# COMPOSITION AND METHOD FOR REGULATING CELL PROLIFERATION AND CELL DEATH

## FIELD OF THE INVENTION

The present invention relates to a composition comprising a mycobacterial DNA (B-DNA) and a first pharmaceutically acceptable carrier, wherein the B-DNA is effective in inducing a response in responsive cells of an animal. Particularly, the present invention relates to a composition comprising *Mycobacterium phlei*-DNA (M-DNA) and a first pharmaceutically acceptable carrier, wherein the M-DNA inhibits proliferation of and induces apoptosis in responsive cells and stimulates responsive cells, of the immune system to produce bioactive molecules. Methods of making M-DNA and methods of using M-DNA also are disclosed.

## BACKGROUND OF THE INVENTION

In multicellular organisms, the number of cells in tissues is determined by the rate of cell proliferation minus the rate of cell elimination. Apoptosis is a genetically programmed, non-inflammatory, energy-dependent form of cell death in tissue including, but not limited to, adult tissue. Apoptosis consists of four sequential steps: (1) commitment to death by extracellular or intracellular triggers, (2) cell death by activation of intracellular proteases and nucleases, (3) engulfment of the dead cell by other cells, and (4) degradation of the dead cell within the lysozomes of phagocytic cells (Steller H. Science 267:1445-1449, 1995). Aberrations in the regulation of cell proliferation, cell apoptosis, or a combination of the two, are associated with the pathogenesis of a variety of diseases including, but not limited to, cancer, neurodegeneration, autoimmunity and heart disease.

Apoptosis can be initiated by ligands which bind to cell surface receptors including, but not limited to, Fas (CD95) (French et al. Journal of Cell Biology 133:355-364, 1996) and tumor necrosis factor receptor 1 (TNFR1). FasL binding to Fas and TNF binding to TNFR1 initiate intracellular signaling resulting in the activation of cysteine aspartyl proteases (caspases) that initiate the lethal proteolytic cascade of apoptosis execution, which is associated with nuclear DNA-fragmentation, release of nuclear matrix proteins (NuMA) and loss of cell substrate contact (Muzio et al. Cell 85:817-827, 1996).

Apoptosis also can be induced by intracellular proteins including, but not limited to, p53/p21 regulators (Levine, A. Cell 88:323-331, 1997). p53/p21 act as a transcription factors to activate expression of apoptosis-mediating genes, including, but not limited to, genes encoding proteins that generate free radicals that, it turn, damage the cell's mitochondria, whose contents leak out into the cytoplasm and activate apoptotic caspases (Polyak et al. Nature 389:300-305, 1997).

Cancer is an aberrant net accumulation of atypical cells that results from an excess of proliferation, an insufficiency of apoptosis, or a combination of the two. Mutations in apoptosis-related genes such as, but not limited to, Fas, TNFR1 and p53/p21 each have been implicated in the pathogenesis of cancers (Levine A. Cell 88:323-331, 1997; Fisher D. Cell 78:529-542, 1994). Apoptosis is important not only to the pathogenesis of cancer, but also to their likelihood of resistance to anti-cancer therapies.

Resistance to apoptosis induction has emerged as an important category of multiple drug resistance (MDR), one that likely explains a significant proportion of treatment failures. MDR, the simultaneous resistance to structurally and functionally unrelated chemotherapeutic agents, can be both inherent and acquired. That is, some cancers never respond to therapy, whereas other cancers, initially sensitive to therapy, develop drug resistance. As chemotherapeutic agents rely primarily on induction of apoptosis in cancer cells for their therapeutic effect, drug resistance, which diminishes the effectiveness of chemotherapeutic agents, leads directly or indirectly to reduced apoptosis and is generally associated with poor prognosis in a variety of cancers.

Cytolysis is the complete or partial destruction of a cell and is mediated by the immune system. As used herein, monocytes, macrophages and leukocytes are included in the immune system. Activated macrophages and monocytes produce bioactive molecules that accelerate, amplify and modulate responses in responsive cells of an animal. By produce is meant synthesize and secrete. These bioactive molecules include, but are not limited to, cytokines and reactive oxygen species.

Cytokines, include, but are not limited to, interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12) and GM-CSF. IL-12, alone and in combination with other cytokines, promotes the maturation of leukocytes including, but not limited to, B-lymphocytes, CD4+ T-cells, CD8+ T-cells, and NK cells, and induces the secretion of interferon-gamma. IL-12 is reported to have anti-cancer activity in some cancer cell (Stine et al. Annals NY Academy of Science 795:420-421, 1996; Chen et al. Journal of Immunology 159:351-359, 1997). This activity includes, but is not limited to, activation of specific cytolytic T-lymphocytes, activation of natural killer (NK) cells and induction of the anti-angiogenic proteins IP-10 and MiG. IP-10 inhibits cancer growth and metastasis, inhibits cancer-induced neovascularization, and further activates NK cells (Angillo et al. Annals NY Academy of Sciences 795:158-165, 1996). GM-CSF is

reported to have pro-cancer activity is some cancer cell (Hawkyard et al. Journal of Urology 150:514-518, 1993).

Reactive oxygen species include, but are not limited to, nitric oxide, superoxide radicals and hydroxyl radicals. Nitric oxide, superoxide radicals and hydroxyl radicals, among other activities, induce apoptosis and cytolysis in responsive target cells.

Natural and synthetic preparations of biological and chemical origin including, but not limited to, preparations from bacteria, have been used to stimulate or to inhibit responsive cells in an animal. Preparations of cell wall from *Mycobacterium* species have been used to treat diseases including, but not limited to, cancers (U.S. Patent No. 4,503,048). However, the therapeutic benefits obtained using such preparations are variable and inconsistent, and appear to depend on the method by which the preparation is prepared, purified and delivered, and on the stability of the preparation.

Prior art anti-cancer agents have proven to be less than adequate for clinical applications. Many of these agents are inefficient (Bischoff et al. Science 274:373-376, 1996) or toxic, have significant side effects (Lamm et al. Journal of Urology 153:1444-1450, 1995), result in development of drug resistance or immunosensitization, and are debilitating for the recipient. Moreover, many of these agents depend on Fas, TNFR1 or p53/p21 for their effectiveness.

Therefore, there is a need for a novel therapeutic agent that inhibits proliferation of and induces apoptosis in cancer cells, and that stimulates responsive cells of the immune system to produce cytokines and reactive oxygen species. This therapeutic agent should be useful as an anti-cancer agent and as an adjunct to other anti-cancer agents. By adjunct is meant useful with other anti-cancer agents to increase treatment effectiveness. Moreover, such a therapeutic agent should be simple and relatively inexpensive to prepare, its activity should be reproducible among preparations, its activity should remain stable over time, and its effects on cancer cells should be achievable with dose regimens that are associated with minimal toxicity.

## SUMMARY OF THE INVENTION

The present invention satisfies the above need by providing a therapeutic composition comprising a mycobacterial DNA (B-DNA) and a first pharmaceutically acceptable carrier, wherein the composition is effective in inducing a response in responsive cells of an animal. Particularly, the present invention provides a therapeutic composition comprising a *Mycobacterium phlei*-DNA (M-DNA) and a first pharmaceutically acceptable carrier, wherein the responses include, but are not limited to, inhibition of proliferation of and induction of apoptosis in responsive cells including, but not limited to cancer cells, and stimulation of responsive cells of the immune system to produce bioactive molecules.

The M-DNA composition is simple and relatively inexpensive to prepare, its activity is reproducible among preparations, it remains stable over time, and it is effective at dose regimens that are associated with minimal toxicity.

M-DNA is prepared from intact *Mycobacterium phlei* (*M. phlei*) by digestion with lysozyme, proteinase K and sodium dodecyl sulfate, and phenol extraction and ethanol precipitation (*M. phlei*-DNA). Alternatively, M-DNA is prepared by disrupting *M. phlei*, collecting the solid components, and preparing a M-DNA-*M. phlei* cell wall complex (MCC). DNase-free reagents are used to minimize M-DNA degradation during preparation. M-DNA is prepared from the MCC by phenol extraction and ethanol precipitation (MCC-DNA).

The M-DNA composition, comprising M-DNA and a first pharmaceutically acceptable carrier, is administered to an animal in a dosage sufficient to inhibit proliferation of and induce apoptosis in responsive cells, and to simulate responsive cells of the immune system to produce bioactive molecules. Such first pharmaceutically acceptable carriers include, but are not limited to, liquid carriers, solid carriers and both. Liquid carrier include, but are not limited to, aqueous carriers, non aqueous carrier, or both. Solid carriers include, but are not limited to, chemically synthesized carriers and natural carriers. Aqueous carriers include, but are not limited to water, saline and physiological buffers. Non-aqueous carriers include, but are not limited to, oils or other hydrophobic liquid formulations and liposomes. Natural carriers include, but are not limited to, deproteinized, delipidated, washed *M. phlei* cell wall, wherein the M-DNA is complexed on the *M. phlei* cell wall (MCC).

Further, the M-DNA composition can be administered in a second pharmaceutically acceptable carrier. Such second pharmaceutically acceptable carriers include, but are not limited to, liquid carriers and solid carriers. Again, liquid carriers include aqueous carriers, non-aqueous carriers, or both. Again solid carriers include chemically synthesized carriers and natural carriers.

The M-DNA composition of the present invention is useful for preventing, treating and eliminating a disease. It is particularly useful for treating a disease mediated by undesired and uncontrolled cell proliferation, such as a cancer. The M-DNA composition also is effective as an adjunct to enhance the effectiveness of other anti-cancer agents. Such agents include, but are not limited to, drugs, immunostimulants, antigens, antibodies, vaccines, radiation and chemotherapeutic, genetic, biologically engineered and chemically synthesized agents, and agents that target cell death molecules for activation or inactivation and that inhibit proliferation of and induce apoptosis in responsive cells.

Accordingly it is an object of the present invention to provide a composition and method that induces a therapeutic response in responsive cells of an animal.

Another object of the present invention is to provide a composition and method that inhibits proliferation of responsive cells.

Another object of the present invention to provide a composition and method that induces apoptosis in responsive cells.

Another object of the present invention is to provide a composition and method that induces apoptosis independent of Fas.

Another object of the present invention is to provide a composition and method that induces apoptosis independent of TNFR1.

Another object of the present invention is to provide a composition and method that induces apoptosis independent of p53/p21.

Another object of the present invention is to provide a composition and method that induces apoptosis independent of drug resistance.

Another object of the present invention is to provide a composition and method that induces caspase activity in responsive cells.

Another object of the present invention is to provide a composition and method that induces responses in responsive cells of the immune system to produce bioactive molecules.

Another object of the present invention is to provide a composition and method that induces responsive cells of the immune system to produce bioactive molecules.

Another object of the present invention is to provide a composition and method that induces responsive cells of the immune system to produce cytokines.

Another object of the present invention is to provide a composition and method that induces responsive cells of the immune system to produce IL-6.

Another object of the present invention is to provide a composition and method that induces responsive cells of the immune system to produce IL-10.

Another object of the present invention is to provide a composition and method that induces responsive cells of the immune system to produce IL-12.

Another object of the present invention is to provide a composition and method that induces responsive cells of the immune system to produce reactive oxygen species.

Another object of the present invention is to provide a composition and method that is effective to prevent a cancer in an animal.

Another object of the present invention is to provide a composition and method that is effective to treat a cancer in an animal.

Another object of the present invention is to provide a composition and method that is effective to eliminate a cancer in an animal.

Another object of the present invention is to provide a composition and method that is effective as an adjunct to other anti-cancer therapies.

Another object of the present invention is to provide a composition and method that is effective as an adjunct to chemical anti-cancer agents

Another object of the present invention is to provide a composition and method that is effective as an adjunct to biological anti-cancer agents.

Another object of the present invention is to provide a composition and method that is effective as an adjunct to biologically engineered anti-cancer agents.

Another object of the present invention is to provide a composition and method that is effective as an adjunct to anti-cancer vaccines.

Another object of the present invention is to provide a composition and method that is effective as an adjunct to nucleic acid based anti-cancer vaccines.

Another object of the present invention is to provide a composition and method that is effective as an adjunct to radiation therapy.

Another object of the present invention is to provide a composition and method that induces terminal differentiation of incompletely differentiated cells.

Another object of the present invention is to provide a composition that can be prepared in large amounts

Another object of the present invention is to provide a composition that can be prepared relatively inexpensively.

Another object of the present invention is to provide a composition that has reproducible activity among preparations.

Another object of the present invention is to provide a composition that remains stable over time.

Another object of the present invention is to provide a composition that maintains its effectiveness over time.

Another object of the present invention is to provide a composition that is minimally toxic to the recipient.

Another object of the present invention is to provide a composition that will not cause anaphylaxis in the recipient.

Another object of the present invention is to provide a composition that does not sensitize the recipient to tuberculin skin tests.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiment and the appended claims.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Oligonucleotide distribution of *M. phlei*-DNA and of MCC-DNA .

FIG. 2. Inhibition of proliferation of HT-1376. HT-1197, B-16 F1, THP-1, RAW 264.7, Jurkat, HL-60, HL-60 MX-1 cancer cells by *M. phlei*-DNA (2A) and

MCC-DNA (2B), calf thymus-DNA (2A & 2B) and herring sperm-DNA (2A & 2B). Results are the mean ± SD of 3 independent experiments.

FIG. 3. Inhibition of proliferation of HT-1376, HT-1197, B-16 F1, THP-1, RAW 264.7, Jurkat, HL-60, HL-60 MX-1 cancer cells by MCC. Results are the mean ± SD of 3 independent experiments

FIG. 4. Inhibition of proliferation of human leukemic THP-1 monocytes by *M. phlei*-DNA, MCC-DNA, MCC and hIL-12. Results are the mean ± SD of 3 independent experiments.

FIG. 5. Inhibition of proliferation of HT-1197 (5A) and HT-1376 (5B) human bladder cancer cells by MCC and LPS. Results are the mean ± SD of 3 independent experiments.

FIG. 6. Induction of DNA fragmentation in human leukemic THP-1 monocytes by PBS, herring sperm-DNA and untreated and DNase I treated *M. phlei*-DNA and MCC . Results shown are for 1 of 3 experiments, each of which gave similar results.

FIG. 7. Induction of DNA fragmentation in human leukemic THP-1 monocytes by PBS, untreated and DNase I treated herring sperm-DNA and *M. phlei*-DNA and by hIL-12. Results shown are for 1 of 3 experiments, each of which gave similar results

FIG. 8. Induction of DNA fragmentation in HT-1197 (8A) and HT-1376 (8B) human bladder cancer cells by MCC and hIL-12. Results shown are for 1 of 3 experiments, each of which gave similar results.

FIG. 9. NuMA release from human leukemic THP-1 monocytes by MCC, *M. phlei*-DNA, MCC-DNA and herring sperm-DNA. Results are the mean ± SD of 3 independent experiments.

FIG. 10. Release of NuMA from human leukemic THP-1 monocytes by untreated and DNase I treated *M. phlei* DNA, MCC-DNA and MCC. Results are the mean ± SD of 3 independent experiments.

FIG. 11. NuMA release from HT-1376 and HT-1197 human bladder cancer cells with increasing concentrations of MCC. Results are the mean ± SD of 3 independent experiments.

FIG. 12 NuMA release from HT-1197 (12A) and HT-1376 (12B) human bladder cancer cells with 1 μg/ml MCC or with 100 μg/ml MCC over 48 h. Results are the mean ± SD of 3 independent experiments.

FIG. 13. NuMA release from Jurkat cells incubated with PBS, CH-11 antibodies, ZB4 antibodies, *M. phlei*-DNA, CH-11 antibodies + *M. phlei*-DNA and ZB4 antibodies + *M. phlei*-DNA

FIG. 14. NuMA release from human leukemic THP-1 monocytes by PBS, *M. phlei*-DNA, sonicated, B*st*U 1 digested, autoclaved and methylated *M. phlei*-DNA.

FIG. 15. Anti-cancer activity of MCC and of DNase I treated MCC in line 10 hepatoma in guinea pigs. Results are the mean ± SD of 7 animals in each experimental group.

FIG 16. Percent LDH release from HT-1197 and HT-1376 human bladder cancer cells as an indicator of MCC cytotoxicity. Results are the mean ± SD of 3 independent experiments.

FIG. 17. MCC stimulation of IL-6, IL-12 and GM-CSF production by HT-1197 and HT-1376 human bladder cancer cells, human THP-1 monocytes, murine macrophages, murine RAW 264.7 monocytes and murine spleen cells. Results are the mean ± SD of 3 independent experiments.

FIG. 18. Effect of antibodies to CD14 receptors on MCC-DNA and on MCC induced IL-12 production by human THP-1 monocytes.

Fig. 19. Effect of cytochalasin D on *M. phlei*-DNA, MCC-DNA and MCC stimulated IL-12 by human THP-1 monocytes.

Fig. 20. Effect of untreated and DNase treated MCC-DNA, MCC and Regressin® on IL-12 production by murine macrophages.

FIG. 21. MCC stimulation of NO production by murine RAW 264.7 monocytes.

FIG. 22. Stimulation of IL-6, IL-10, IL-12 and GM-CSF in CD-1 mice by intraperitoneal injection of 50 mg/kg MCC.

FIG. 23. Stimulation of IL-12 production in CD-1 mice by intravenous injection of 6.6 mg/kg MCC.

FIG. 24. Stability of MCC during 6 months of storage.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a therapeutic composition, comprising a bacterial deoxyribonucleic acid (DNA) and a first pharmaceutically acceptable carrier, wherein the composition is effective in inducing a response in responsive cells of an animal, including a human. More particularly, the present invention relates to a composition comprising a mycobacterial DNA (B-DNA) and a first pharmaceutically acceptable carrier, wherein the composition is effective in inducing a response in responsive cells of an animal. Most particularly the present invention relates to a composition comprising *M.phlei* DNA (M-DNA) and a first pharmaceutically acceptable carrier, wherein the composition is effective in inhibiting proliferation of and inducing apoptosis in responsive cells, and in stimulating responsive cells of the immune system to produce bioactive molecules.

