectively. The combined treatment with O-LM resulted in a differential response observed in transformed and normal cells. As shown in Fig. 7a, addition of O-LM to SOJ-6 cells enhanced the cytotoxic effect of 5-FU in all doses tested. In addition, O-LM, when added alone to cell cultures, significantly reduced cell survival. The EC_{50} value for 5-FU when combined with O-LM was 0.8 ± 0.2 μM.

TABLE I. Food and Water Consumption of Control and O-LM-Treated Male Rats

Age (months)	Food consumption (g/100 g weight)			Water consumption (mL/100 g weight)		
	Control	O-LM	O-LM (×10)	Control	O-LM	O-LM (×10)
4	7.1−9.0	7.2−9.7	7.3−9.8	7.0−9.5	7.1−9.2	7.1−9.2
10	7.0−8.0	7.1−8.4	7.2−8.7	7.0−9.1	7.5−9.5	7.5−9.6

TABLE II. Hematological and Clinical Laboratory Parameters Determined in Control and in O-LM-Treated Rats

Parameter (Units)	Mean Value ± SD		
	Control	O-LM	O-LM (×10)
Erythrocytes ($10^6/mm^3$)	8.0 ± 1.1	7.9 ± 0.40	8.3 ± 1.2
Hematocrit (%)	44.7 ± 3.7	43.1 ± 1.6	43.9 ± 2.1
Hemoglobin (g/dL)	15.4 ± 1.3	14.9 ± 1.1	14.8 ± 1.1
Mean conc. Hb corp (%)	34.3 ± 1.0	33.8 ± 1.5	34.8 ± 1.6
Mean Hb corp (pg)	19.2 ± 1.2	19.8 ± 1.2	19.5 ± 1.3
Mean vol. corp (μ^3)	55.0 ± 3.6	56.0 ± 2.6	55.9 ± 2.4
Leucocytes ($10^3/mm^3$)	11.5 ± 2.6	10.9 ± 3.0	10.9 ± 2.2
Neutrophils (%)	13.9 ± 4.0	14.8 ± 4.5	14.1 ± 3.5
Lymphocytes (%)	82.2 ± 4.6	81.0 ± 5.0	81.8 ± 5.1
Monocytes (%)	2.1 ± 0.7	1.9 ± 1.0	1.8 ± 1.1
Eosinophils (%)	1.5 ± 0.8	1.3 ± 1.1	1.4 ± 1.0
Basophils (%)	0	0	0
Platelets ($10^3/mm^3$)	845 ± 159	810 ± 199	821 ± 131

However, when normal HBL-100 cells were cultured in the presence of O-LM and further treated with 5-FU, a protective effect was observed at all doses tested (Fig. 7b), showing a significantly higher EC_{50} value of 20 ± 3 μM for the cytotoxic drug.

DISCUSSION

Data obtained clearly demonstrated the protective effect exerted in vivo by the combination of oligoelements Mn, Se, and Zn plus LM in animals receiving high-dose cytostatic treatment. Global survival significantly increased from 59% in Control groups to 84.3% in O-LM-treated rats. Furthermore, in vitro O-LM showed a different action applied to normal or transformed cells.

Results of in vivo studies showed that LD_{50} for Control rats was 14.1-fold the basic dose versus 18.5-fold for O-LM-treated animals, indicating a significant enhancement in tolerance to cytostatic drugs. The highest proportion of deaths in the Control group occurred between the 7th and 12th days after CMF administration; it was related to sepsis in 80% of cases, mainly associated to acute

TABLE III. Effect of Oligoelements Plus LM on Proliferation of Human Normal and Transformed Cell Lines

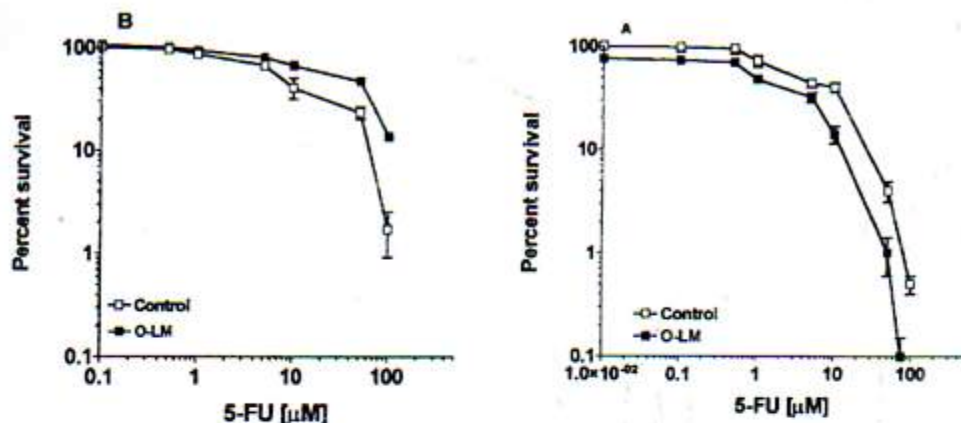| Cell line	Proliferation (%)
SOJ-6	60[a]
PANC-1	34[b]
MCF-7	60[a]
WM35	55[b]
WM983	58[b]
HBL-100	120[c]

Fig. 7. Dose–response curves for cells treated with different concentrations of 5-FU in the presence (■) or absence (□) of O-LM. Cell survival is expressed as the relation between the number of colonies in 5-FU-treated cultures and colonies in Control plates. (A) SOJ-6 transformed cell line. EC_{50} value for 5-FU, 6 ± 2 µM; for 5-FU combined with O-LM, 0.8 ± 0.2 µM ($P < 0.01$). (B) HBL-100 normal cell line. EC_{50} value for 5-FU, 6 ± 2 µM; for 5-FU combined with O-LM, 20 ± 3 µM ($P ± 0.01$).

