Mononuclear cells release low molecular weight factors with anti-cancer activity: A lower level of production by cells of cancer patients

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Abstract. Following the clinical observations that tumor metastases are extremely rare in striated muscles we defined recently a low molecular weight factor which is released by muscle cells (muscle factor, MF) and possesses specific anti-proliferative activity against tumor cells. We demonstrate that peripheral blood mononuclear cells constitutively release low molecular weight factor (LMF) similar to the MF which is capable of inhibiting *in vitro* the proliferation of carcinoma, melanoma, leukemia and lymphoma cell lines. The proliferation of normal cells such as bone marrow or fibroblasts was not inhibited but slightly stimulated following incubation with the LMF. Biochemical purification of this factor by several HPLC steps revealed that the inhibitory activity against tumor cells was concentrated within two definitive peaks. The LMF affects tumor cell growth by arresting them in the G_0/G_1 of the cell cycle and its activity is species and tumor non-specific. *In vivo* studies in melanoma-bearing mice revealed that the LMF inhibited melanoma growth when given either intraperitoneally or orally. Mononuclear cells from cancer patients with different malignancies (non-Hodgkin lymphoma, malignant melanoma, colon carcinoma and carcinoma of the rectum) secreted lower level of LMF in comparison to healthy subjects. The capability of the LMF to inhibit tumor cell growth and promote normal cell proliferation combined with its bioavailability *in vivo* may lead to its potential therapeutic and diagnostic use.

Introduction

Peripheral blood mononuclear cells are known to secrete a panel of cytokines which are capable of inhibiting directly or indirectly the proliferation of tumor cells. These anti-proliferative cytokines include the interferons, TGF-ß, interleukin-1, TNF, Oncostatin-M, LIF, all are glyco-proteins with a molecular weight within the range of 15-30 kDa (1-4).

We recently found a novel anti-proliferative factor, which is released by muscle cells derived from primary cultures of newborn rats, and that unlike the known anti-proliferative cytokines, is non-proteinacious and has a molecular weight of less than 3 kDa (5). The rationale to look for an anti-cancer activity in muscle cells came from clinical observations that tumor metastases are extremely rare in striated muscles. The factor designated by us as MF inhibited both *in vitro* and *in vivo* the proliferation of various tumor cell lines including melanoma, sarcoma, and carcinoma. Although of a rat origin, the MF affected both human and murine tumor cells but did not inhibit proliferation of normal cells. Cell cycle analysis revealed that the MF exerted a cytostatic rather than a cytotoxic effect on tumor proliferating cells by arresting them in the G_0/G_1 phase of the cell cycle.

Since thymus and spleen are also very rarely sites of metastases we presumed that a similar cytokine such as the MF may be secreted by lymphocytes and monocytes which consist most of the population in these organs.

In the present study we show the ability of peripheral blood mononuclear cells of healthy subjects to release a similar factor to the MF capable of inhibiting the proliferation of tumor cells *in vitro* and *in vivo*. However, mononuclear cells of patients with different types of malignancies release the factor in a much lower level.

Materials and methods

Blood samples. Twenty ml of heparinized blood were collected from 29 healthy volunteers and from 34 patients with different malignancies: non-Hodgkin's lymphoma (n=5), malignant melanoma (n=7), colon carcinoma (n=10), carcinoma of the rectum (n=12). All the cancer patients had histologically established diagnosis. Blood samples were drawn from the patients following surgical treatment of the

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primary disease, or after carrying out a diagnostic procedure. None of the patients has been exposed to chemotherapy or to immunotherapy.

Preparation of mononuclear cell conditioned medium (MNC CM). Mononuclear cells (MNC) were fractionated from heparinized blood using Ficoll-Hypaque gradient. $2x10^6$ mononuclear cells were cultured in phosphate buffered saline (PBS), pH 7.4 for 20 h and at the end of the incubation period the supernatant was collected, centrifuged and filtered through 0.2 μ sterile filter. Since based on our previous work (5) we already knew that the MF is detected in a lower than 3 kDa molecular weight fraction, the supernatant was subjected to ultrafiltration through Amicon membrane with a molecular cut-off of 3 kDa and kept at -20°C until assayed.