The M-DNA composition of the present invention is simple and relatively inexpensive to prepare, its activity is reproducible among preparations and remains stable

over time. Further, it is minimally, if at all, toxic to the recipient, does not cause a positive tuberculin reaction in the recipient and rarely causes an anaphylactic response in the recipient even upon repeated administration.

Many bacterial species can be used to practice the present invention including, but not limited to, *Coryneform* bacterial, *Corynebacterium* species, *Rhodococcus* species, *Eubacterium* species, *Bordetella* species, *Escherichia* species, *Listeria* species, *Nocardia* species and *Mycobacterium* species. Preferably, the bacterial-DNA is prepared from a *Mycobacterium* species including, but not limited to, *M. phlei, M. smegmatis, M. fortuitum, M. kansaasii, M. tuberculosis, M. bovis, M. vacciae and M. avium.* Most preferably, the mycobacterial-DNA is prepared from the *Mycobacterium* species *M. phlei.*

Methods to increase the therapeutic activity of M-DNA include, but are not limited to, chemically supplementing or biotechnologically amplifying stimulatory sequences or confirmations of DNA derived from the same or different bacterial species, or using bacterial plasmids containing appropriate stimulatory sequences or confirmations of DNA derived from the same or different bacterial species. Other methods to increase the therapeutic activity of M-DNA include, but are not limited to, complexing the M-DNA to synthetic or biological carriers or coupling the M-DNA to tissue-type or cell-type directed ligands or antibodies.

M-DNA is provided in a first pharmaceutically acceptable carrier which may be prepared by bringing into association the M-DNA and its carrier. Preferably, the M-DNA composition is prepared by uniformly and intimately bringing into association the M-DNA with a liquid carrier, with a solid carrier, or with both. Liquid carriers include, but are not limited to, aqueous carriers, non-aqueous carriers, or both. Solid carriers include, but are not limited to, chemically synthesized and natural carriers

For administration in an aqueous carrier, M-DNA is suspended in water, in saline or in a pharmaceutically acceptable buffer by techniques including, but not limited to, mixing, sonication and microfluidization.

For administration in a non-aqueous carrier, M-DNA is emulsified with a neutral oil such as, but not limited to, a diglyceride, a triglyceride, a phospholipid, a lipid, an oil and mixtures thereof, wherein the oil contains an appropriate mix of polyunsaturated and saturated fatty acids. Examples include, but are not limited to, soybean oil, canola oil, palm oil, olive oil and myglyol, wherein the number of fatty acid carbons is between 12 and 22 and wherein the fatty acids can be saturated or unsaturated. Optionally, charged lipid or phospholipid can be suspended in the neutral oil.

For administration in a natural carrier such as, but not limited to, MCC, the M-DNA is preserved and complexed on the *M. phlei* cell wall during preparation of the MCC. In MCC, the amount of M-DNA is enriched relative to the amount of M-DNA in an

intact *M. phlei* cell, and the M-DNA is more accessible to the responding cells than is the M-DNA within an intact *M. phlei* cell. It is to be understood that methods disclosed for using M-DNA can be used also for MCC.

The M-DNA composition, comprising M-DNA and a first pharmaceutically acceptable carrier, may further be administered in a second pharmaceutically acceptable carrier, The M-DNA composition and second pharmaceutically acceptable carrier formulations may be prepared by uniformly and intimately bringing into association the M-DNA composition with a liquid carrier, with a solid carrier, or with both. Liquid carriers include, but are not limited to aqueous carriers, non-aqueous carriers, or both. Solid carriers include, but are not limited to, chemically synthesized carriers and natural carriers.

Both the M-DNA and the M-DNA composition may be administered as an oil emulsion, water in oil emulsion, or water-in-oil-in-water emulsion. Further, they may be administered in carriers including, but not limited to, liposomes, site-specific emulsions, long-residence emulsions, sticky-emulsions, microemulsions, nanoemulsions, microparticles, microspheres, nanospheres, nanoparticles and minipumps, and with various natural or synthetic polymers that allow for sustained release of the M-DNA, the minipumps or polymers being implanted in the vicinity of where drug delivery is required. Polymers and their use are described in, for example, Brem et al. (Journal of Neurosurgery 74:441-446, 1991). In addition, the M-DNA and the M-DNA composition may be used with any one, all, or any combination of excipients regardless of the first pharmaceutically acceptable carrier or the second pharmaceutically acceptable carrier used to present M-DNA to the responding cells. These excipients include, but are not limited to, anti-oxidants, buffers, and bacteriostats, and may include suspending agents and thickening agents. .

Although not wanting to be bound by the following hypothesis, it is believed that the therapeutic effects of M-DNA include, but are not limited to, initiation of intracellular signaling resulting in apoptosis of responsive cells, and stimulation of cytokine and reactive oxygen species production resulting in cytolysis and in apoptosis of responsive cells. Apoptosis and cytolysis, both individually and in combination, have both anti-cancer activity and adjunct activity. That is, the M-DNA composition of the present invention may be used alone as an anti-cancer agent, or may be used before, at the same time as, or after another anti-cancer agent to increase treatment effectiveness.

The M-DNA composition is administered in an amount effective to induce a therapeutic response. The dosage of M-DNA administered will depend on the condition being treated, the particular formulation, and other clinical factors such as weight and condition of the recipient and route of administration. Preferably, the amount of M-DNA administered is from about 0.00001 mg/kg to about 100 mg/kg per dose, more preferably from about 0.0001 mg/kg to about 50 mg/kg per dose and most preferably from about 0.001 mg/kg to about 10 mg/kg per dose.

When M-DNA is administered as MCC, preferably the DNA content of the MCC is between about 0.001 mg DNA/100 mg dry MCC and about 90 mg DNA/100 mg dry MCC, more preferably between about 0.01 DNA/100 mg dry MCC and about 40 mg DNA/100 mg dry MCC, most preferably between about 0.1 mg DNA/100 mg dry MCC and about 30 mg DNA/100 mg dry MCC. Also it is preferable that the protein content of the *M. phlei* cell wall be less than about 2 mg/100 mg dry MCC and that the fatty acid content be less than about 2 mg/100 mg dry MCC.

Further, when M-DNA is administered as MCC, the amount of MCC is preferably from about 0.00001 mg/kg to about 100 mg/kg per dose, more preferably from about 0.0001 mg/kg to about 50 mg/kg MCC per dose and most preferably from about 0.001 mg/kg to about 10 mg/kg MCC per dose.

Routes of administration include, but are not limited to, oral, topical, subcutaneous, intra-muscular, intra-peritoneal, intra-venous, intra-dermal, intra-thecal, intra-lesional, intra-tumoral, intra-bladder, intra-vaginal, intra-ocular, intra-rectal, intra-pulmonary, intra-spinal, transdermal, subdermal, placement within cavities of the body, nasal inhalation, pulmonary inhalation, impression into skin and electrocorporation.

Depending on the route of administration, the volume per dose is preferably about 0.001 ml to about 100 ml per dose, more preferably about 0.01 ml to about 50 ml per dose and most preferably about 0.1 ml to about 30 ml per dose. The M-DNA composition can be administered in a single dose treatment or in multiple dose treatments on a schedule and over a period of time appropriate to the cancer being treated, the condition of the recipient and the route of administration.

Administration of M-DNA is not an immunization process but a therapeutic treatment that prevents, treats or eliminates a disease including, but not limited to cancers. Such cancer include, but are not limited to, squamous cell carcinoma, fibrosarcoma, sarcoid carcinoma, melanoma, mammary cancer, lung cancer, colorectal cancer, renal cancer, osteosarcoma, cutaneous melanoma, basal cell carcinoma, pancreatic cancer, bladder cancer, ovarian cancer, leukemia, lymphoma and metastases derived therefrom.

MCC retains its effectiveness after sonication and autoclaving, which reduce M-DNA base-pair length, and after GpC methylation, which abolishes the activity of the palindromic oligonucleotide sequence, purine-purine-C-G-pyrimidine-pyrimidine.

Moreover, the unexpected and surprising ability of M-DNA to induce apoptosis in various cancer cells lines including, but not limited to, Fas abnormal, p52/21 abnormal and drug resistant cancer cells lines addresses a long felt unfulfilled need in the medical arts, and provides an important benefit for animals, including humans.

The following examples will serve to further illustrate the present invention without, at the same time, however, constituting any limitation thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments,

modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

EXAMPLE 1

*Preparation of MCC from Mycobacterium phlei*

MCC was prepared from *Mycobacterium phlei* (strain 110). *M. phlei* was obtained from the Institut fur Experimental Biologie and Medizin, Borstel, Germany, and was stored as a suspension in sterile milk at -60° C. The *M. phlei* was cultured on Petragnani medium (Difco Labs, Detroit, MI) and was grown in Bacto AC broth (Difco Labs) for 10 to 20 days. The cells were harvested by centrifugal sedimentation. All reagents used in the following procedure were selected to enhance conservation of the *M. phlei* DNA.

About 400 grams of moist cell mass was placed into an autoclaved blender with a capacity of about 1200 ml. The cell mass was mixed at high speed for between 30 to 60 sec. After mixing, 6 ml of DNase-free Tween 80 (Sigma Chemical Co., St. Louis, MO) and between 200 and 400 ml of autoclaved water were added to the cell mixture. The entire cell suspension was again mixed in the blender at low speed for about 10 sec.

Cell disruption was accomplished by sonication. Five hundred ml of cell suspension, wherein the cells comprised about 50% to 70% of the volume, were placed in a one liter autoclaved beaker and sonicated. The sonicate was stored in an autoclaved flask on ice during the fractionation process. Unbroken cells were remove by low speed centrifugation. The supernatant from the low speed centrifugation was centrifuged for 1 h at 27,500 g at 15° C and the supernatant from this centrifugation was discarded.

The sediment from the 27,000 g centrifugation was transferred to an autoclaved blender and suspended in autoclaved deionized water by mixing at low speed. This suspension was again centrifuged at 27,000 g at 15° C for 1 h and the supernatant was again discarded. The sediment was suspended in autoclaved deionized water and spun for 5 min at 350 g to sediment any remaining unbroken cells. The supernatant was decanted and centrifuged at 27,000 g for 1 h at 15° C to sediment the crude cell wall fraction.

The crude cell wall fraction was deproteinized by digestion with proteolytic enzymes, care being taken to use DNase-free reagents where possible to optimize the amount of DNA in the preparation and to preserve the structure of the DNA in the preparation. The crude cell wall fraction derived from about 400 g of whole cells was suspended in 1 liter of 0.05 M DNase-free Tris-HCl, pH 7.5, by mixing at low speed. After the crude cell wall fraction was thoroughly suspended, 50 mg of DNase-free trypsin (Sigma Chemical Co) was added and the suspension was stirred using a magnetic stirring bar at 35° C for 6 h. Following trypsin treatment, 50 mg of DNase-free pronase

(Amersham Canada Limited, Oakville, Ontario) were added to each liter of trypsin-treated crude cell wall suspension. The suspension was stirred using a magnetic stirring bar for 12 to 18 h at 35° C.

After proteolytic digestion, the crude cell wall fraction was delipidated with detergent and phenol. To each liter of suspension, 60 g of DNase-free urea (Sigma Chemical Co.), 2.0 ml of DNase-free 100% phenol or 150 ml of 90% w/v phenol (Sigma Chemical Co.) were added. The flask containing the suspension was covered loosely with aluminum foil, warmed to 60°-80° C and stirred for 1 hr. The suspension was spun for 10 min at 16,000 g. The supernatant fraction and the fluid beneath the pellet were discarded. The pellet was washed 3 times by resuspension in about 1 liter of autoclaved deionized water and centrifuged for 10 min at 16,000 g.

The washed, deproteinized, delipidated MCC was lyophilized by transferring the suspension to an autoclaved lyophilizing flask with a small amount of autoclaved water. One 300 ml lyophilizing flask was used for each 30 grams of wet cell complex starting material. The MCC suspension was shell frozen by rotating the flask in ethanol cooled with solid carbon dioxide. After the content of the flask was frozen, the flask was attached to a lyophilization apparatus (Virtis Co. Inc., Gardiner, NY) and lyophilized. After lyophilization, the sample was transferred to an autoclaved screw-cap container and stored at -20° C in a desiccator jar containing anhydrous calcium sulfate.

Unless stated otherwise, the lyophilized MCC was resuspended in autoclaved deionized water or in a pharmaceutically acceptable DNase-free buffer such as, but not limited to, saline and PBS, and emulsified by sonication. Optionally, the emulsified MCC mixture was homogenized by microfluidization at 15,000-30,000 psi for one flow-through. The MCC suspension was either processed under aseptic conditions or was sterilized by autoclaving.

## EXAMPLE 2

*Purification of M-DNA from MCC and from M. phlei*

MCC was prepared as in Example 1. M-DNA was purified from MCC (MCC-DNA) by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (Short Protocols in Molecular Biology, 3rd Edition, Ausubel et al. Eds., John Wiley & Sons Inc., New York, USA). Unexpectedly, we found that at least about 3.6% of the dry weight of MCC is extractable M-DNA.

M-DNA was purified from *M. phlei* (*M. phlei*-DNA) by suspending the *M. phlei* (strain 110) in 5 ml of DNase-free 50 mM Tris-HCl, 5 mM EDTA, pH 8.0, adding DNase-free lysozyme (Sigma Chemical Co.) to a concentration of 1 mg/ml and incubating for 90 min at 37°C. DNase-free Proteinase K (Life Technologies, Burlington, Ontario, Canada) was added to a concentration of 0.1 mg/ml, DNase-free sodium dodecyl sulfate (BioRad, Richmond, CA) was added to a concentration of 1% and the incubation was

continued for 10 min at 65° C. The M-DNA was phenol/chloroform/isoamyl alcohol extracted and ethanol precipitated.

Unless stated otherwise, the M-DNA was sonicated in autoclaved deionized water or in a DNase-free pharmaceutically acceptable buffer such as, but not limited to, saline and PBS. MCC, MCC-DNA and *M. phlei*-DNA do not contain endotoxins as determined using a Limulus amebocyte lysate QCL-1000 kit (BioWhittaker, Walkersville, MD).

EXAMPLE 3

*Preparation of bacterial-DNA-bacterial cell wall complex and of bacterial DNA from other bacterial species.*

Bacterial DNA-bacterial cell wall complex is prepared from *M. smegmatis, M. fortuitous, Nocardia rubra, Nocardia asteroides, Cornybacterium parvum, M. kansaasii, M tuberculosis and M. bovis* as in Example 1. Bacterial-DNA is purified from bacterial DNA-bacterial cell wall complex and from intact bacteria as in Example 2.

EXAMPLE 4

*DNase treatment*

MCC-DNA, M-phlei-DNA and MCC, each containing 1 μg of M-DNA, and Regressin® (US Patent No. 4,744,9840) were digested with 1 international unit (IU) of RNase-free DNase I (Life Technologies) for 1 h at 25° C in 20 mM Tris HCl, pH 8.4, 2 mM $MgCl_2$ and 50 mM KCl. DNase I was inactivated by the addition of EDTA to a final concentration of 2.5 mM and heating for 10 min at 65° C. DNase I digests both single stranded and double stranded DNA. Digestion with DNase I results in almost total degradation of DNA. Regressin® (Bioniche, Inc. London, Ontario, Canada) is a formulation containing 1 mg mycobacterial cell wall extract, 20 μl mineral oil NF in 1 ml PBS and 0.5% v/v of Tween 80.

EXAMPLE 5

*Comparison of MCC-DNA and M. phlei-DNA*

MCC-DNA and *M. phlei*-DNA were electrophoresed in a 5% agarose gel (3h; 100V) using procedures known to those skilled in the art. The DNA molecular weight distribution was analyzed by gel photo scanning using 1D Main Program software (Advance American Biotechnology, Fullerton, CA)

As shown in Fig. 1, *M. phlei*-DNA contains a broad range of oligonucleotides between about 5 and about 10,000 base pairs (bp), as well as genomic DNA (>12,000 bp). MCC-DNA contains a range of oligonucleotides between about 5 and about 250 base pairs, 1 major peak at about 40 base pairs, and little genomic DNA.

EXAMPLE 6

Cells and reagents

All cell lines, except OC2 and SW260, were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured in the medium recommended by the ATCC. OC2 and SW260 were obtained from Dr. J.K. Collins (University College Cork, Cork, Ireland) and were cultured in DMEM medium supplemented with 10% FCS. Table 1 shows the cell lines, their origins and their properties.

TABLE 1

Cell lines

| CELL LINE | ORIGIN | PROPERTIES |
|---|---|---|
| THP-1 | Human acute monocytic leukemia | |
| HL-60 | Human promyelo-cytic leukemia | |
| HL-60 MX-1 | Human promyelo-cytic leukemia | Atypical drug resist-ance to mixoxantrone |
| RAW 264.7 | Murine monocytic leukemia | |
| JURKAT | Human T lymphoblast | |
| HT-1376 | Human bladder carcinoma | Mutation in p53/p21 MDR |
| HT-1197 | Human bladder carcinoma | |
| B-16-F1 | Murine melanoma | |
| SW260 | Human colon adenocarcinoma | FAS-L resistance |
| OC2 | Human esophageal carcinoma | |
| LS1034 | Human cecum carcinoma | Conventional MDR |

Murine macrophages were obtained from female CD1 mice injected intra-peritoneally with 1.5 ml sterile Brewer's thioglycolate broth (Difco, Detroit, MI). The peritoneal exudate (> 85% macrophages) was harvested at day 4, washed by centrifugation in HBSS and cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 20 mM HEPES (Life Technologies). The cells were allowed to adhere for 18 h after which non-adherent cells were removed by gentle washing with warm medium.