renal tubular necrosis and bone marrow aplasia. A small percent of animals (8%) died between the 13th and 60th days; in these cases deaths were attributed to cardiac or central circulatory causes with scarce histological alterations. However, in O-LM rats, mortality due to generalized sepsis between the 7th and 12th days decreased drastically from 80% to 20%. Deaths in the treated group were related to acute renal tubular necrosis and bone marrow aplasia. In O-LM-treated rats, no deaths were recorded after the 13th day post-CMF injection. The lower percent of mortality indicates a protective effect of O-LM, and it may be explained in part by the enhancement of immune response that can be attributed to the O-LM combination. We have previously reported that the administration of O-LM in rats bearing mammary adenocarcinomas increased host anti-tumor response, clearly observed in tumor microenvironment [23].

At the time of death or when sacrificed at 60th day post-CMF injection, Control rats invariably presented either bone marrow aplasia in its earlier phase or chronic myeloid leukemia in its last phase. In contrast, there were no cases of myeloid leukemia in O-LM-treated rats. These observations are in agreement with the chemopreventive action reported for oligoelements, which exert an antioxidant effect protecting cells from oxidizing radicals [24]. It has been shown that fewer DNA chain fractures are induced by carcinogens following Se pre-treatment [25]. Furthermore, studies carried out in our laboratory showed that O-LM exerts a preventive action on the carcinogenic effect of nitroso-N-methylurea (NMU). Administration of this combination significantly reduced the development of NMU-induced mammary adenocarcinomas in female Sprague-Dawley rats. A significant decrease in tumoral incidence, from 100% to 20%, and an increase in animal survival, from 205 to 385 days ($P < 0.0001$ Kaplan-Meier, log-rank test) was determined when comparing O-LM-treated animals to Control groups [16]. Similar results were obtained employing a tumor model induced in nude mice by the inoculation of human PANC-1 cell line [15].

An important observation was the marked whole body weight recovery of rats treated with O-LM and receiving CMF doses 2- to 10-fold the basic dose. In addition, the toxicological studies clearly demonstrated that O-LM treatment did not produce any toxic or undesirable effect when administered to normal animals. Whole body weight, water and food consumption, and laboratory parameters remained unchanged after O-LM administration also with a dose 10-fold higher than the one employed in the experimental work. In agreement, histological studies further confirmed that the proposed therapeutic combination does not produce any toxic effect on normal tissues. The toxicological effect of Se has been report; in preliminary toxicology studies we have determined the LD_{50} for sodium selenite in Sprague-Dawley rats to be 3.40 mg/kg BW for males and 5.80 mg/kg BW for females. It is important to note that this value is approximately 10^4-fold higher than the concentration employed to obtain the therapeutic and protective effect of O-LM described in the present work.

As we have previously observed in experimental tumors, O-LM combination produces an inhibitory effect on proliferation of transformed cells [15]. In the present study, all malignant cell lines tested showed a similar response in vitro: a significant decrease in the number and size of colonies formed. On the other hand, in the HBL-100 normal cell line, O-LM produced an increase in proliferation, although not statistically significant. The inhibitory effect on cell proliferation may be a consequence of the induction of apoptosis or an arrest on cell cycle progression. As it is known, the increase of cAMP levels produces arrest in cell growth and plays an important role in cell differentiation [21,26,27]. Experiments carried out in our laboratory demonstrated that the combination O-LM produces a 3-fold increase in cAMP intracellular levels when studied in vitro employing the same cell lines (data not shown, manuscript in preparation).

Furthermore, in vitro studies clearly demonstrated that O-LM treatment produced a protective effect on normal cells against 5-FU while it enhanced the cytotoxic effect on malignant cells. The results of the experiments carried out with normal HBL-100 cells showed that for all the 5-FU concentrations tested the viability of cells increased when O-LM was added to the medium. However, enhancement of cytotoxic effect was observed in transformed SOJ-6 cells when O-LM was added to these cultures, significantly decreasing cell survival. Tobey demonstrated that in vitro Zn and Se has a protective effect on normal cells, which allow a 10-fold increase in the chemotherapeutic dose in pretreated versus non-pretreated cultures and up to a 16-fold increase when associated with Se and Cu through an independent mechanism of metallothionein synthesis [28,29]. It has been demonstrated in vitro that Se is unable to protect atypical cells against anti-neoplastic drugs and that Zn exhibits a similar inability [28,29]. In the present work we observed an increase of almost 4-fold in the EC_{50} value for 5-FU in normal cells when treated with O-LM, from 6 ± 2 µM to 20 ± 3 µM. These results are in agreement with the response observed in vivo, where normal tissues, such as bone marrow, rapidly recovered from the toxicity of chemotherapeutic drugs in animals receiving O-LM.

One of the major limitations to alkylating agents as chemotherapeutic drugs is their toxicity for normal cells. Enhancing the resistance of normal cells to al-

kylating agents selectively could allow more effective dose levels without an increase of toxicity for normal cells.

CONCLUSIONS

. The results presented in this work are in agreement with our hypothesis that O-LM exerts its therapeutic action through a complex mechanism: it enhances the immune response and also modulates cellular growth and metabolism. While several mechanisms have been proposed to explain the action of oligoelements and of PLA$_2$ contained in LM, we consider that the underlying molecular mechanism of the effects produced by the combination O-LM that were described in this work must be further investigated. This treatment is undoubtedly a promising future therapy that makes it possible to significantly increase the doses of cytotoxic drugs that are usually employed in the treatment of different types of cancer.

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The Journal of
Trace Elements in
Experimental Medicine

Volume 16, Number 1 was mailed the week of January 27, 2003.