Tumor cell lines. Tumor cell lines from murine rat or human origin were used. The murine cell lines included B-16-F10 melanoma cells, DA-3 breast carcinoma cells and the BB88 mouse leukemia (erythroleukemia induced by the Friend murine leukemia virus). The human cell lines were SK-28 melanoma, MCF-7 breast carcinoma and K562 chronic myelogenous leukemia. All the cell lines were purchased from the American Type Tissue Culture Collection, Rockville, MD (ATCC). The Nb2-11C rat lymphoma cell line was kindly given by A. Gertler from the Agriculture Faculty, Rehovot, Israel (6). The cells were routinely maintained in RPMI medium containing 10% fetal calf serum and twice weekly transferred to a freshly prepared medium.

Normal cells. Murine bone marrow cells derived from the femur of C57BL/6J mice and fibroblasts derived from primary cultures of newborn rat skeletal muscle, served as normal proliferating cells and were prepared as described previously by us (5).

Cell proliferation assays. Cell count assay. The effect of mononuclear CM on the proliferation of rat Nb2-11C lymphoma cells was monitored by cell counting. The Nb2-11C cells were synchronized in the G_0/G_1 phase prior to cultivation with the CM by transferring the cells to horse serum (Biological Industries, Beit Haemek, Israel) supplemented medium for overnight incubation. $3x10^5$ cells/ml were cultured in 24-well plates in 1 ml RPMI medium containing 5% horse serum and two-fold dilution of 3 kDa ultrafiltrated MNC CM. Since the MNC CM consisted of PBS, two-fold dilution of PBS was added to synchronized Nb2-11C cells and served as control. Cell proliferation was initiated by addition of human growth hormone (Biotechnology General, Rehovot, Israel) to a final concentration of 2 ng/ml. The cultures were incubated at 37°C at 5% CO_2 and cells were counted in Coulter Counter 48 h later. Inhibition of cell proliferation was calculated as follows:

$$\% \text{ of inhibition} = \frac{\text{cell count of sample} - \text{cell count without hormone x100}}{\text{control cell count with hormone} - \text{cell count without hormone}}$$

This assay with the Nb2-11C cells was later used as the biological assay to detect inhibition of proliferation during the fractionation procedures.

[^{3}H]-Thymidine incorporation assay. $1x10^4$ of each murine or human tumor cell line or normal cells were incubated with RPMI medium containing 10% FCS and 75% 3 kDa ultrafiltrated MNC CM in 96 microtiter plates for 48 h. Since the CM consisted of PBS, cultures containing tumor cells suspended in RPMI medium, 75% PBS and 10% FCS served as control. During the last 6 h of incubation, each well was pulsed with 1 μCi [^{3}H]-thymidine. The cells were harvested and the [^{3}H]-thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ, USA).

Cell cycle analysis. Flow cytometric analysis of the cell cycle of K-562 myelogenous leukemia or SK-28 human melanoma cells was carried out by propidium iodide staining (7). Briefly, $1x10^6$/ml of each cell type was cultured in RPMI medium containing 10% FCS and 50% 3 kDa ultrafiltrated MNC CM and were incubated for 24 h at 37°C in a CO_2 incubator. Cells cultured in RPMI medium containing 50% PBS and 10% FCS served as control. At the end of the incubation period, the cells were washed three times with PBS and resuspended in staining buffer containing 0.1% bovine serum albumin (BSA), 50 mg/ml propidium iodide, 0.1% Triton X-100 and 1 mg/ml RNAse (boiled for 10 min). Samples were examined after 30 min of staining on a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA, USA).

Fractionation and partial purification of the inhibitory activity in the mononuclear cell CM. Following the ultrafiltration of the MNC CM through an Amicon membrane with a molecular cut-off of 3 kDa, the filtrate was subjected to fractionation through four types of HPLC columns. In each purification step, fractions were collected and biologically assayed for their ability to inhibit cell proliferation of the Nb2-11C lymphoma cells. Each fraction was lyophilized prior to testing, suspended in RPMI medium and filtered through 22 μ sterile filter.

The first column was a preparative RP-HPLC with a diameter of 2" and length of 200 mm. The column was loaded with Merck Lichrosphere-C18 particles with a diameter of 12 μ and a porosity of 60A. In each round, 800 ml of the above filtrate was loaded and the column was eluted with a gradient of acetonitrile at a rate of 2%/min, until a level of 60% of acetonitrile was reached. The rate of elution was 100 ml/min and fractions of 200 ml were collected. A small portion (5 ml) from each fraction was dried and then used to test biological activity.