Murine spleen cells were prepared by gentle teasing through sterile stainless steel screens. Cell suspensions were layered on Lympholyte-M cell separation media (CedarLane, Hornby, Ontario, Canada) and centrifuged at 2200 rpm for 30 min to remove red blood cells and dead cells. These cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 20 mM HEPES (Life Technologies).

Unless stated otherwise, cells were seeded in 6 well flat-bottom tissue culture plates at concentrations between 3 X $10^5$ and $10^6$ cells/ml and were maintained at 37° C in a 5% CO2 atmosphere.

Calf thymus-DNA, herring sperm-DNA and *Escherichia coli* lipopolysaccharide (LPS) were obtained from Sigma Chemical Co. Recombinant human IL-12 (hIL-12) was obtained from R&D Systems (Minneapolis, MN).

EXAMPLE 7

*Inhibition of cell proliferation*

Cell proliferation was determined using dimethylthiazol-diphenyltetrazolium bromide (MTT) reduction (Mosman et al. Journal of Immunological Methods 65:55-63, 1983). Briefly, 100 µl of MTT (Sigma-Aldrich) dissolved in PBS at 5 mg/ml was added into the wells of the plates. After 4 h, acid-isopropanol, 1 ml of 0.04 N HCl in isopropanol, was added and reduced MTT was measured at a wavelength of 570 nm.

HT-1376, HT-1197, B-16 F1, THP-1, RAW 264.7, Jurkat, HL-60 and HL-60 MX-1 cells were incubated for 24 h with from 0 µg/ml to 10 µg/ml of *M. phlei*-DNA, MCC-DNA, herring sperm-DNA and calf thymus-DNA. *M. phlei*-DNA (Fig. 2A) and MCC-DNA (Fig. 2B) inhibited proliferation in each of the cancer cell lines tested in a dose-dependent manner, whereas herring sperm-DNA (Figs 2A & 2B) and calf thymus-DNA (Figs 2A & 2B) did not inhibit proliferation of any of the cell lines tested.

HT-1376, HT-1197, B-16 F1, THP-1, RAW 264.7, Jurkat, HL-60 and HL-60 MX-1 cells were incubated for 24 h with from 0 µg/ml to 10 µg/ml of MCC. MCC inhibited proliferation in each of the cancer cell lines tested in a dose dependent manner (Fig. 3).

Human leukemic THP-1 monocytes were incubated for 24 h with from 0 µg/ml to 10 µg/ml of *M. phlei*-DNA, MCC-DNA, MCC and hIL-12. *M. phlei*-DNA, MCC-DNA and MCC inhibited proliferation in a dose-dependent manner. hIL-12 did not inhibit proliferation (Fig. 4).

HT-1197 (Fig. 5A) and HT-1376 (Fig. 5B) human bladder cancer cells were incubated for 20 h with from 0 to 100 µg/ml of MCC and LPS. MCC inhibited proliferation. LPS did not inhibit proliferation.

*M. phlei*-DNA, MCC-DNA and MCC, wherein the first pharmaceutically acceptable carrier is *M. phlei* cell wall, inhibit proliferation of each of the cancer cell lines tested. In contrast, LPS, which is a nonspecific immunostimulant reported to induce apoptosis in some cancer cell lines (Izquierdo et al. Anticancer Drugs 7:275-2801996); hIL-12, which is a cytokine reported to induce apoptosis in some cancer cell lines (Stine et al. Annals NY Academy of Science 795:420-421, 1996) and DNA from calf thymus and herring sperm do not inhibit proliferation of any of the cancer cell lines tested. These data show that *M*-DNA is responsible for the inhibition of proliferation in the cancer cell lines

tested and that other DNAs (herring sperm-DNA and calf thymus-DNA) cannot replace M-DNA. These data also show that M-DNA inhibition of cell proliferation does not result from nonspecific immunostimulation (LPS) or from cytokine activity (hIL-12). Moreover, among the cancer cells inhibited are p53/p21 abnormal and drug resistant HT-1376 human bladder cancer cells and atypical drug resistant HL-60 MX-1 human promyelocytic leukemia cells.

EXAMPLE 8

*Induction of apoptosis as indicated by DNA fragmentation*

Fragmentation of cellular DNA into nucleosome-sized fragments is characteristic of cells undergoing apoptosis. Nucleosome-sized fragments are DNA fragments possessing a difference of about 200 base-pairs in length as determined by agarose gel electrophoresis (Newell et al. Nature 357:286-289, 1990). To assess DNA fragmentation, non-adherent cells were collected by centrifugation at 200 g for 10 min. Pellets of non-adherent cells and the remaining adherent cells were lysed with 0.5 ml of hypotonic lysing buffer (10 mM Tris buffer, 1 mM EDTA, 0.2% Triton X-100, pH 7.5). The lysates were centrifuged at 13,000 g for 10 min and the supernatants, containing fragmented DNA, were precipitated overnight at -20° C in 50% isopropanol and 0.5 M NaCl. The precipitates were collected by centrifugation and were analyzed by electrophoresis in 0.7% agarose gels for 3 h at 100V.

A suspension culture of human leukemic THP-1 monocytes were incubated for 48 h with PBS and with 1 μg/ml of untreated or of DNase I treated *M. phlei*-DNA and MCC and with untreated herring-sperm-DNA (Fig. 6). *M. phlei*-DNA (lane 2) and MCC (lane 4) induced significant DNA fragmentation, whereas PBS (lane 1), DNase I treated *M phlei*-DNA (lane 3), DNase I treated MCC (lane 5) and herring sperm-DNA (lane 6) did not induce DNA fragmentation. A 123-bp DNA ladder (Life Technologies) was used to determine the molecular weight of the nucleosome-sized DNA fragments (lane L).

A suspension culture of THP-1 monocytes were incubated for 48 h with PBS and with 1 μg/ml of untreated or DNase I treated *M. phlei*-DNA and herring sperm-DNA and with hIL-12 (Fig. 7). *M. phlei*-DNA induced significant DNA fragmentation (lane 5), whereas DNase I treated *M phlei*-DNA (lane 4), herring sperm-DNA (lane 3), DNase I treated herring sperm-DNA (lane 2), hIL-12 (lane 1) and PBS (lane 6) did not induce DNA fragmentation. A 123-bp DNA ladder (Life Technologies) was used to determine the molecular weight of the nucleosome-sized DNA fragments (lane L).

HT-1197 and HT-1376 human bladder cancer cells were incubated for 48 h with 1 μg/ml MCC or with hIL-12 (Fig. 8). MCC induced significant DNA fragmentation in non-adherent HT-1197 (Fig. 8A, lane 2) and HT-1376 (Fig. 8B, lane 2) cells, but not in adherent HT-1197 (Fig. 8A, lane 3) and HT-1376 (Fig. 8B, lane 3) cells. PBS (Fig. 8A & 8B, lane 5), hIL-12 (Fig. 8A & 8B, lane 4), DNase I-treated MCC (Fig. 8A & 8B, lane 7)

and untreated cells (Fig. 8A & 8B, lane 1) did not induce DNA fragmentation in non-adherent HT-1197 or HT-1376 cells. A 123-bp DNA ladder (Life Technologies) was used to determine the molecular weight of the nucleosome-sized DNA fragments (Fig. 8A & 8B, lane L).

*M. phlei*-DNA and MCC, wherein M-DNA is preserved and complexed on *M. phlei* cell wall, induce apoptosis in human leukemic THP-1 monocytes and in HT-1197 and HT-1376 human bladder cancer cells, whereas herring sperm-DNA, hIL-12, and DNase I treated *M. phlei* DNA and MCC and do not induce apoptosis in these cells. These data show that *M*-DNA is responsible for the induction of apoptosis in the cancer cell lines tested, that the oligonucleotides of the M-DNA must be intact (DNase I treatment) and that other DNAs (herring sperm-DNA) cannot replace M-DNA. These data also show that M-DNA induction of apoptosis does not result from nonspecific immunostimulation (LPS).

EXAMPLE 9

*Induction of apoptosis as indicated by solubilization of nuclear mitotic protein apparatus (NuMA)*

Striking morphological changes in the cell nucleus caused by the solubilization and release of NuMA are characteristic of apoptosis. To determine solubilization and release of NuMA, media from the cultured cells were removed and centrifuged at 200 g for 10 min. The supernatants were collected and 100 µl of each supernatant was used to quantitate NuMA release in units/ml (U/ml) using a commercial ELISA (Calbiochem, Cambridge, MA) (Miller et al. Biotechniques 15:1042-1047, 1993).

Human leukemic THP-1 monocytes were incubated for 48 h with 0 µg/ml to 10 µg/ml of *M. phlei*-DNA, MCC-DNA, MCC and herring sperm-DNA. *M. phlei*-DNA, MCC-DNA and MCC induced release of NuMA in a dose-dependent manner, whereas herring sperm-DNA did not induce release of NuMA (Fig. 9). DNase I treatment of *M. phlei*-DNA, MCC-DNA and MCC significantly inhibited their induction of NuMA release from these cells (Fig. 10)

HT-1197 and HT-1376 human bladder cancer cells were incubated with 0 µg/ml to 100 µg/ml of MCC. MCC induced the release of NuMA in a dose-dependent manner (Fig, 11) and in a time-dependent manner (Fig. 12A & 12B). Enhanced release of NuMA was detected within 24 hours after incubation of HT-1197 cells (Fig. 12A) and of HT-1376 (Fig. 12B) cells with 100 µg/ml of MCC.

*M. phlei*-DNA, MCC-DNA and MCC, each of which contain M-DNA, induce apoptosis cancer cells. MCC, wherein the first pharmaceutically acceptable carrier is *M. phlei* cell wall, induces more apoptosis than *M. phlei*-DNA or MCC-DNA. This suggests that the carrier that presents M-DNA to responsive cells effects M-DNA induction of apoptosis.

EXAMPLE 10

*Fas independent induction of apoptosis in Jurkat human T lymphoblast cells by M. phlei-DNA.*

Jurkat human T lymphoblast cells were incubated for 1 h with PBS, with 1 µg/ml CH-11 antibody, an antibody that binds to Fas and induces apoptosis (+ control) (Coulter-Immunotech, Hialeah, FL) or with 1 µg/ml ZB4 antibody, an antibody that binds to Fas and inhibits apoptosis (- control) (Coulter-Immunotech). *M. phlei*-DNA, 1 µg/ml, was added and NuMA release was determined after 48 h.

As shown in Fig. 13, *M. phlei*-DNA induced apoptosis both in the absence of and in the presence of ZB4. These data demonstrate that induction of apoptosis by *M. phlei*-DNA is independent of Fas.

EXAMPLE 11

*Summary of the effects of M phlei-DNA , MCC-DNA and MCC on cell proliferation and on apoptosis*

Table 2 summarizes the effects of *M phlei*-DNA, MCC-DNA and MCC on inhibition of proliferation of and on induction of apoptosis in cancer cells as determined by DNA fragmentation, NuMA release and flow cytometric analysis in human and murine cancer cell lines.

TABLE 2

Inhibition of proliferation and induction of apoptosis in human and murine cancer cell lines

| Cells | Inhibition of proliferation | Nucleosome-sized DNA | NuMA released | Flow cytometric analysis |
|---|---|---|---|---|
| THP-1 | yes | yes | yes | ND*. |
| HL-60 | yes | yes | yes | ND. |
| HL-60 MX-1 | yes | yes | yes | ND. |
| RAW 264.7 | yes | yes | yes | ND. |
| JURKAT | yes | yes | yes | ND. |
| HT-1376 | yes | yes | yes | ND. |
| HT-1197 | yes | yes | yes | ND |
| B-16-FI | yes | yes | ND. | ND. |
| SW260 | ND. | ND. | ND. | yes |
| OC2 | ND. | ND | ND, | yes |
| LS1034 | yes. | ND. | yes | ND. |

* ND = Not Done

*M. Phlei*-DNA, MCC-DNA and MCC, wherein the first pharmaceutically acceptable carrier is *M. phlei* cell wall and wherein the M-DNA is complexed on the cell wall, inhibit proliferation of and induce apoptosis in each of the cancer cell lines tested. These cancer cell lines include atypical drug resistant HL-60 MX-1 human promyelocytic leukemia, p53/p21 abnormal and drug resistant HT-1376 human bladder, Fas abnormal

SW260 human colon, and conventional drug resistant LS1034 human cecum carcinoma cells.

EXAMPLE 12

*MCC activation of caspase-3 in human leukemic THP-1 monocytes*

Caspase 3 is a key enzyme in the apoptotic pathway downstream of Fas-FasL signaling. To determine if MCC can by-pass Fas and directly activate the caspase cascade in cancer cells, the effect of MCC on caspase-3 activity was assayed in human leukemic THP-1 monocytes.

THP-1 monocytes (2 X $10^7$ cells) were incubated for 48 h with MCC (100 µg/ml). The THP-1 cells were lysed in 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA and 10% glycerol. Caspase-3 activity was determined with a commercial ELISA (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), using the included substrate, inhibitor and purified caspase-3 enzyme. Results are expressed as optical densities read at 405 nm.

TABLE 3

MCC (100 µg/ml) activation of caspase 3 activity in human leukemic THP-1 monocytes

| Incubation | *p*-nitroanalide absorbance, 405 nm | |
|---|---|---|
| | O.D. x $10^{-1}$, 3 hours incubation | O.D. x $10^{-1}$, 6 hours incubation |
| THP-1 alone | 0.19 | 0.06 |
| THP-1 + MCC cell extract | 0.44 | 0.20 |
| THP-1 + MCC treated with DNase cell extract | 0.12 | 0.11 |
| Purified Caspase-3 | 0.87 | 0.58 |
| Purified Caspase-3 + caspase-3 inhibitor | 0.00 | 0.00 |
| THP-1 + MCC cell extract + Caspase-3 inhibitor | 0.00 | 0.00 |

As shown in Table 3, incubation with MCC resulted in a 232% (3 h) and 333% (6 h) increase in caspase-3 and caspase-3 like activity in human leukemic THP-1 monocytes. MCC induction of caspase-3 and caspase-3 like activity was abolished by DNase I treatment of MCC. Specificity of MCC induction of caspase-3 and caspase-3 like activity was demonstrated using caspase-3 inhibitor. Addition of caspase-3 inhibitor to MCC-treated THP-1 cell extract completely abolished measurable activity. The ability of MCC to specifically and directly induce caspase-3 and caspase-3 like activity in human leukemic THP-1 monocytes is totally unexpected. To specifically stimulate caspase-3 and caspase-3 like activity, MCC must enter the cells by one or more mechanisms, and initiate the lethal proteolytic cascade of apoptosis execution.

EXAMPLE 13

*Effect of tamoxifen on MCC inducted apoptosis in human leukemic THP-1 monocytes*

Human leukemic THP-1 monocytes were incubated for 90 min in control medium or in medium containing 10 µg/ml tamoxifen (Sigma-Aldrich), an anti-estrogen used in the palliative treatment of advanced breast cancer. Cells were washed extensively with ice-cold medium (2X), resuspended to about $10^6$ cells/ml in medium and incubated for 48 h with 0, 1, 10 and 100 µg/ml of MCC. Apoptosis was quantitated by measuring NuMA.

TABLE 4

Effect of tamoxifen on MCC induced apoptosis in human leukemic THP-1 monocytes determined by NuMA release in U/ml.

| | +MCC 1 µg/ml | +MCC 10 µg/ml | +MCC 100 µg/ml | + medium only |
|---|---|---|---|---|
| Control | 174.6 | 260.0 | 237.2 | 174.6 |
| Tamoxifen (10 µg/ml) | 354.9 (↑ 50.8%) | 406.2 (↑ 36.0%) | 410.0 (↑ 42.1%) | 284.7 |

As shown in Table 4, preincubation in tamoxifen significantly increased MCC induced apoptosis at each of the MCC concentrations used. These data demonstrate that MCC, wherein M-DNA is complexed on *M. phlei* cell wall, can be used as an adjunct to other anti-cancer agents to increase treatment effectiveness.

EXAMPLE 14

*Modification of M- phlei-DNA by methylation, sonication and autoclaving*

Nucleic acid preparations from *bacillus Calmette-Guerin* (BCG) inhibit cancer growth (US patent 4,579,941; Tokunaga et al. Microbiology and Immunology 36:55-666, 1992). The active constituent in BCG nucleic acid has been identified as the palindromic oligonucleotide sequence purine-purine-C-G-pyrimidine-pyrimidine (CG motif). Cytosine methylation with CpG methylase abolishes the activity of this DNA (Krieg et al. Nature 374:546-549, 1995). Therefore, the effect of CpG methylation on the ability of *M. phlei*-DNA to induce apoptosis was determined.

*M. phlei*-DNA, 1 µg, was methylated using 2.5 U of CpG *Sss* I methylase (New England Biolabs, Mississauga, Ontario, Canada) in 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 160 µM S-adenosylmethionine for 1 h at 37° C. Native and methylated *M. phlei*-DNA were subjected to cleavage by *Bst*U I restriction endonuclease (New England Biolabs) for 1 h at 60° C in 50 mM NaCl, 10 mM Tris HCl, 10 mM $MgCl_2$ and 1 mM DTT, pH 7.9. Electrophoretic analysis in 0.5% agarose gel for 3 h at 100 V showed that native *M. phlei*-DNA was digested by B*st*U I restriction

endonuclease, whereas methylated *M.* phlei-DNA was not digested by *BstU* I restriction endonuclease. This confirmed that methylation of the *M. phlei*-DNA was complete.

As shown in Fig. 14, methylation did not modify *M. phlei* induced NuMA release from human leukemic THP-1 monocytes. These data demonstrate that, unlike BCG, CG-motifs are not necessary apoptosis induction by *M. phlei*-DNA.

The base pair length of *M phlei*-DNA (1 µg) was reduced by sonication for 15 sec or for 20 min on ice in a Model W-38 ultrasonic processor (HeatSystems-Ultrasonics, Inc.), by digestion with BstU I restriction endonuclease, or by autoclaving at 121° C for 30 min (Castle Sybron MDT, Dubuque, Iowa). As shown in Fig. 14, sonication, *Bst*U I digestion and autoclaving, each of which reduced base pair length to the range of about 5 base pairs to about 250 base pairs, did not effect *M. phlei*-DNA induction of NuMA release from THP-1 monocytes. These results demonstrate that *M. phlei*-DNA induces apoptosis in cancer cells, even at short oligonucleotide length (about 5 base pairs to about 250 base pairs).

Human leukemic THP-1 monocytes were incubated for 48 h with untreated *M. phlei*-DNA and MCC and with *M. phlei*-DNA and MCC autoclaved for 30 min at 121° C. Autoclaving, which reduces base pair length of DNA, did not affect the ability of *M. phlei*-DNA or of MCC to inhibit of proliferation of (Table 5A) or to induce apoptosis in (Table 5B) these cells.

Table 5A

Effect of autoclaving on MCC and M. phlei-DNA inhibition of proliferation

| | Non-autoclaved % inhibition | Autoclaved % inhibition |
|---|---|---|
| MCC 1 µg/ml | 87 ± 4 | 84 ± 10 |
| MCC 10 µg/ml | 68 ± 1 | 74 ± 6 |
| MCC 100 µg/ml | 59 ± 7 | 64 ± 6 |
| *M. phlei* -DNA 1 µg/ml | 88 ± 8 | 84 ± 11 |
| *M. phlei* -DNA 10 µg/ml | 80 ± 8 | 74 ± 6 |
| *M. phlei* -DNA 100 µg/ml | 68 ± 4 | 64 ± 5 |

Table 5B

Effect of autoclaving on MCC and M. phlei-DNA induction of apoptosis as determined by NuMA release in U/ml

| | Non-autoclaved | Autoclaved |
|---|---|---|
| MCC 1 µg/ml | 298.0 | 277.1 |
| MCC 10 µg/ml | 325.9 | 322.4 |
| MCC 100 µg/ml | 339.9 | 357.3 |
| *M. phlei* -DNA * 100 µg/ml | 261.4 | 268.4 |

| | | |
|---|---|---|
| *M. phlei* -DNA 10 µg/ml | 317.2 | 278.4 |
| *M. phlei* -DNA 1 µg/ml - | 306.8 | 285.8 |

EXAMPLE 15

*MCC inhibits cancer growth in vivo*

MCC and DNase I treated MCC were emulsified to a final concentration of 1 mg/ml in PBS containing 2% w/v mineral oil and 0.02% w/v Tween 80 (Fisher Chemical Co.) by sonication at 4° C for 5 min. (Heat Systems-Ultrasonics, Inc.).

Line 10 hepatoma cells, syngeneic for strain-2-guinea pigs were rapidly thawed, washed by centrifugation and resuspended in M199 medium to a concentration of $10^6$ cells/ml. One-tenth ml containing 1.5 X$10^6$ cells was injected intra-dermally into the flanks of 3 month-old strain 2 guinea pigs. Treatment was initiated 6 to 7 days post-injection, when the cancers were between about 0.5 and about 0.8 cm in diameter. Seven animals were treated with emulsification buffer alone (control), 7 with emulsification buffer containing MCC, and 7 with emulsification buffer containing DNase I treated MCC. The emulsions were instilled directly into the cancer and surrounding normal tissue. One-half ml of emulsion was administered at 0 h and at 6 h for a total volume of 1 ml containing 1 mg of MCC or of DNase I treated MCC.

Cancer diameters (longest diameter + shortest diameter) were recorded weekly for 3 weeks. Cancer volumes were calculated in $mm^3$ as 0.5 x a (longest diameter) x $b^2$ (shortest diameter) and the increase in cancer volume relative to day 0 of treatment was calculated for each guinea pig. Statistical analysis was done using 2-way ANOVA with replicates (PHARM/PCS version 4.2, MCS, Philadelphia, PA). Differences in treatment were considered significant at $p \leq 0.05$.

As shown in Fig. 15, with control emulsion, cancer volume increased by about 22-fold by week 3, whereas with MCC, cancer growth was significantly inhibited compared to control emulsion (Fig. 15, Table 6). With DNase I treated MCC, cancer growth was not significantly different from control (Fig. 15, Table 6).

TABLE 6

Two-way ANOVA with replicates

| Treatment comparison | p value |
|---|---|
| Control vs MCC | $p<0.01$ |
| Control vs MCC+DNase | Not significant |
| MCC vs MCC+DNase | $p<0.01$ |

These data show that instillation of MCC at the site of a tumor results in tumor regression. Moreover, the significant ($p<0.01$) difference in inhibition of cancer

growth between MCC and DNase I treated MCC shows that undegraded M-DNA is necessary for the anti-cancer activity of MCC *in vivo*.

EXAMPLE 16

*MCC cytotoxicity*

Cell cytotoxicity is characterized by the loss of plasma membrane integrity and release of cytoplasmic enzymes such as, but not limited to, LDH (Wyllie et al. International Review of Cytology 68: 251-306, 1980; Phillips et al. Vaccine, 14:898-904, 1996). Human bladder cancer cells release LDH when treated with cytotoxic agents (Rahman M. Urology International 53:12-17, 1994).

To assess the cytotoxicity of MCC, HT-1197 and HT-1376 human bladder cancer cells were incubated for 48 h with from 0 μg/ml to 100 μg/ml of MCC or with lysing buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100, pH 7.5) as a control for total LDH release (Filion et al. Biochim Biophys Acta 1329:345-356, 1997). LDH release into the culture supernatant was determined using a commercial assay (Sigma-Aldrich).

As determined by LDH release, MCC was not cytotoxic to HT-1197 or to HT-1376 cells (Fig. 17). The absence of cytotoxicity demonstrates that MCC acts directly to inhibit proliferation of and to induce apoptosis in cancer cells.

EXAMPLE 17

*Stimulation of cytokine synthesis in vitro*

IL-12 is reported to have anti-cancer activity in some cancer cells (Voest et al. Journal National Cancer Institute 87:581-586, 1995; Stine et al. Annals NY Academy of Science 795:420-421, 1996), whereas GM-CSF is reported to have pro-cancer activity in some cancer cells (Hawkyard et al. Journal of Urology 150:514-518, 1993). Moreover, some cancer cells are reported to secrete cytokines (De Reijke et al. Urology Research 21:349-352, 1993; Bevers et al. British Journal of Urology 80:35-39, 1997). Therefore, the effect of MCC on IL-6, IL-12 and GM-CSF synthesis by HT-1197 and HT 1376 human bladder cancer cells and by human THP-1 monocytes, murine macrophages, murine RAW 264.7 monocytes, and murine spleen cells was determined.

Cytokine production was determined in pg/ml in 100 μl of culture supernatant using the appropriate commercial ELISA (BioSource, Camarillo CA). The IL-12 ELISA measures both IL-12 p70 complex and free p40 subunit.

HT-1197 and HT-1376, THP-1, macrophage, RAW 264.7, and murine spleen cells were incubated for 48 h with 1 μg/ml MCC. As shown in Fig. 1, MCC stimulated production of IL-6 and of IL-12 by human monocytes and murine macrophages, but not by human bladder cancer cells, murine monocytes or spleen cells. MCC did not stimulate GM-CSF production of in any of the cancer cells tested.

These data show that MCC, wherein *M. phlei* cell wall is the first pharmaceutically acceptable carrier, stimulates production of the anti-cancer cytokines IL-6

and IL-12 by human monocytes and murine macrophages. MCC does not stimulate cytokine production by human bladder cancer cells. MCC does not stimulate production of the pro-cancer cytokine GM-CSF.

EXAMPLE-18

*Effect of untreated and DNase I treated M. phlei-DNA, MCC-DNA, MCC and Regressin® on IL-12 production by human THP-1 monocytes.*

Human THP-1 monocytes were incubated for 48 h with *M-phlei*-DNA, MCC-DNA, MCC and Regressin®, before treatment with DNase I, after treatment with DNase I, and after addition of M-DNA to DNase I treated *M. phlei*-DNA, MCC-DNA and MCC.

TABLE 7

IL-12 production in pg/ml by human THP-1 monocytes

| Treatment | IL-12 production, pg/ml culture supernatant | | | |
|---|---|---|---|---|
| | *M. phlei*-DNA 1 μg/ml | MCC-DNA 1 μg/ml | MCC 1 μg/ml | Regressin® 1 μg/ml |
| None | 407 | 676 | 1222 | 196 |
| DNase | 178 | 367 | 729 | 200 |
| DNase + 1 μg DNA | 424 | 658 | 783 | Not Done |

As shown in Table 7, *M phlei*-DNA, MCC-DNA and MCC each stimulated THP-1 monocytes to produce IL-12. MCC stimulated more IL-12 production than *M. phlei*-DNA or MCC-DNA. Regressin® stimulated minimal IL-12 production. DNase I treatment reduced by about 50% *M. phlei*-DNA, MCC-DNA and MCC stimulated IL-12 production. Regressin® was not affected by DNase I treatment. Addition of *M. phlei*-DNA to DNase I-treated *M. phlei* and of MCC-DNA to DNase I treated MCC-DNA restored their stimulation of IL-12 production. Addition of MCC-DNA to DNase I-treated MCC did not restore its stimulation of IL-12 production.

That MCC, wherein *M. phlei* cell wall is the first pharmaceutically acceptable carrier, stimulates more IL-12 production than *M. phlei-DNA* or MCC-DNA suggests that the carrier used to present the M-DNA to responsive cells effects M-DNA stimulated IL-12. That DNase I treatment, which degrades the M-DNA, reduces significantly *M. phlei*-DNA, MCC-DNA and MCC stimulated IL-12 production suggests that the oligonucleotide form of M-DNA must be preserved for optimal stimulation of IL-12 production. That addition of M-DNA to DNase I treated MCC did not restore its stimulation of IL-12 production suggests that the manner in which the M-DNA is complexed on the *M. phlei* cell wall in MCC is important for optimal stimulation of IL-12 production by human monocytes.

EXAMPLE 19

*Effect of autoclaving on M. phlei-DNA and MCC stimulation of IL-12 production by human THP-1 monocytes*

Human THP-1 monocytes were incubated for 48 h with MCC and *M. phlei*-DNA and with MCC and *M. phlei*-DNA autoclaved for 30 min in sterile water.

TABLE 8

Effect of autoclaving on MCC and *M. phlei*-DNA stimulation of IL-12 production in pg/ml by THP-1 monocytes

| | Non-autoclaved IL-12 (pg/ml) | Autoclaved IL-12 (pg/ml) |
|---|---|---|
| MCC 1 µg/ml | 1017.4 | 905.2 |
| MCC 10 µg/ml | 1061.6 | 1076.8 |
| *M. phlei* -DNA 1 µg/ml | 902.0 | 1088.6 |
| *M. phlei* -DNA 10 µg/ml | 1027.1 | 949.5 |

As shown in Table 8, autoclaving, which reduces DNA base-pair size, did not affect MCC or *M. phlei*-DNA stimulation of IL-12 production by monocytes.

EXAMPLE 20

*Effect of CD14 antibody treatment on MCC -DNA and MCC stimulated IL-12 production by human THP-1 monocytes*

Human THP-1 monocytes were incubated with PBS or with 10 µg/ml of anti-CD14 antibody (clone MY4, Coulter-Immunotech) for 1 h. Then, 5 µg/ml of MCC-DNA or of MCC were added and the incubation was continued for 48 h. CD14 antibodies, which bind to CD14 receptors on the cell surface, caused about an 85% decrease in MCC-DNA stimulated IL-12 production and about a 20% decrease in MCC stimulated IL-12 production (Fig. 18).

EXAMPLE 21

*Effect of cytochalasin D on M. phlei-DNA, MCC-DNA and MCC stimulated IL-12 production by human THP-1 monocytes*

Human THP-1 monocytes were incubated for 48 h with PBS or with 1 µg/ml of *M. phlei*-DNA, MCC-DNA or MCC in the absence of and in the presence of 10 µg/ml of cytochalasin D (Sigma Chemical Co.). Cytochalasin D, which inhibits phagocytosis, caused a 64% decrease in *M-phlei*-DNA, a 50% decrease in MCC-DNA and a 55% decrease in MCC stimulated IL-12 production (Fig. 19).

Although not willing to be bound by the following hypothesis, but based on the data shown in Figs 18 & 19, it is believed that *M. phlei*-DNA, MCC-DNA and MCC interact with monocytes by multiple mechanism. Fig. 18 suggests that they interact with the GPI-linked membrane receptor CD14 and are internalized. This mechanism is more specific for soluble *M. phlei*-DNA and MCC-DNA than for insoluble MCC. Fig. 19

suggests that they interact with phagocytic receptors, such as the scavenger receptor, and are internalized. This mechanism is more specific for insoluble MCC than for soluble *M. phlei*-DNA and MCC-DNA.

EXAMPLE 22

*Effect of CG sequence and of MCC on IL-12 production by human THP-1 monocytes*

Nucleic acid preparations from BCG, are reported to stimulate lymphocyte proliferation, secretion of IL-6 and IL-12 by B-lymphocytes, secretion of IL-12 by monocytes, secretion of IL-6 and interferon-gamma by T-lymphocytes and secretion of interferon-gamma by NK cells (Klinman et al. Proceeding of the National Academy of Science USA 93:2879-2883, 1996).

As the active constituent of BCG nucleic acid has been identified as the CG motif, human THP-1 monocytes were incubated for 48 h with 0.5, 1 and 5 µg/ml of MCC or of 5'-GCTAGACGTTAGCGT-3' DNA sequence prepared by solid phase synthesis using an automated DNA synthesizer.

TABLE 9

Effect of CG-containing oligonucleotide and of MCC on IL-12 production in pg/ml by THP-1 monocytes.

| | 5 µg/ml | 1 µg/ml | 0.5 µg/ml |
|---|---|---|---|
| GCTAGACGTTAGCGT | Undetectable | Undetectable | Undetectable |
| MCC | Not Done | 1239 | Not Done |

As shown in Table 9, the CG-containing oligonucleotide sequence, did not stimulate IL-12 production at any of the three concentrations tested, whereas MCC at 1 µg/ml had a significant stimulatory effect on IL-12 production.

EXAMPLE 23

*Effect of heat treatment and of DNase I treatment on M. phlei-DNA, MCC-DNA, MCC and Regressin® stimulation of IL-12 production by murine macrophages*

Murine peritoneal macrophages were incubated for 48 h with *M. phlei*-DNA, MCC-DNA, MCC and Regressin® and with *M. phlei*-DNA, MCC-DNA and Regressin®, which had been heated at 100° C for 10 minutes and then cooled in ice for 2 minutes.

TABLE 10

IL-12 production in pg/ml by murine macrophages.

| Treatment | IL-12 production, pg/ml supernatant | | |
|---|---|---|---|
| | 12.5 µg/ml | 5.0 µg/ml | 0.1 µg/ml |
| *M. phlei*-DNA | 228 | 207 | 140 |
| *M phlei*-DNA heat-treated | 278 | 222 | 164 |
| MCC-DNA | Not Done | 176 | 164 |
| MCC-DNA heat-treated | Not Done | 235 | 131 |
| MCC | Not Done | 745 | Not Done |
| Regressin® | 110 | 96 | 80 |
| Regressin® heat-treated | 93 | 82 | 79 |

As shown in Table 10, at a concentration of 5 µg/ml, IL-12 production was stimulated most by MCC, less by *M. phlei*-DNA and MCC-DNA and least by Regressin®. Heat treatment of *M. phlei*-DNA, MCC-DNA and Regressin® had no significant effect on their stimulation of IL-12 production. Although not shown, heat treatment of MCC caused a slight, but significant, increase in IL-12 production.

Murine peritoneal macrophages were incubated for 48 h with untreated and with DNase I treated MCC-DNA, MCC and Regressin® (Fig. 20). IL-12 production was stimulated most by MCC, less by MCC-DNA and least by Regressin®. DNase I treatment of MCC-DNA and of MCC significantly reduced their stimulation of IL-12 production by murine macrophages. DNase I treatment of Regressin® had no effect on its activity. These data again suggest that, as with monocytes (Example 18), the oligonucleotide structure of M-DNA must be preserved for optimal stimulation of IL-12 production by murine macrophages.

EXAMPLE 24

*Effect of M-phlei-DNA, MCC-DNA, MCC and Regressin® on nitric oxide (NO) production by murine peritoneal macrophages.*

Macrophage activation stimulates production of reactive oxygen species including, but not limited to, nitric oxide (NO), superoxide radicals and hydroxyl radicals These reactive oxygen species induce cytolysis and apoptosis in responsive cells and, therefore, have anti-cancer activity.

Murine peritoneal macrophages were incubated for 48 h with 0.1, 5.0 or 12.5 μg/ml of *M-phlei*-DNA, MCC-DNA, MCC and Regressin®. NO production was measured in nmol/L by reaction of $NO_2^-$ with Griess reagent using 100 μl of culture supernatant.

TABLE 11

Effect of *M-phlei*-DNA, MCC-DNA, MCC and Regressin® on NO production by murine macrophages.

| Treatment | NO production, nmol/L | | |
|---|---|---|---|
| | 12.5 μg/ml | 5.0 μg/ml | 0.1 μg/ml |
| *M. phlei*-DNA | 18.9 | 8.3 | 2.6 |
| MCC-DNA | Not Done | 3.0 | 1.8 |
| MCC | Not Done | 36.2 | Not Done |
| Regressin® | 1.6 | 2.6 | 0.1 |

As shown in Table 11, at 5 μg/ml, MCC stimulated significantly more NO production than *M. phlei*-DNA, MCC-DNA or Regressin®.

Murine macrophages were incubated for 48 h with 1 μg/ml untreated and DNase I treated MCC and with untreated *M. phlei*-DNA and MCC-DNA.