The active fractions were dried in a rotary evaporator, dissolved in water, filtered and injected into a Supelco-C18 5 μ column (4.6x250 mm) with porosity of 80A. The flow rate was 1 ml/min. The eluent was a water/acetonitrile gradient between 0-60%. The rate of the gradient was 3%/min, i.e. reaching 60% after 20 min. One ml fractions were collected, tested for biological activity and the active fractions were dried in a concentrating centrifuge, dissolved in 50 mM formic acid for the next chromatography stage on a Polyhydroxyethyl A column (9.4x200 mm), 5 μ, (Poly LC, Columbia, MD). The eluent was 50 mM formic acid solution. 200 μl obtained from

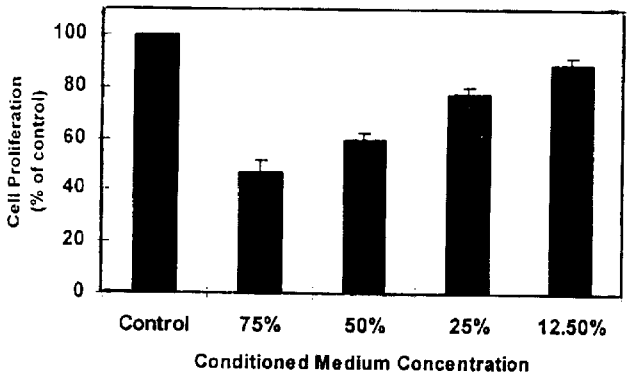

Figure 1. Inhibitory dose dependent effect of the 3 kDa mononuclear cell conditioned medium on the proliferation of synchronized Nb2-11c rat lymphoma cells. Proliferation was evaluated by cell count.

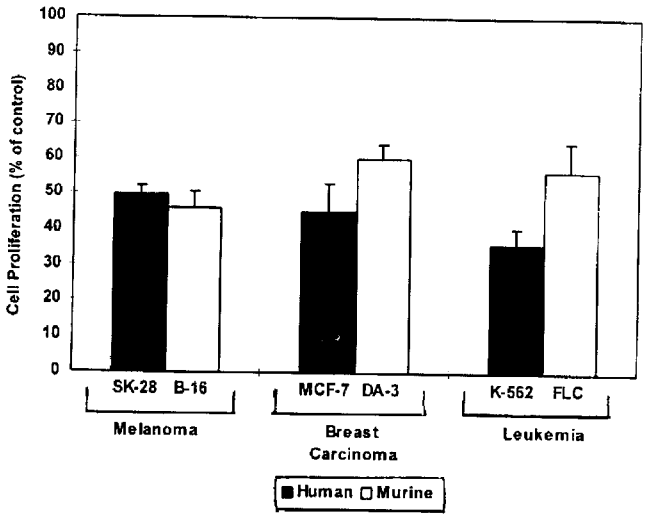

Figure 2. Inhibitory effect of the 3 kDa ultrafiltrated mononuclear cell conditioned medium on the proliferation of various human and murine cell lines. Proliferation was evaluated by [^{3}H]-thymidine uptake.

the previous stage was injected into the column and chromatographed at a flow rate of 0.5 ml/min and 0.5 ml fractions were collected. The fractions were dried in a concentrating centrifuge, biologically tested for activity and further purified on a Hypersil Cyanopropylsilane column, 5 µ (4.6x250) (Shandon, USA). 200 µl was injected into the column with a water/acetonitrile gradient of 0-60% and a flow rate of 1 ml/min.

In vivo studies. The 3 kDa ultrafiltrated MNC CM was tested for its ability to inhibit the development of B-16-F10 melanoma in C57BL/6J mice. Each group included 20 mice which were intravenously inoculated with $2x10^5$ B-16-F10 melanoma cells. The mice were treated twice daily by 0.5 ml of the 3 kDa ultrafiltrated MNC CM either by intraperitoneal or by per os administration. Eighteen days later the mice were sacrificed, the lungs were excised and melanoma tumor foci were counted using a dissecting microscope.