TABLE 12

Stimulation of NO production in murine macrophages by MCC, DNase I treated MCC, MCC-DNA and *M. phlei*-DNA

| | Experiment #1 NO (nmol/ml) | Experiment #2 NO (nmol/ml) |
|---|---|---|
| MCC | 43.7 | 30.7 |
| MCC + DNase I (1 U) | 0.0 | 2.1 |
| MCC-DNA | 2.6 | 2.1 |
| *M. phlei*-DNA | 0.0 | 1.6 |

As shown in Table 12, M-DNA, complexed on M. phlei cell wall, as MCC stimulated significant NO production. DNase I treatment of MCC, which degrades the M-DNA, abolished MCC stimulation of NO production. MCC-DNA and *M. phlei*-DNA stimulated minimal NO production. These data demonstrate that both the intact oligonucleotide structure of the M-DNA and the carrier that presents these nucleotides to the macrophages are important for optimal stimulation of NO production.

EXAMPLE 25

*Effect of MCC on the production of nitric oxide (NO) by murine RAW 264.7 monocytes*

Murine RAW 264.7 monocytes were incubated for 24 h with 0.5 to 95 µg/ml of MCC. Increasing concentrations of MCC stimulated increasing amounts of NO production (Fig. 21). This was unexpected as receptors for NO induction are not optimally expressed on monocytes and, therefore, NO production is not usually associated with monocytes. Under the same conditions, Regressin® did not stimulate NO production.

EXAMPLE 26

*Stimulation of cytokine synthesis in vivo*

Four groups of CD-1 mice, each containing 5 mice, were injected intraperitoneally with 50 mg/kg of MCC. Blood was collected at 0, 3, 6 and 24 h after injection and concentrations (pg/ml) of IL-6, IL-10, IL-12 and GMSF in the sera were determined at 0, 3, 6 and 24 h post-injection (Fig. 22 ). With intraperitoneal MCC, sera concentrations of IL-6, IL-10 and IL-12 were significantly increased at 3 and 6 h post-injection, and declined to approximately control values (0 h) at 24 h post injection. Sera concentrations of GM-CSF remained at about control values (0 h) at 3, 6 and 24 h post-injection.

Five groups of CD-1 mice, each containing 5 mice, were injected intravenously with 6.6 mg/kg of MCC. Blood was collected at 0, 3, 6 and 24 h after injection and concentrations (pg/ml) of IL-10 and IL-12 in the sera were determined at 0, 3, 6 and 24 h post-injection (Fig. 23). With intravenous MCC, sera concentrations of IL-12 were significantly increased at 3 and 6 h post-injection, and declined to approximately control values (0 h) at 24 h post injection. Sera concentrations of IL-10 remained at about control values (0 h) at 3, 6 and 24 h post-injection.

These data demonstrate that *in vivo* administration of M-DNA, wherein the M-DNA is complexed on the M. phlei cell wall, as MCC stimulates production of the anti-cancer cytokines IL-6, IL-10 and IL-12, but not the pro-cancer cytokine GM-CSF. Further, these data demonstrate that the amount of MCC administered and the route by which it is administered both effect the ability of MCC to stimulate cytokine production *in vivo* .

Four groups of CD-1 mice, each containing 4 mice, were injected intraperitoneally with untreated and with DNase I treated *M. phlei*-DNA and MCC. After 3 h, the mice were sacrificed, blood was collected by cardiac micropuncture and the concentration (pg/ml) of IL-12 in the sera was measured (Table 13).

TABLE 13

Effect of MCC and of *M. Phlei*-DNA ± DNase I treatment on IL-12 production in pg/ml by CD-1 mice

| | MCC | | MCC+DNase | % inhibition |
|---|---|---|---|---|
| mouse #1 | 255 | mouse #5 | 126 | 49% |
| mouse #2 | 180 | mouse #6 | 57 | 68% |
| mouse #3 | 146 | mouse #7 | 121 | 17% |
| mouse #4 | 199 | mouse #8 | 143 | 28% |
| average | 195±46 | | 111±38 | 40.5±22.6% |

| | *M. phlei*-DNA | | *M. phlei*-DNA +DNase | % inhibition |
|---|---|---|---|---|
| mouse #9 | 135 | mouse #13 | 110 | 19% |
| mouse #10 | 283 | mouse #14 | 146 | 48% |
| mouse #11 | 118 | mouse #15 | 121 | 82% |
| mouse #12 | 270 | mouse #16 | 169 | 37% |
| average | 195±46 | | 111±38 | 46.5±26.5% |

As shown in Table 13, *in vivo* administration of MCC and of *M. phlei*-DNA stimulate production of the anti-cancer cytokine IL-12. After DNase I treatment, MCC stimulated IL-12 production decreased 40.5% and *M. phlei*-DNA stimulated IL-12 production decreased 46.5%. This demonstrates that the oligonucleotide structure of M-DNA must be preserved for optimal stimulation of IL-12 production *in vivo*.

EXAMPLE 27

*MCC stability*

MCC at 1 mg/ml was stored as a sterile suspension in 0.85% w/v NaCl in the dark at 4° C or 6 months. Mean particle diameter was calculated using photon correlation spectroscopy (N4 Plus, Coulter Electronics Inc.). The MCC suspension was diluted with 0.85% w/v NaCl to a particle count rate between 5 $x10^4$ and $10^6$ counts/sec. Mean particle diameter was calculated in size distribution processor mode (SDP) using the following conditions: fluid refractive index 1.33, temperature 20° C, viscosity 0.93 centipoise, angle of measurement 90.0°, sample time 10.5 µs, and sample run time 100 sec. Potential, the electric charge at the hydrodynamic interface between the particles and the bulk solvent, was measured in a Delsa 440SX (Coulter Electronics Inc.) using the

following conditions: current 0.7 mA, frequency range 500 Hz, temperature 20°C, fluid refractive index 1.33, viscosity 0.93 centipoise, dielectric constant 78.3, conductivity 16.7 ms/cm, on time 2.5 sec, off time 0.5 sec, and sample run time 60 sec.

As shown in Fig. 24, MCC charge and MCC diameter remained relatively unchanged during 6 months of storage. Moreover, MCC stimulation of IL-12 production and MCC induction of apoptosis in THP-1 monocytes remained unchanged during 6 months of storage.

EXAMPLE 28

*MCC-DNA and MCC treatment of human colon cancer*

Human colon cancer cells (ICM12C) are established as an ectopic solid tumor in the subcutaneous tissues of immunodeficient athymic nude mice (nu/nu mice) and the mice are divided into 5 groups. Group 1 receives vehicle alone. Group 2 receives MCC-DNA. Group 3 receives DNase I treated MCC-DNA. Group 4 receives MCC. Group 5 receives DNase I treated MCC. Cancer mass is measured before treatment and weekly during 4 weeks of treatment. Group 2 mice and Group 4 mice show regressin of cancer mass.

EXAMPLE 29

*M. phlei-DNA and MCC treatment of human ovarian cancer*

Human ovarian cancer cells (36M2) are established as ascites in the peritoneal cavity of immunodeficient athymic nude mice (nu/nu mice) and the mice are divided into 5 groups. Group 1 receives vehicle alone. Group 2 receives *M-phlei*-DNA. Group 3 receives DNase I treated *M-phlei*-DNA. Group 4 receives MCC. Group 5 receives DNase I treated MCC. Cancer mass is measured before treatment and weekly during 4 weeks of treatment. Group 2 mice and Group 4 mice show inhibition of ascitic cancer cell proliferation.

EXAMPLE 30

*MCC treatment of canine transmissible venereal cancer*

Dogs with veneral cancers (VT) are divided into 4 groups. Group 1 receives vincristine. Group 2 receives vincristine combined with methotrexate and cyclophosphamide. Group 3 receives MCC-DNA, complexed with and presented on a carrier, wherein the carrier is mycobacterial cell wall (MCC). Group 4 receives vincristine and MCC. Cancer mass is measured before treatment and weekly during 12 weeks of treatment. Group 4 dogs show regression of VT.

EXAMPLE 31

Suspension in aqueous carrier.

M-DNA is suspended in a first pharmaceutically acceptable carrier and is sonicated at 20% output for 5 minutes (Model W-385 Sonicator, Heat Systems-Ultrasonics Inc). Optionally, the sonicated composition is homogenized by microfluidization at 15,000-30,000 psi for one flow-through (Model M-110Y; Microfluidics, Newton, MA).






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We claim:

1. A composition effective for inducing a response in responsive cells of an animal, comprising:

a. a mycobacterial-DNA (B-DNA), and

b. a first pharmaceutically acceptable carrier.

2. The composition of claim 1, wherein the B-DNA is *M. phlei* DNA (M-DNA).

3. The composition of claim 1, wherein the first pharmaceutically acceptable carrier is selected from the group consisting of an aqueous carrier, a non-aqueous carrier and a mycobacterial cell wall.

4. The composition of claim 3, wherein the first pharmaceutically acceptable carrier in an aqueous carrier.

5. The composition of claim 3, wherein the first pharmaceutically acceptable carrier is a mycobacterial cell wall.

6. The composition of claim 5, wherein the mycobacterial cell wall is *M. phlei* cell wall.

7. The composition of claim 1, further comprising a second pharmaceutically acceptable carrier.

8. The composition of claim 7, wherein the second pharmaceutically acceptable carrier is selected from the group consisting of an aqueous carrier and a non-aqueous carrier.

9. The composition of claim 1, wherein the response is selected from the group consisting of inhibition of proliferation of responsive cells, induction of apoptosis in responsive cells, and stimulation of responsive cells of the immune system to produce bioactive molecules.

10. The composition of claim 9, wherein the response is inhibition of proliferation of the responsive cells.

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11. The composition of claim 10, wherein the responsive cells are cancer cells.

12. The composition of claim 9, wherein the response is induction of apoptosis in the responsive cells.

13. The composition of claim 12, wherein the induction of apoptosis is independent of Fas.

14. The composition of claim 12, wherein the induction of apoptosis is independent of p53/p21.

15. The composition of claim 14, wherein the induction of apoptosis is independent of drug resistance.

16. The composition of claim 9, wherein the responsive cells are cancer cells.

17. The composition of claim 9, wherein the response is stimulation of the responsive cells of the immune system to produce bioactive molecules.

18. The composition of claim 17, wherein the bioactive molecules are selected from the group consisting of cytokines and reactive oxygen species..

19. The composition of claim 18, wherein the cytokines are selected from the group consisting of IL-6, IL-10 and IL-12.

20. A method for inducing a response in responsive cells of an animal, wherein a composition comprising:

a. a mycobacterial DNA (B-DNA), and

b. a first pharmaceutically acceptable carrier,

is administered to the animal in need of such administration in an amount effective to induce the response in the responsive cells of the animal.

21. The method of claim 20, wherein the B-DNA is *M. Phlei* DNA (M-DNA).

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22. The method of claim 20 wherein the first pharmaceutically acceptable carrier is selected from the group consisting of an aqueous carrier, a non-aqueous carrier and a mycobacterial cell wall.

23. The method of claim 22, wherein the first pharmaceutically acceptable carrier is an aqueous carrier.

24. The method of claim 22, wherein the first pharmaceutically acceptable carrier is a mycobacterial cell wall.

25. The method of claim 24, wherein the mycobacterial cell wall is *M. phlei* cell wall.

26. The method of claim 20, further comprising a second pharmaceutically acceptable carrier.

27. The method of claim 26, wherein the second pharmaceutically acceptable carrier is selected from the group consisting of an aqueous carrier and a non-aqueous carrier.

28. The method of claim 20, wherein the response is selected from the group consisting of inhibition of proliferation of responsive cells, induction of apoptosis in responsive cells, and stimulation of responsive cells of the immune system to produce bioactive molecules.

29. The method of claim 28, wherein the response is inhibition of proliferation of the responsive cells.

30. The method of claim 29, wherein the responsive cells are cancer cells.

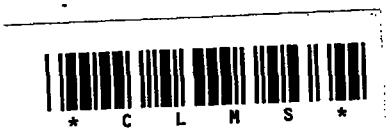
31. The method of claim 28, wherein the response is induction of apoptosis in the responsive cells.

32. The method of claim 31, wherein the induction of the apoptosis is independent of Fas.

33. The method of claim 31, wherein the induction of the apoptosis is independent of p53/p21.

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34. The method of claim 31, wherein the induction of the apoptosis is independent of drug resistance.

35. The method of claim 31, wherein the responsive cells are cancer cells.

36. The method of claim 28, wherein the response is stimulation of the responsive cells of the immune system to produce bioactive molecules.

37. The method of claim 36, wherein the bioactive molecules are selected from the group consisting of cytokines and reactive oxygen species.

38. The method of claim 37, wherein the cytokines are selected from the group consisting of IL-6, IL-10 and IL-12.

39. A method for inducing caspase activity in responsive cells of an animal, wherein a composition comprising:

a. M-DNA, and

b. a first pharmaceutically acceptable carrier, wherein the first pharmaceutically acceptable carrier is *M. phlei* cell wall,

is administered to the animal in need of such administration in an amount effective to induce caspase activity in the responsive cells of the animal.

40. The method of claim 39, further comprising a second pharmaceutically acceptable carrier.

41. The method of claim 40, wherein the second pharmaceutically acceptable carrier is selected from the group consisting of an aqueous carrier and a non-aqueous carrier.

42. The method of claim 39, wherein the responsive cells are cancer cells.

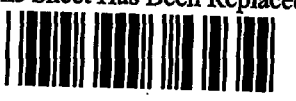
43. A composition for treating a cancer in an animal comprising administering to the animal in need of such treatment a composition, comprising:

a. M-DNA; and

b. a first pharmaceutically acceptable carrier

in an amount effective to treat the cancer.

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44. A method for treating a cancer in an animal, wherein a composition, comprising:

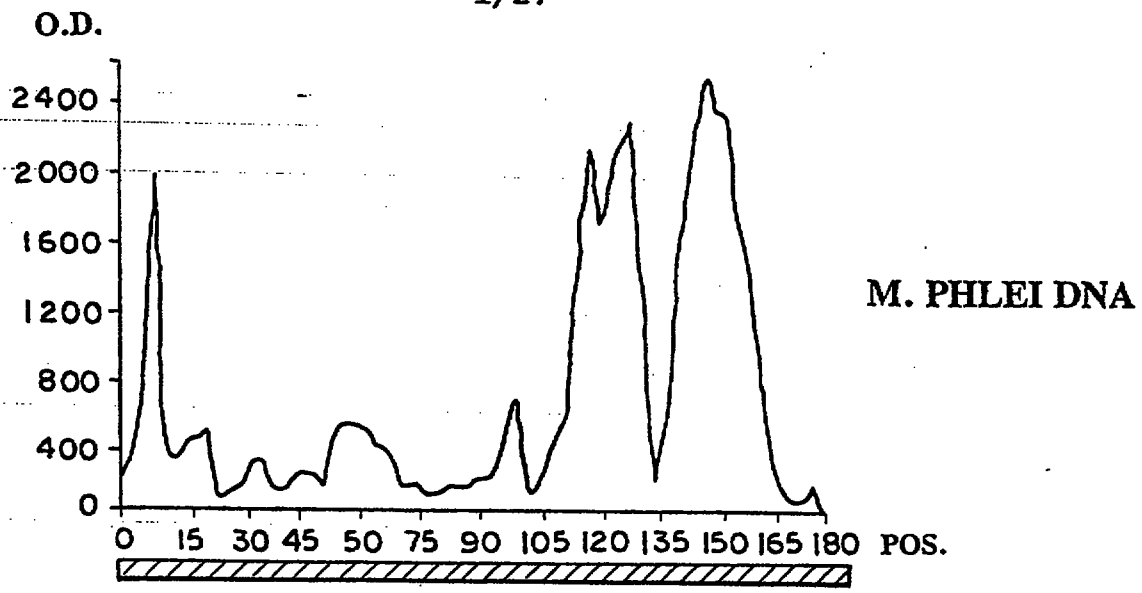
a. a M-DNA; and

c. a first pharmaceutically acceptable carrier

is administered to the animal in need of such treatment in an amount effective to treat the cancer.