Results

Effect of the low molecular weight MNC CM on the proliferation of tumor or normal cells. CM was prepared from human peripheral blood MNC as described above and the 3 kDa ultrafiltrated MNC CM was tested for its capability to inhibit tumor cell proliferation. The cell proliferation of the Nb2-11C cells as measured by cell count showed a dose dependent statistically significant inhibition following incubation with two-fold dilution of the MNC CM (Fig. 1). Fig. 2 depicts the effect of the MNC CM on different types of murine or human tumor cell lines. An inhibition within the range of 38%-62% was seen in the different cell lines tested. Human cells derived from myeloid leukemia origin were the most sensitive to the effect of the MNC CM.

The proliferation of murine fibroblasts or bone marrow cells was not inhibited but rather stimulated following incubation with the 3 kDa ultrafiltrated MNC CM (Fig. 3).

A comparison between the ability of CM derived from cancer patients and healthy volunteers to inhibit the proliferation of the 3 human tumor cell lines is depicted in Fig. 4. Three kDa ultrafiltrated MNC CM derived from healthy subjects inhibited the proliferation of all tumor cell

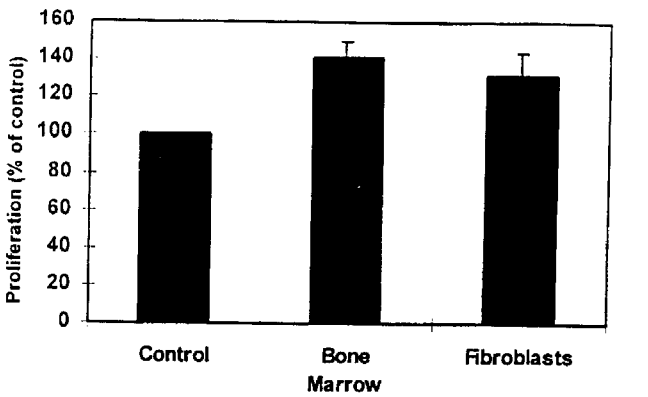

Figure 3. Effect of the 3 kDa ultrafiltrated mononuclear cell conditioned medium on the proliferation of normal murine bone marrow cells and rat fibroblasts. A slight stimulation of normal cell proliferation is seen. Proliferation was evaluated by [^{3}H]-thymidine uptake.

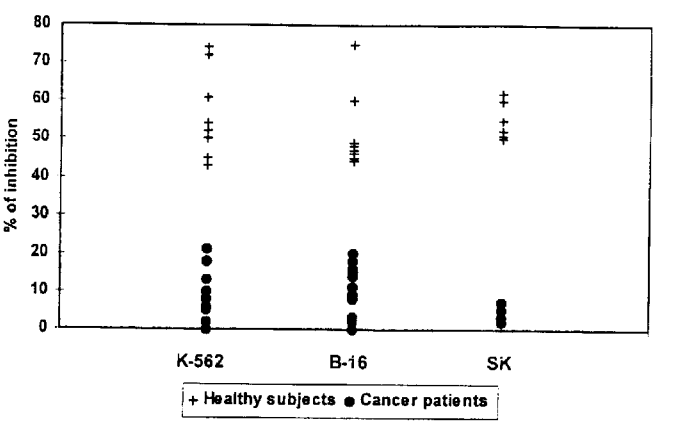

Figure 4. CM derived from healthy subjects inhibits the proliferation of three tumor cell lines, i.e. K-562, B-16 and SK. Much less inhibition is seen by CM derived from patients with cancer.