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O.D.
2400
2000
1600
1200
800
400
0
0 15 30 45 50 75 90 105 120 135 150 165 180
POS.
M. PHLEI DNA

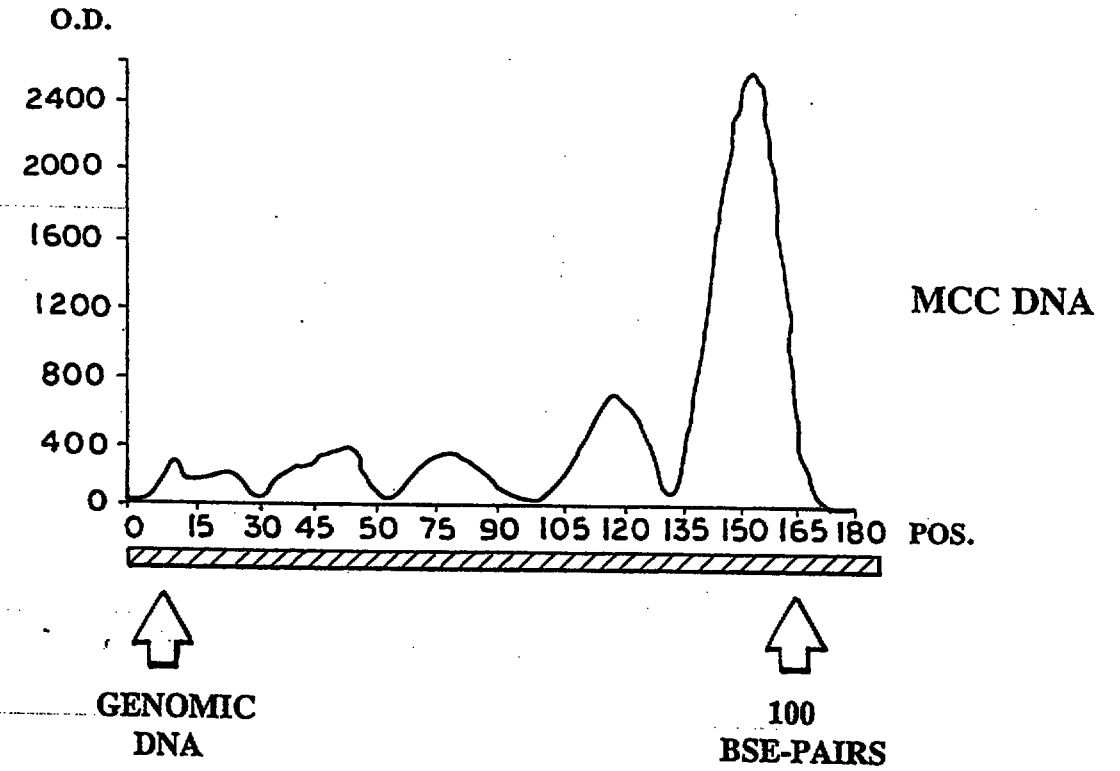

O.D.
2400
2000
1600
1200
800
400
0
0 15 30 45 50 75 90 105 120 135 150 165 180
POS.
MCC DNA
GENOMIC DNA
100 BSE-PAIRS

Fig_1

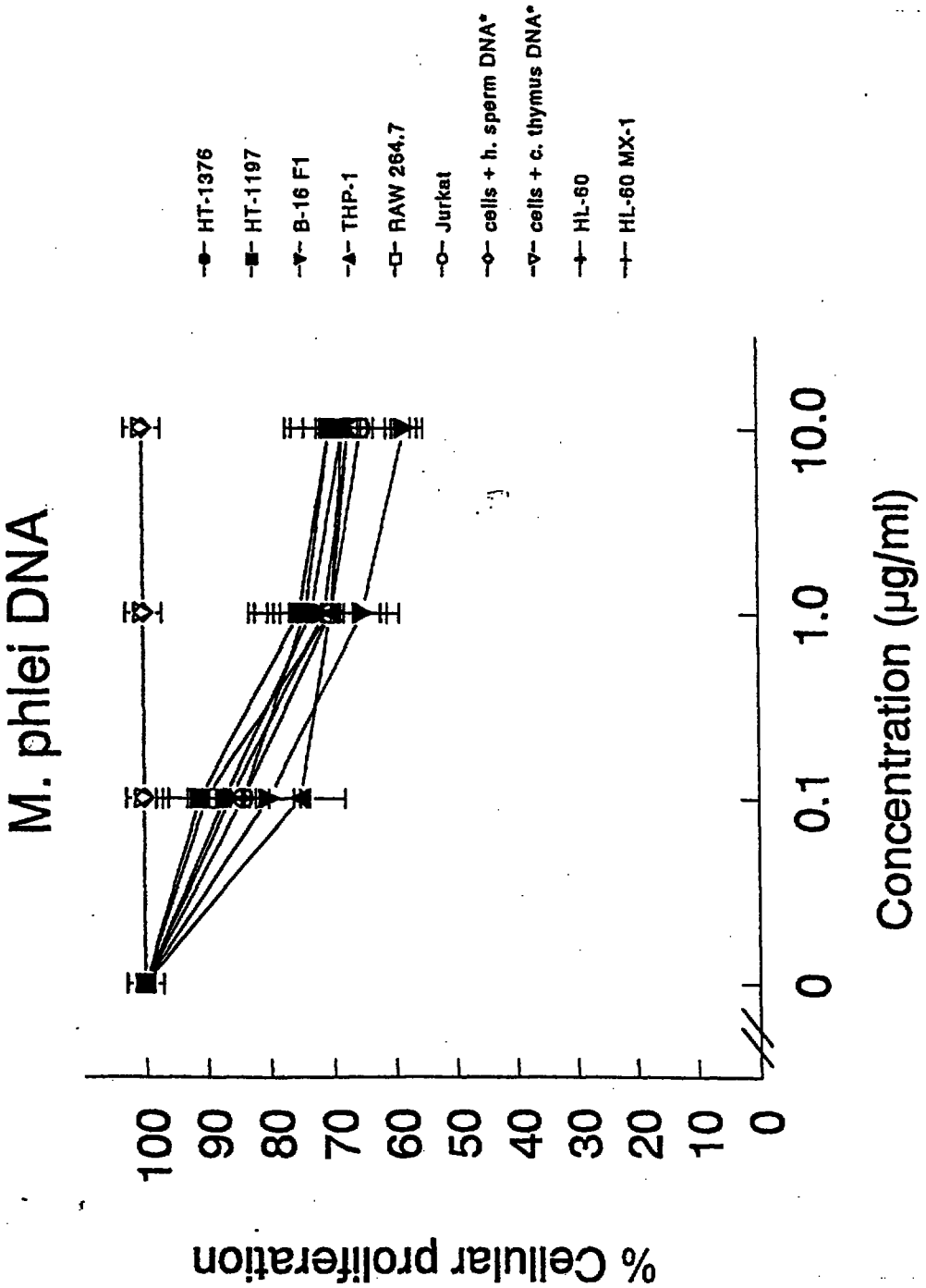

HT-1376
HT-1197
B-16 F1
THP-1
RAW 264.7
Jurkat
cells + h. sperm DNA*
cells + c. thymus DNA*
HL-60
HL-60 MX-1
M. phlei DNA
% Cellular proliferation
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1.0
10.0
Concentration (µg/ml)

FIGURE 2A

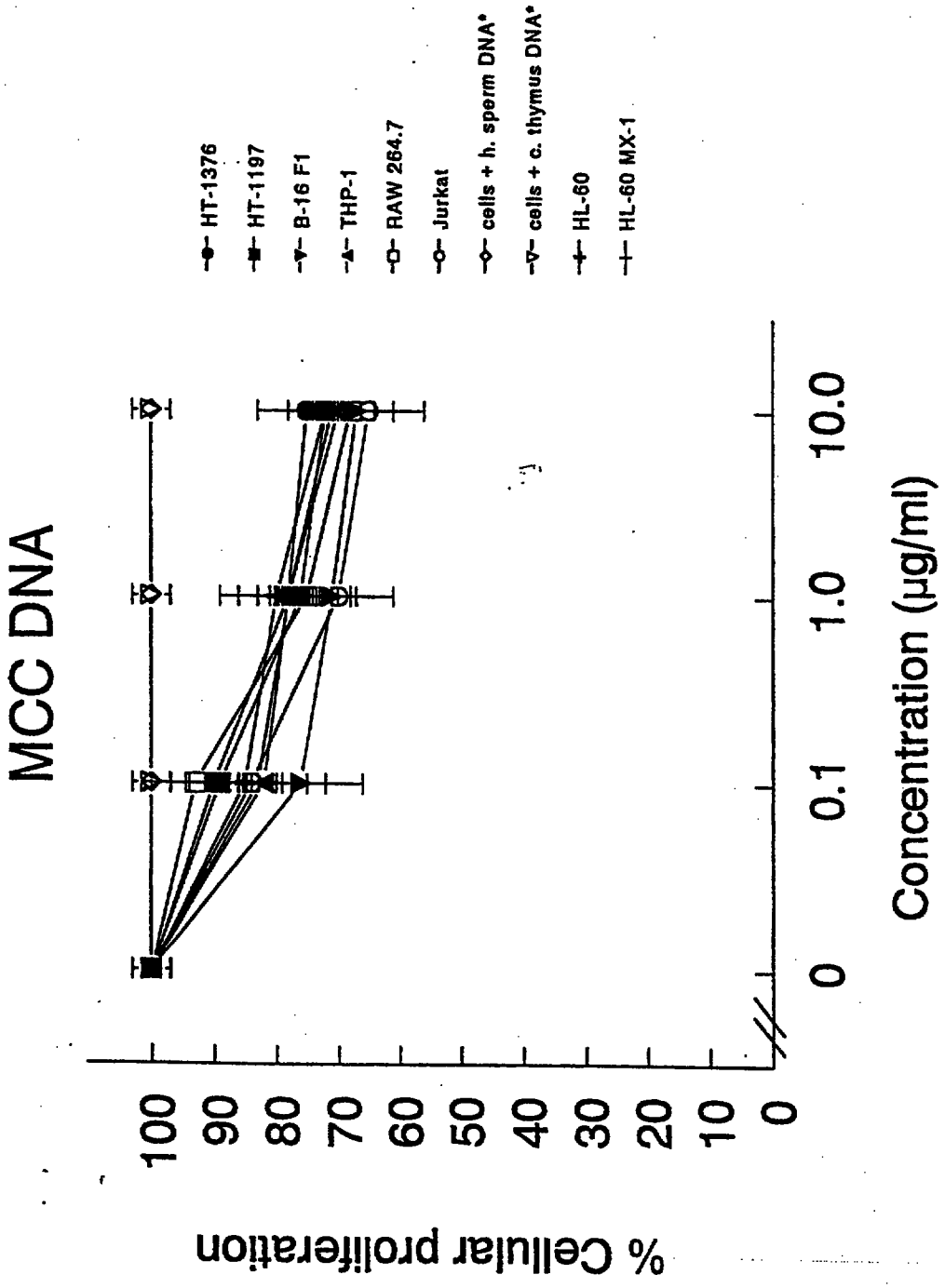

HT-1376
HT-1197
B-16 F1
THP-1
RAW 264.7
Jurkat
cells + h. sperm DNA*
cells + c. thymus DNA*
HL-60
HL-60 MX-1
MCC DNA
% Cellular proliferation
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1.0
10.0
Concentration (µg/ml)

FIGURE 2B

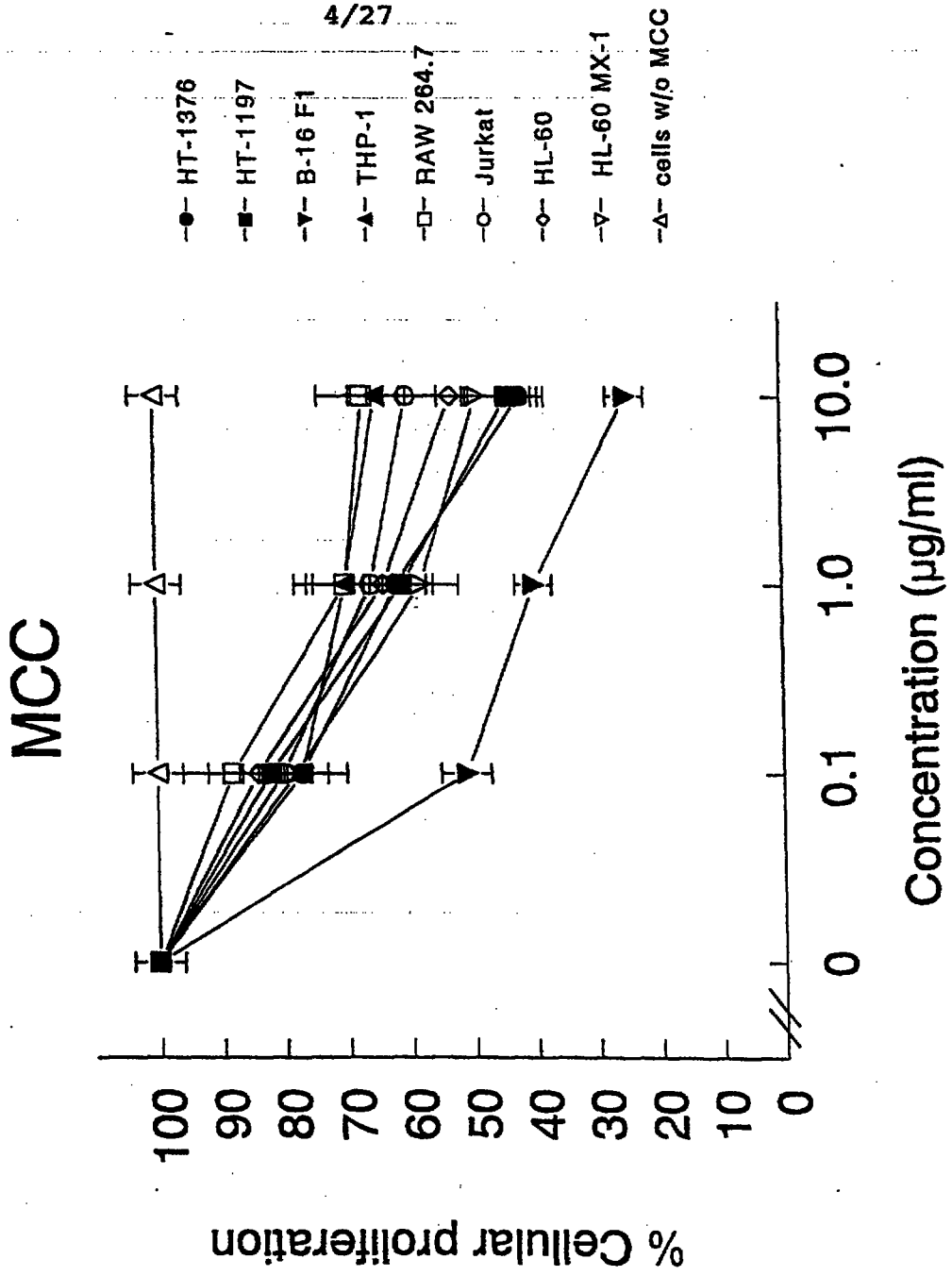

HT-1376
HT-1197
B-16 F1
THP-1
RAW 264.7
Jurkat
HL-60
HL-60 MX-1
cells w/o MCC
MCC
% Cellular proliferation
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1.0
10.0
Concentration (µg/ml)

FIGURE 3

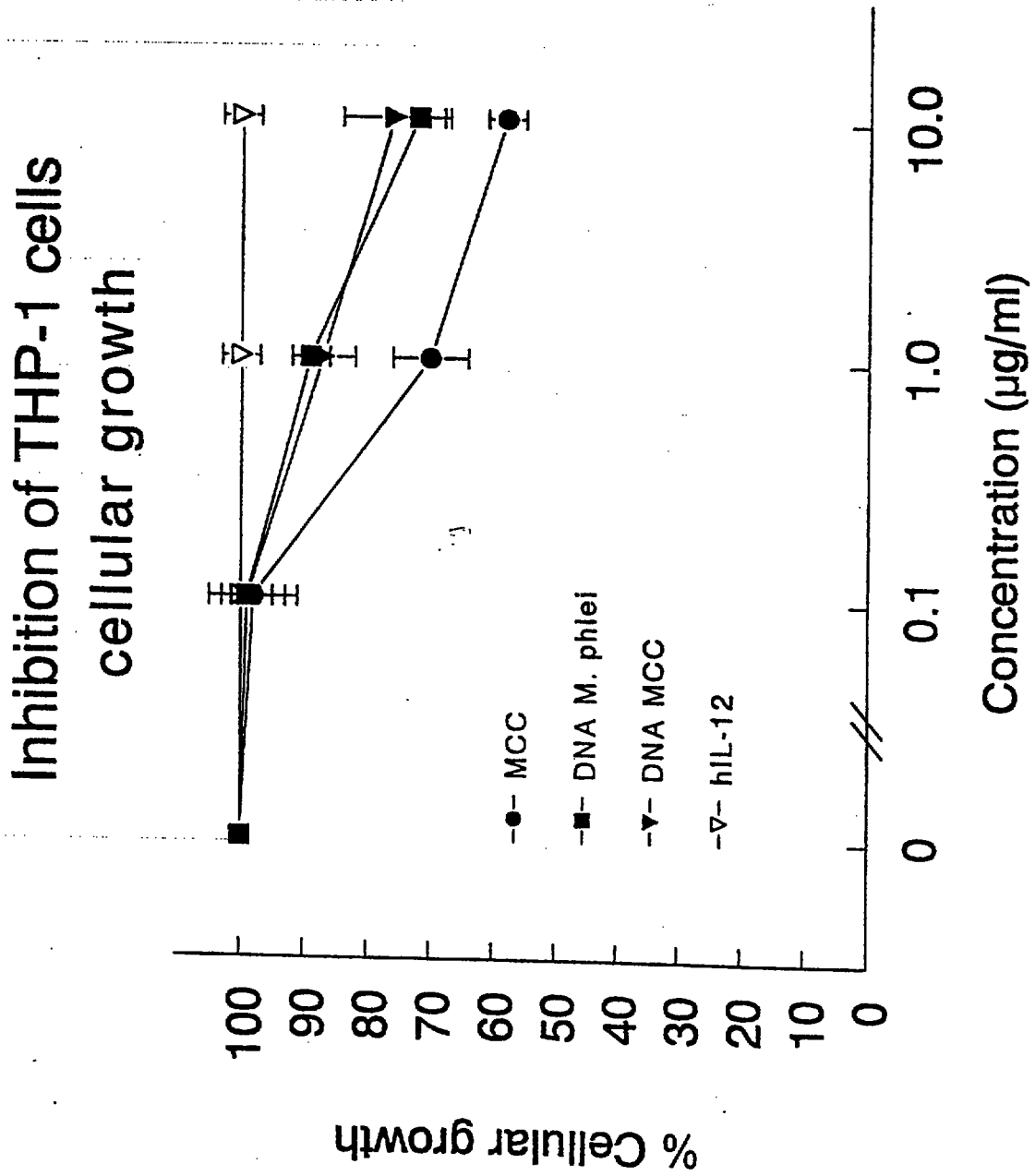

Inhibition of THP-1 cells
cellular growth
MCC
DNA M. phlei
DNA MCC
hIL-12
0
0.1
1.0
10.0
Concentration (µg/ml)
100
90
80
70
60
50
40
30
20
10
0
% Cellular growth

FIGURE 4

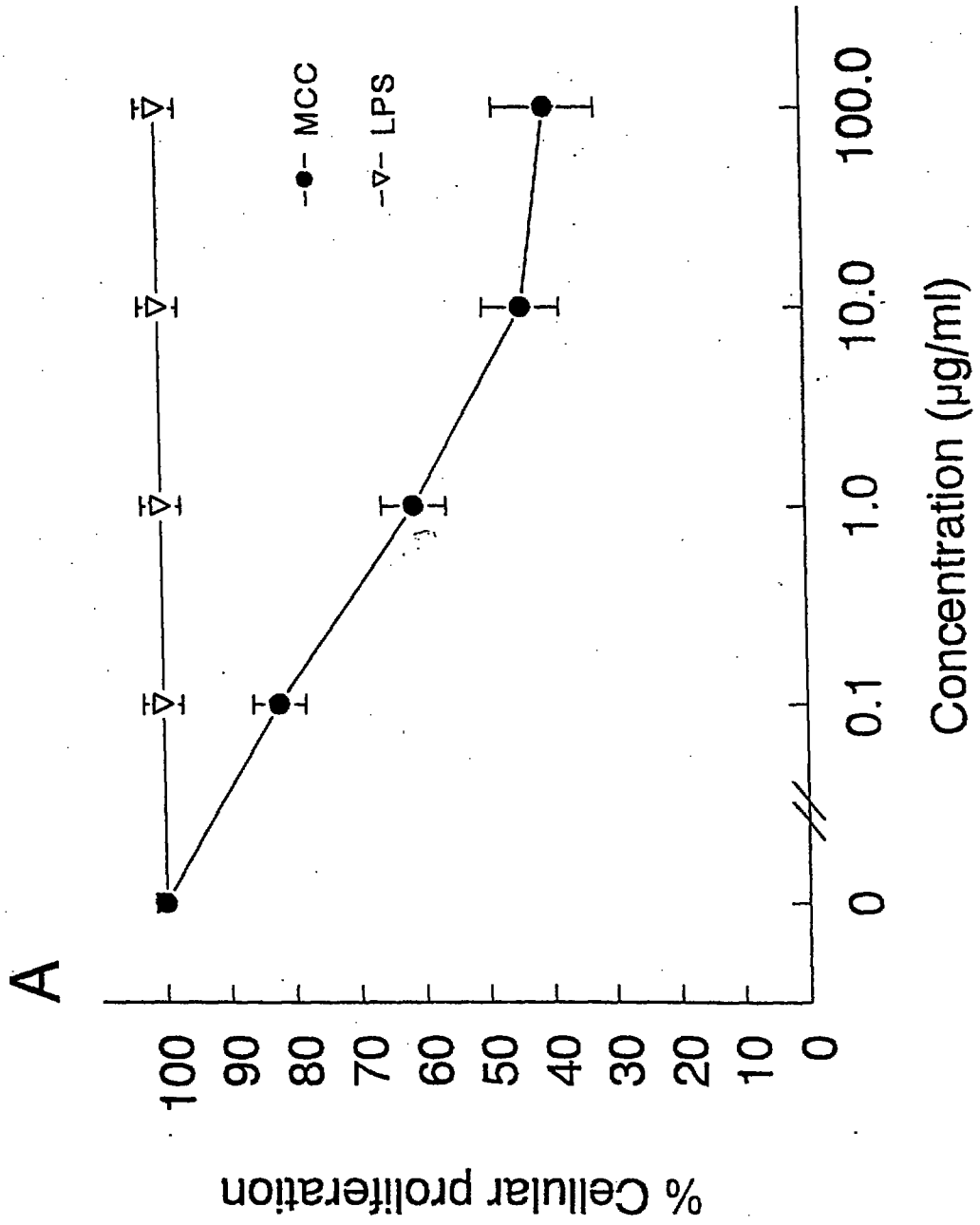

A
MCC
LPS
% Cellular proliferation
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1.0
10.0
100.0
Concentration (µg/ml)

FIGURE 5A

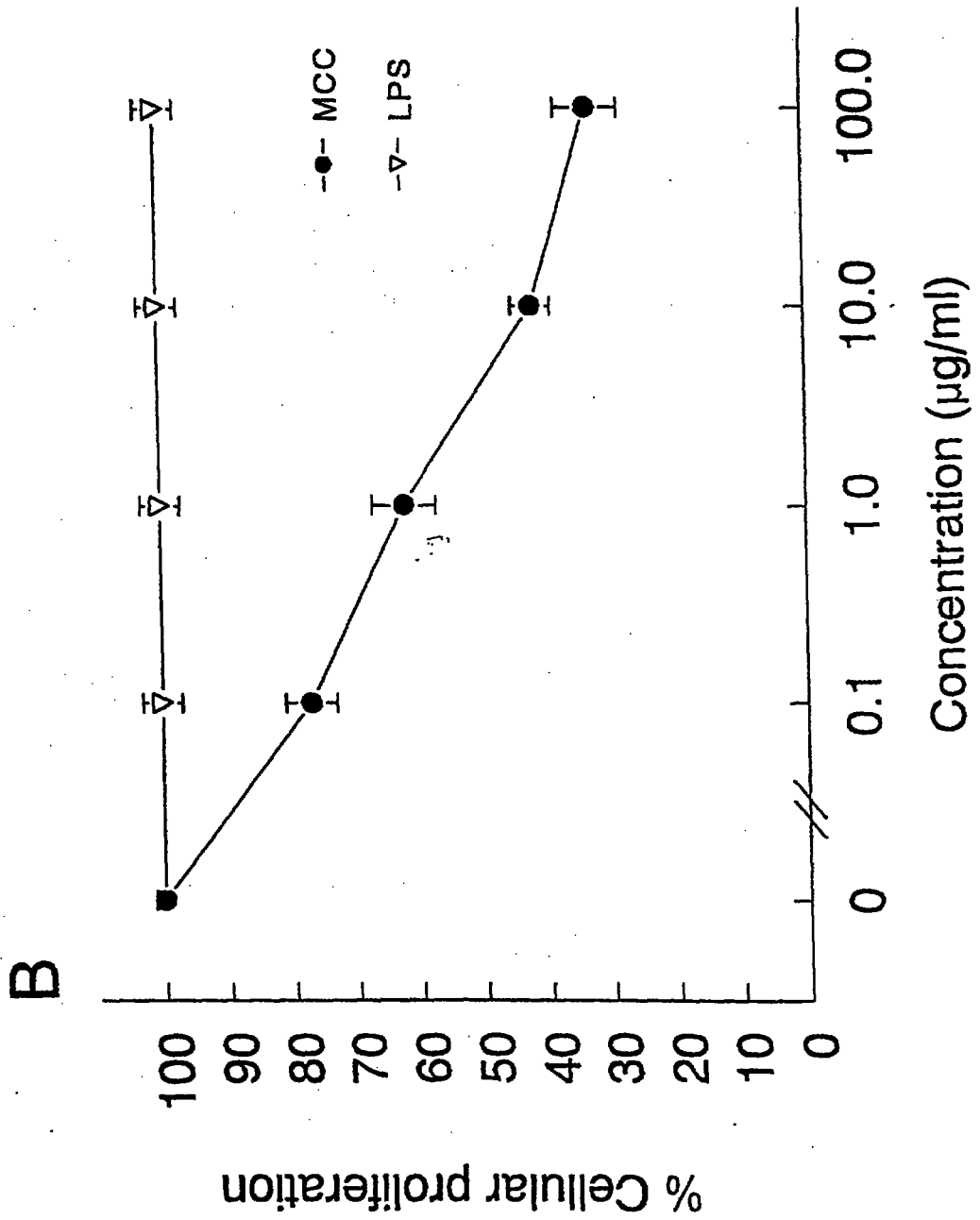
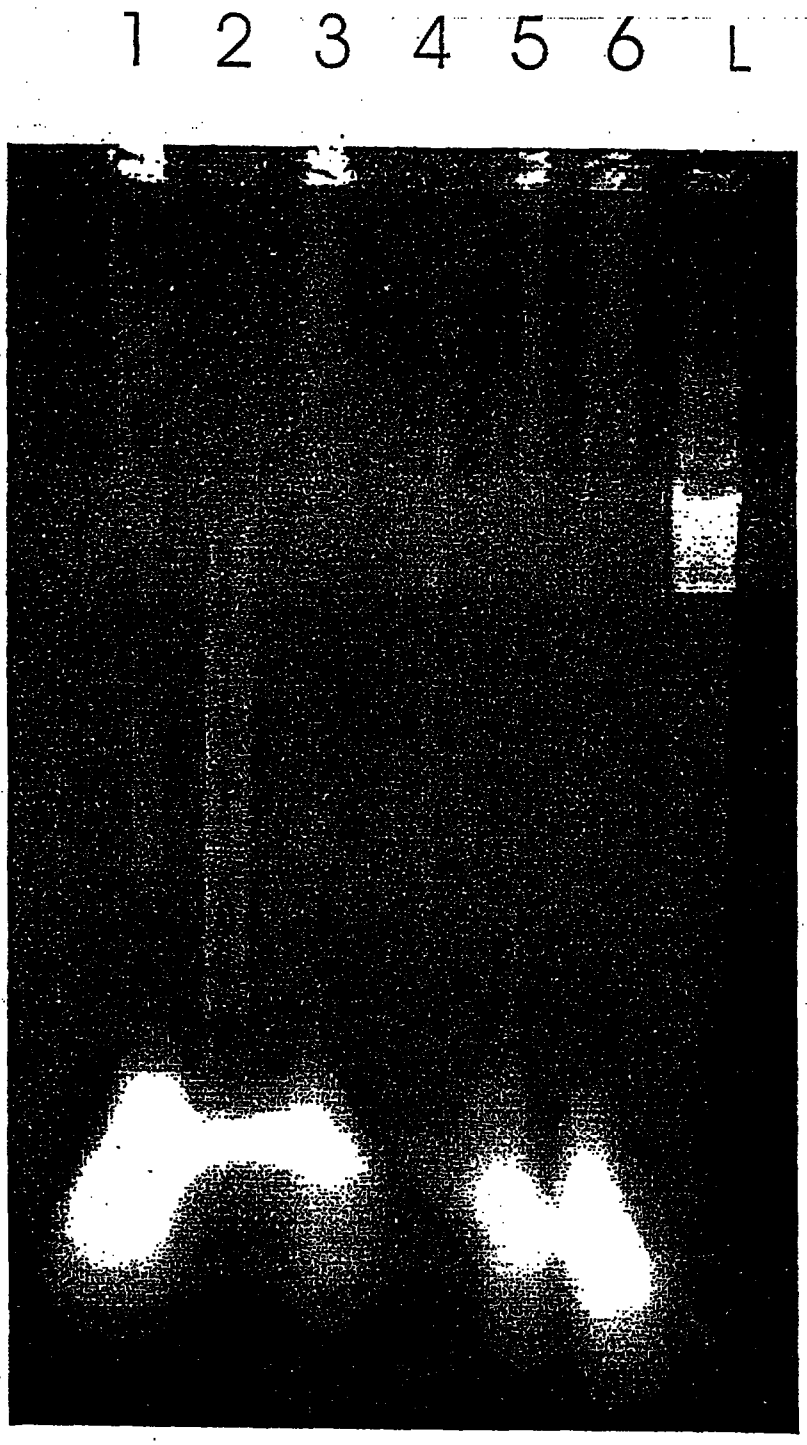

B
% Cellular proliferation
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1.0
10.0
100.0
Concentration (µg/ml)
MCC
LPS

FIGURE 5B

FIGURE 6



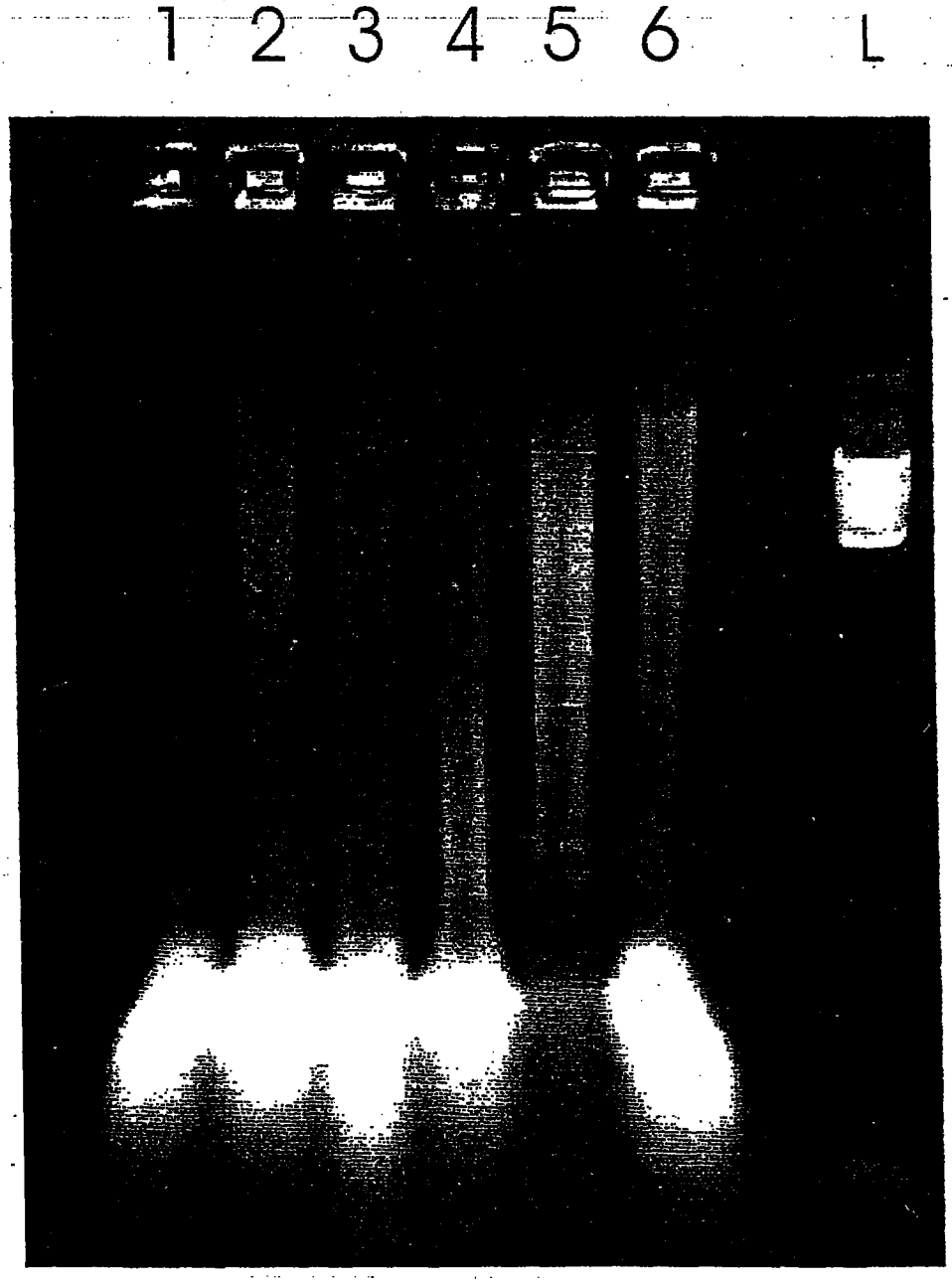



FIGURE 7

FIGURE 8B

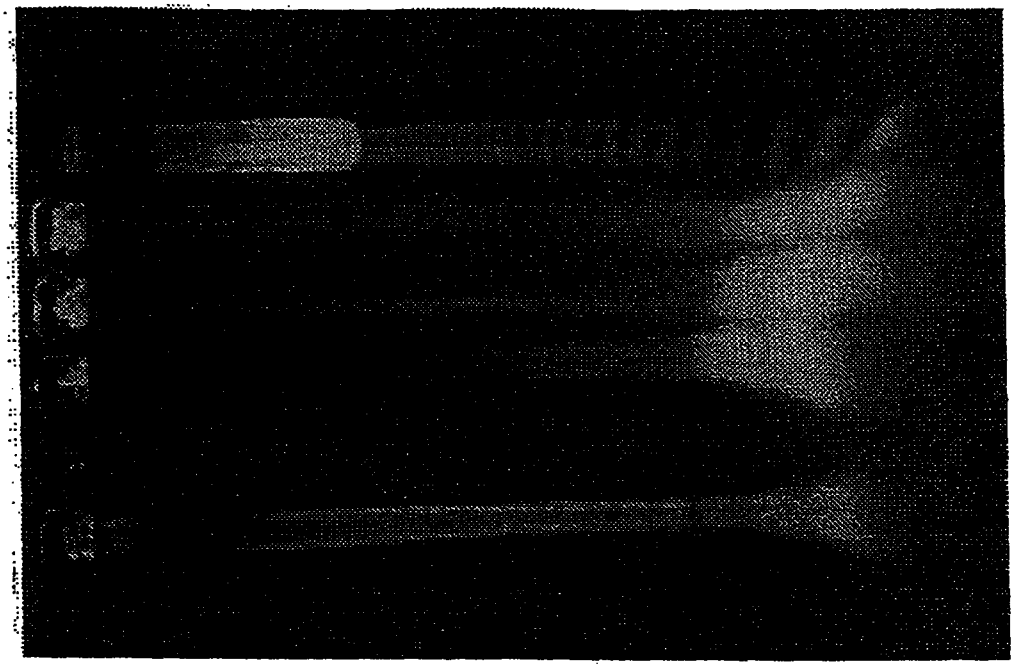
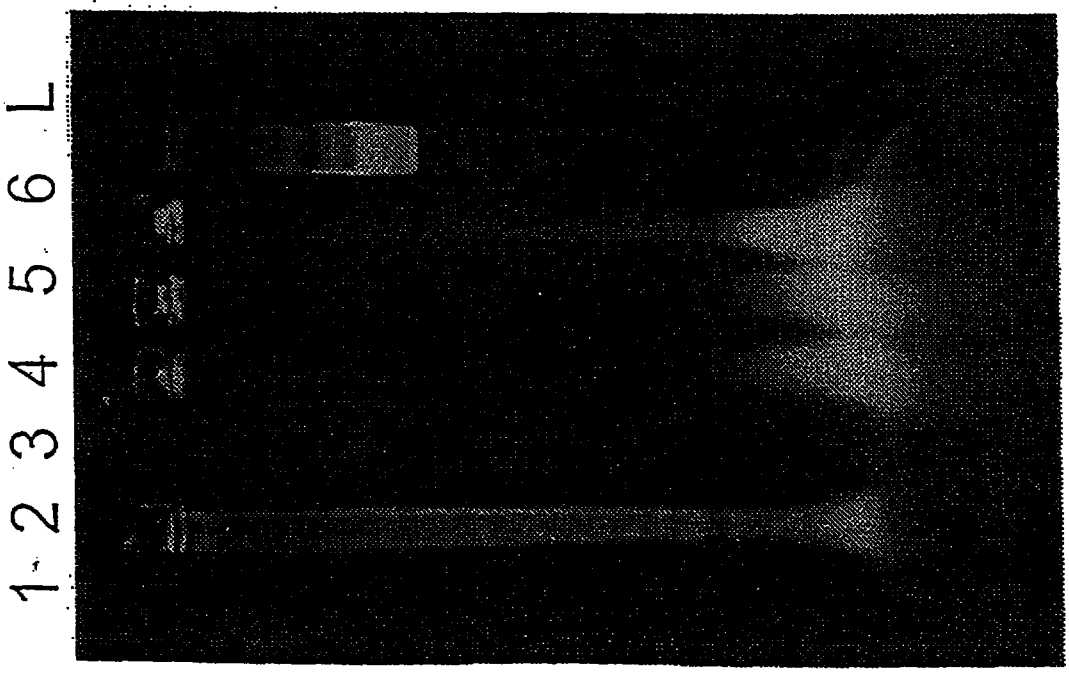