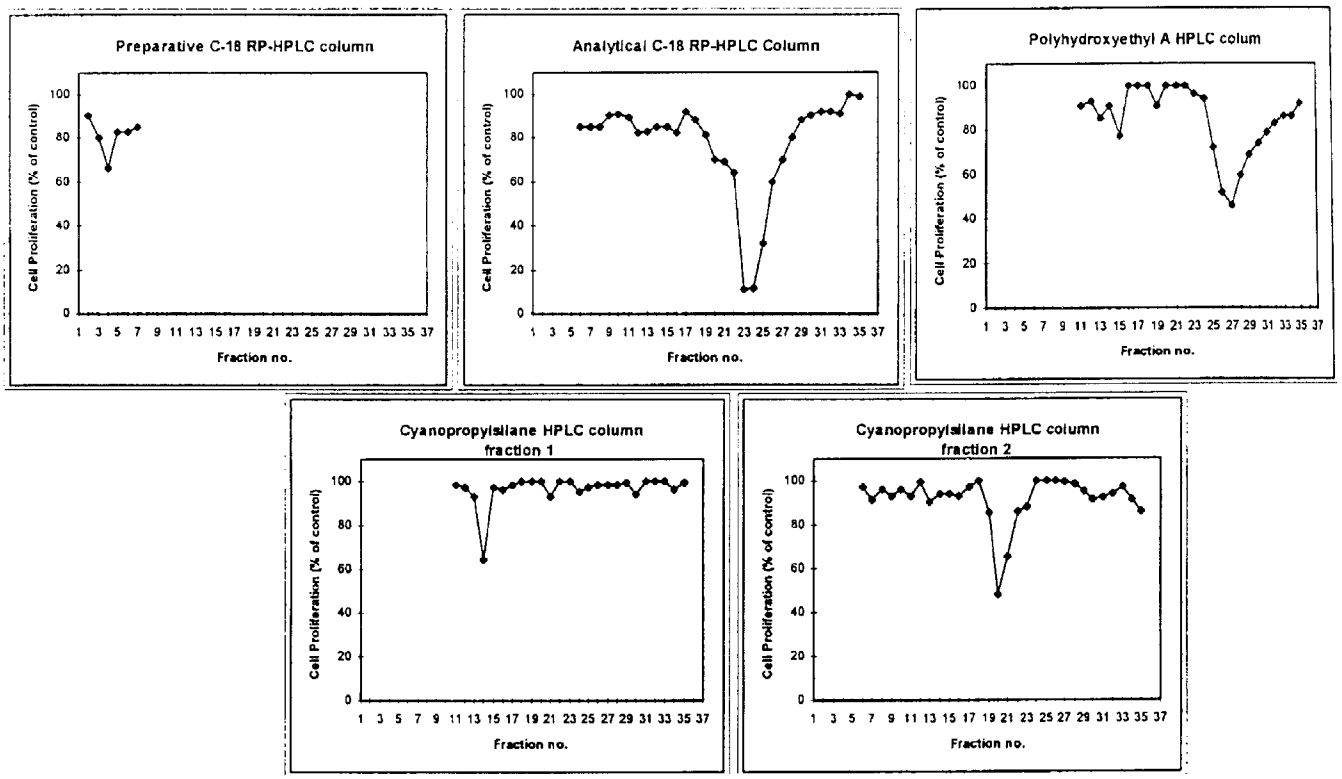

Figure 5. The biological activity of various fractions eluted from the different steps of HPLC regarding their capability to inhibit the proliferation of the Nb2-11C lymphoma cells. Active inhibitory fractions of each column were pooled and subjected to the next column.

Table I. Effect of 3 kDa ultrafiltrated mononuclear cell conditioned medium (MNC CM) on the cell cycle of K562 leukemia cells and SK-28 melanoma cells.

	% G1	% S	% G2
SK-28			
Control	63.1	34.2	2.7
MNC CM	72.4	19.5	9.1
K-562			
Control	32.2	64.1	3.3
MNC CM	42.4	52.8	4.8

lines to a greater extent than the CM derived from patients with cancer. A statistically significant difference ($p<0.0001$) between the tested groups was recorded with no overlap among the inhibitory values between patients and controls.

Effect of the low molecular weight mononuclear cell CM on the cell cycle of tumor cells. Table I summarizes the flow cytometric analysis of K-562 leukemic cells and SK-28 melanoma cells incubated with or without 3 kDa ultrafiltrated MNC CM and stained with propidium iodide after RNAse digestion. The distribution of the cells in the different stages of the cell cycle is presented. An increase in the number of cells in the G_0/G_1 phase was recorded in the cells incubated with MF CM, as compared to those incubated with RPMI medium.

Fractionation of the 3 kDa ultrafiltrated MNC CM derived from healthy volunteers. Fig. 5 depicts the active fractions in each purification step and specifies the percentage of recovery. In the preparative and analytic RP HPLC-C18 column, the active fractions were eluted in an acetonitrile concentration of 16-20%. In the polyhydroxyethyl A column two peaks of inhibitory activity were detected, i.e. one at 17-22 min (fraction A), and the second eluted at 25-32 mins (fraction B). Fraction A from the former column was eluted at acetonitrile concentration of 20-24% and fraction B at 40-60% on the Hypersil cyanopropylsilane column.

In vivo studies. C57BL6J mice were inoculated with B-16 melanoma cells and 18 days later melanoma foci were counted. In the control untreated group 41±7.9 foci were detected while in the group treated with the MNC CM by intraperitoneal or per os administration, 22.5±4.1 and 25.7±6.1 foci respectively were observed.

Discussion

The present study shows that a low-molecular weight factor designated by us as LMF is released spontaneously by mononuclear cells and modulates the proliferation of tumor cells.

Various tumor cells originating from melanoma, carcinoma, leukemia or lymphoma cell lines were markedly inhibited following incubation with the LMF, while normal cells such as bone marrow cells and fibroblasts were even stimulated by the LMF. The specific antiproliferative effect on tumor cells was evidenced by four different methods i.e., decreased cell count,

reduced [^{3}H]-thymidine incorporation, cell cycle arrest in the G_0/G_1 phase and inhibitory effect *in vivo* on tumor cell growth. The LMF was found to be species non-specific since it is released by peripheral blood human mononuclear cells and affects human, rat or murine tumor cells.

The LMF was purified from MNC conditioned serum free buffer using several HPLC columns which yielded a definitive peak with tumor inhibitory activity.

These characteristics of the LMF distinguish it from the known interleukins which are glycoproteins with a molecular weight range of 15-30 kDa, secreted following mitogenic stimulation and possess antiproliferative effect towards tumor cells both *in vitro* and *in vivo*. The preference of the factor described in this study is its stability and low molecular weight which may contribute to its bioavailability. This was reflected in the *in vivo* studies where the efficacy of the LMF against melanoma growth was shown either by peritoneal or by oral administration.

Dekoter *et al* (8) reported recently on the secretion of a low molecular weight factor, designated Reptimed, from rat bone marrow cells which is capable of inhibiting the proliferation of several leukemic cell lines defined as immature hematopoietic progenitor cells. Reptimed did not inhibit stem cell proliferation such as the K562 myeloid leukemia cell line. Reptimed showed some similarity to the LMF described in the present study in that both are low molecular weight factors which are spontaneously released by hematopoietic cells. However, the LMF markedly inhibited the proliferation of the K562 cells while reptimed failed to do so.

The characteristics of the LMF described in the present study are similar to those of the muscle factor (MF) which was recently defined by us. The MF is released spontaneously by newborn skeletal rat muscle cells (5) and possesses similar biological and biochemical characteristics as the LMF. The MF was defined by us following studies which were aimed to address the question why tumor metastases are extremely rare in skeletal muscles. We recently further purified the MF and it was found biochemically and biologically to be identical with the LMF (the two factors were subjected to the same purification procedures as was specified above for the LMF and the inhibitory activity was eluted in the same fractions, unpublished data). The similarity between the factors which are released by muscle cells or mononuclear cells may point towards the existence of humoral physiological factors which are secreted constitutively and play a role in the natural defense against tumor development.

Indeed, MNC derived from patients with various types of malignancies secreted a significantly lower level of LMF in comparison to MNC derived from healthy subjects. It has been recently shown that in cancer patients the production of cytokines, such as IL-10, IFN-γ, GM-CSF and LIF, by whole blood cells after polyclonal activation, was significantly lower than in healthy controls (9). Although biochemically the LMF is different from the known antiproliferative cytokines, it shares with them some biological characteristics such as inhibition of tumor cell proliferation and lower level of production by cells of cancer patients.

Two findings in the present study support the notion that the LMF are physiological factors within the body that play a role in the immune defense against tumor development, i) the lesser ability of MNC from cancer patients to secrete the LMF and, ii) *in vivo* studies with the melanoma bearing mice showed that exogenous supplementation of the LMF to mice inoculated with melanoma cells prevented tumor growth.

The capability of the LMF to inhibit tumor cell growth and promote normal cell proliferation distinguishes them from the known cytotoxic agents which are known to affect normal as well as malignant cell proliferation, thereby limiting their clinical usefulness. These unique characteristics of the LMF combined with the stability of the active components and their efficacy when given *in vivo*, may lead to potential clinical use following their complete characterization. Moreover, the difference in the capability of healthy subjects and cancer patients to produce the LMF may lead to its future use as a diagnostic marker.

Acknowledgements

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