FIGURE 8A



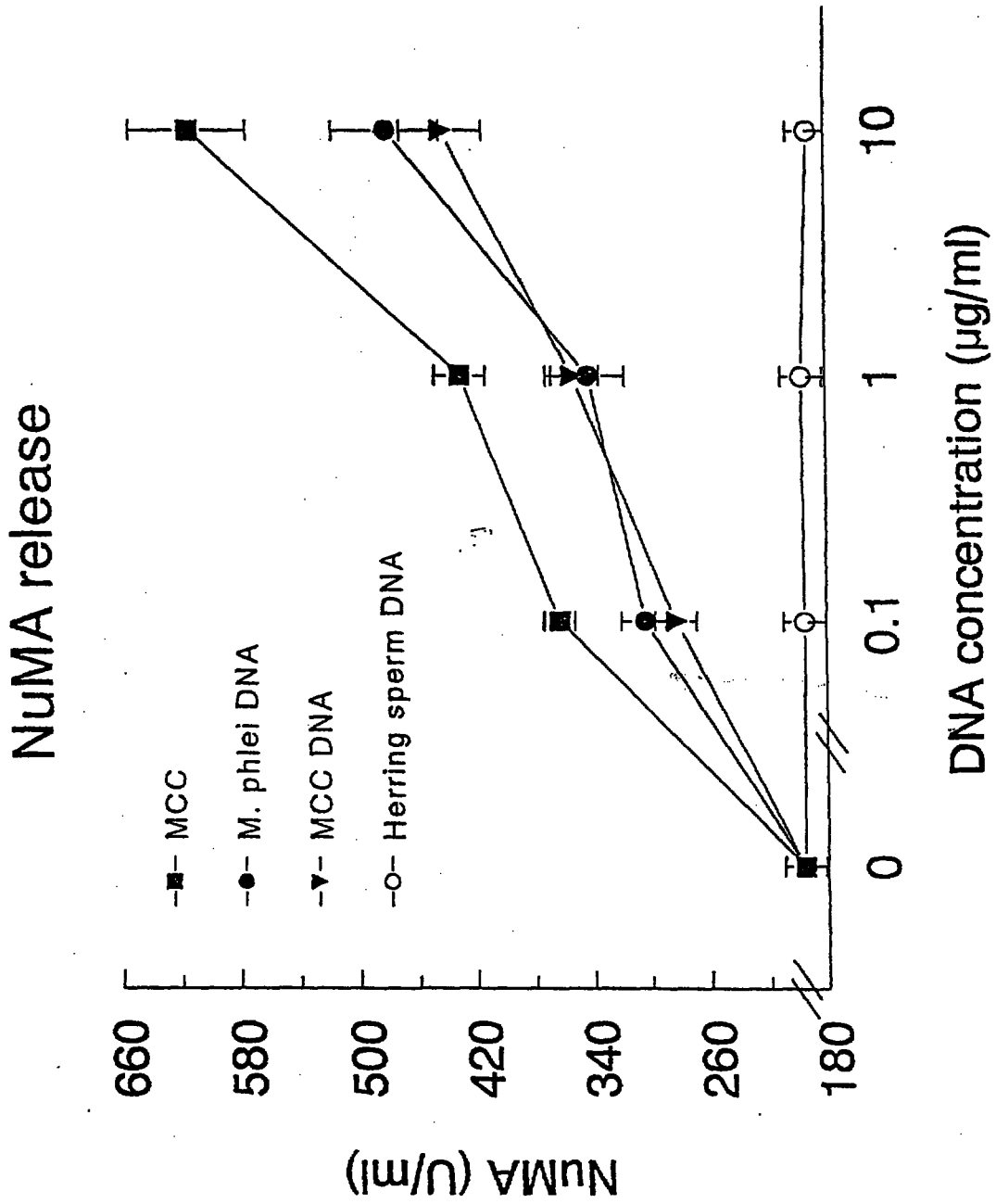

NuMA release
MCC
M. phlei DNA
MCC DNA
Herring sperm DNA
NuMA (U/ml)
660
580
500
420
340
260
180
0
0.1
1
10
DNA concentration (µg/ml)

FIGURE 9

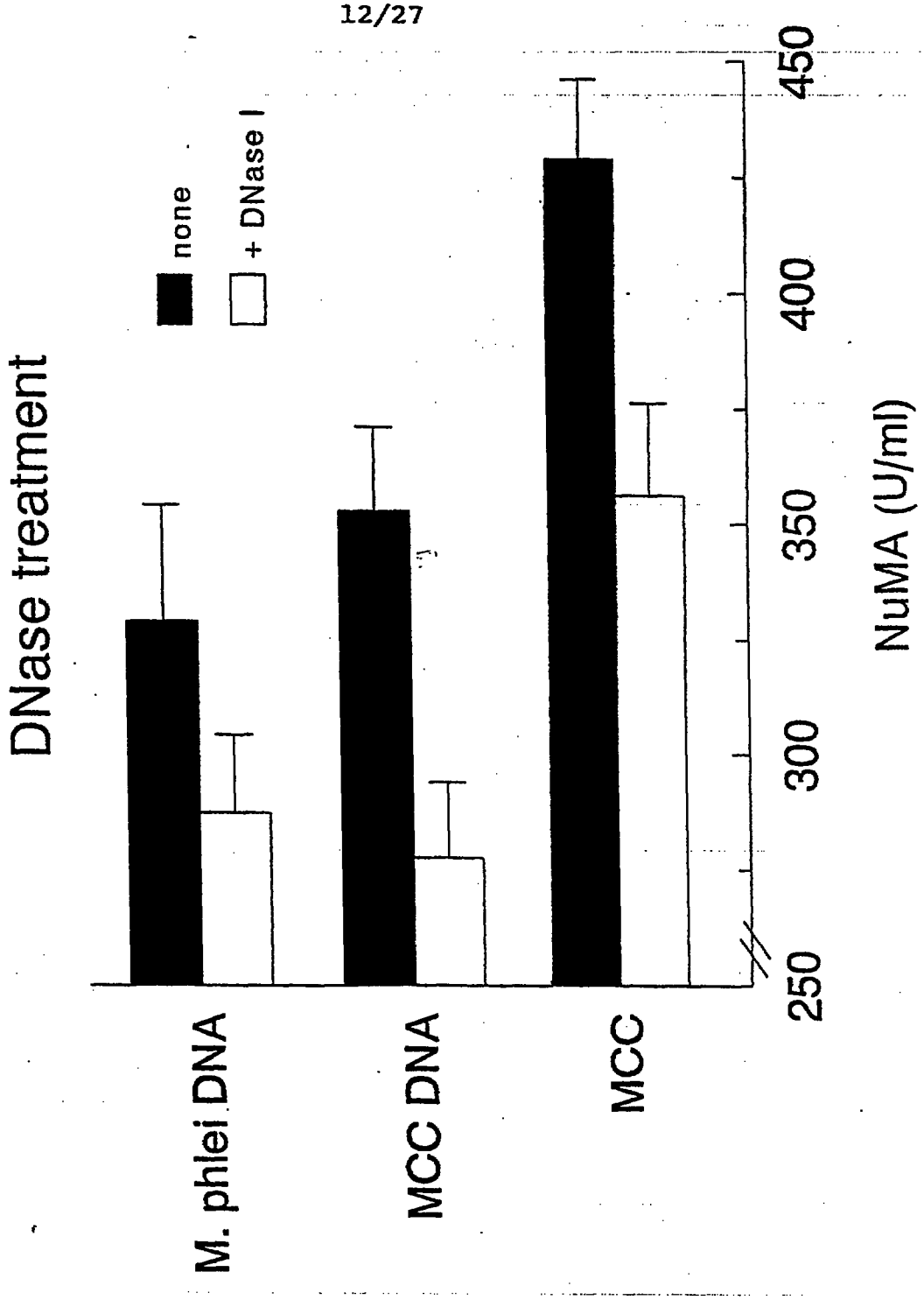

DNase treatment
none
+ DNase I
NuMA (U/ml)
450
400
350
300
250
M. phlei DNA
MCC DNA
MCC

FIGURE 10

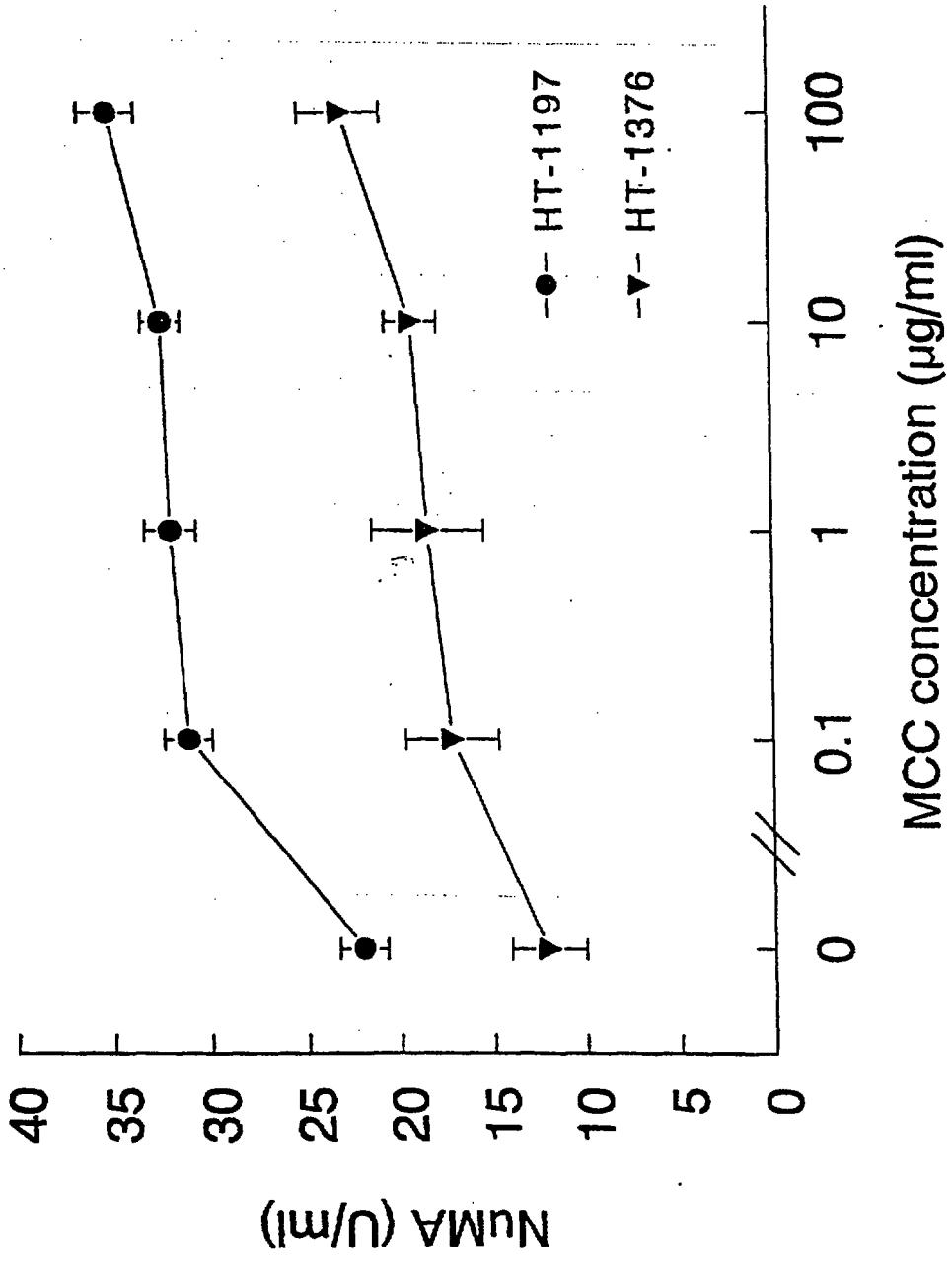

NuMA (U/ml)
40
35
30
25
20
15
10
5
0
0
0.1
1
10
100
MCC concentration (µg/ml)
HT-1197
HT-1376

FIGURE 11

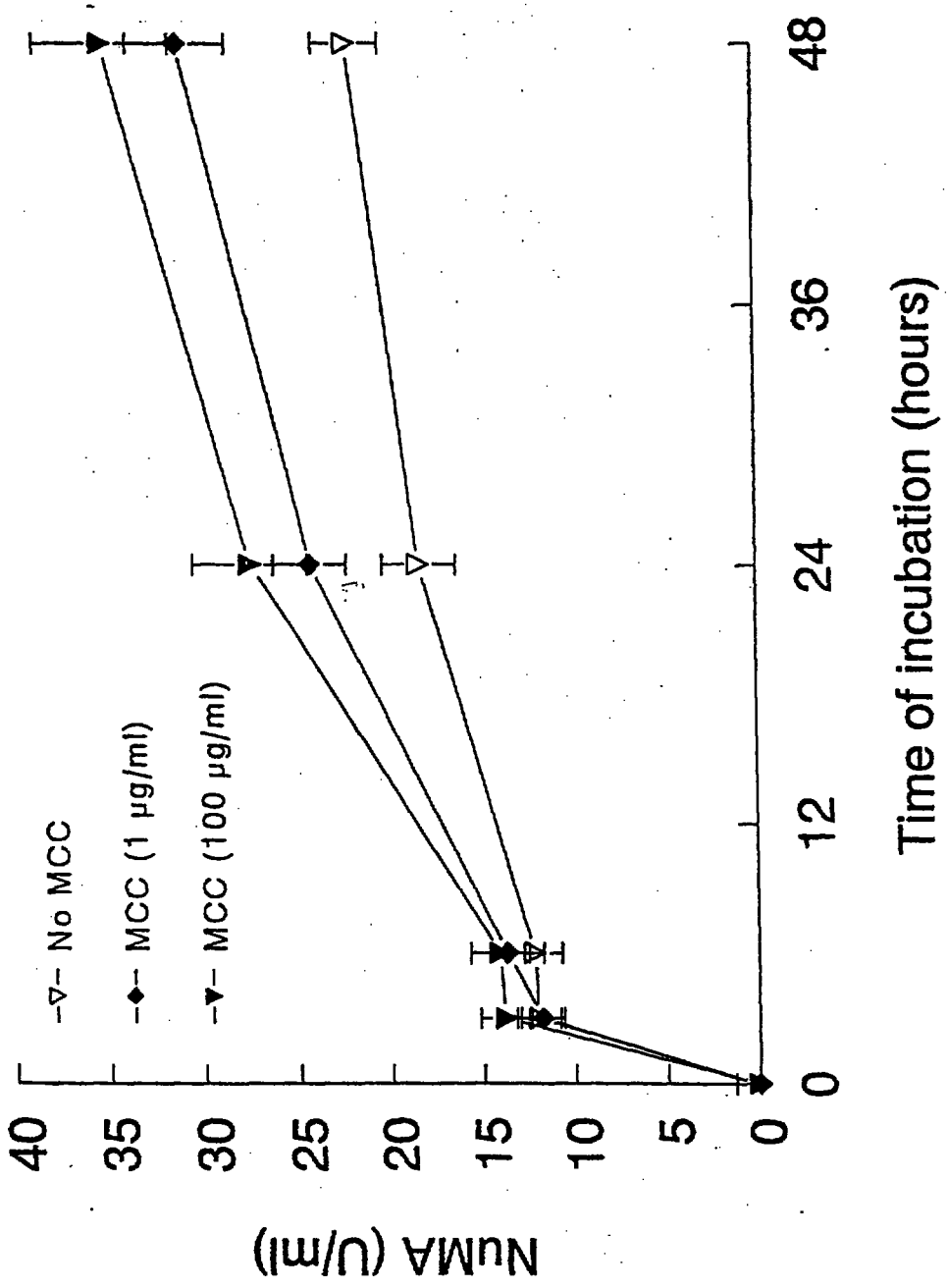

NuMA (U/ml)
40
35
30
25
20
15
10
5
0
0
12
24
36
48
Time of incubation (hours)
No MCC
MCC (1 µg/ml)
MCC (100 µg/ml)

FIGURE 12A

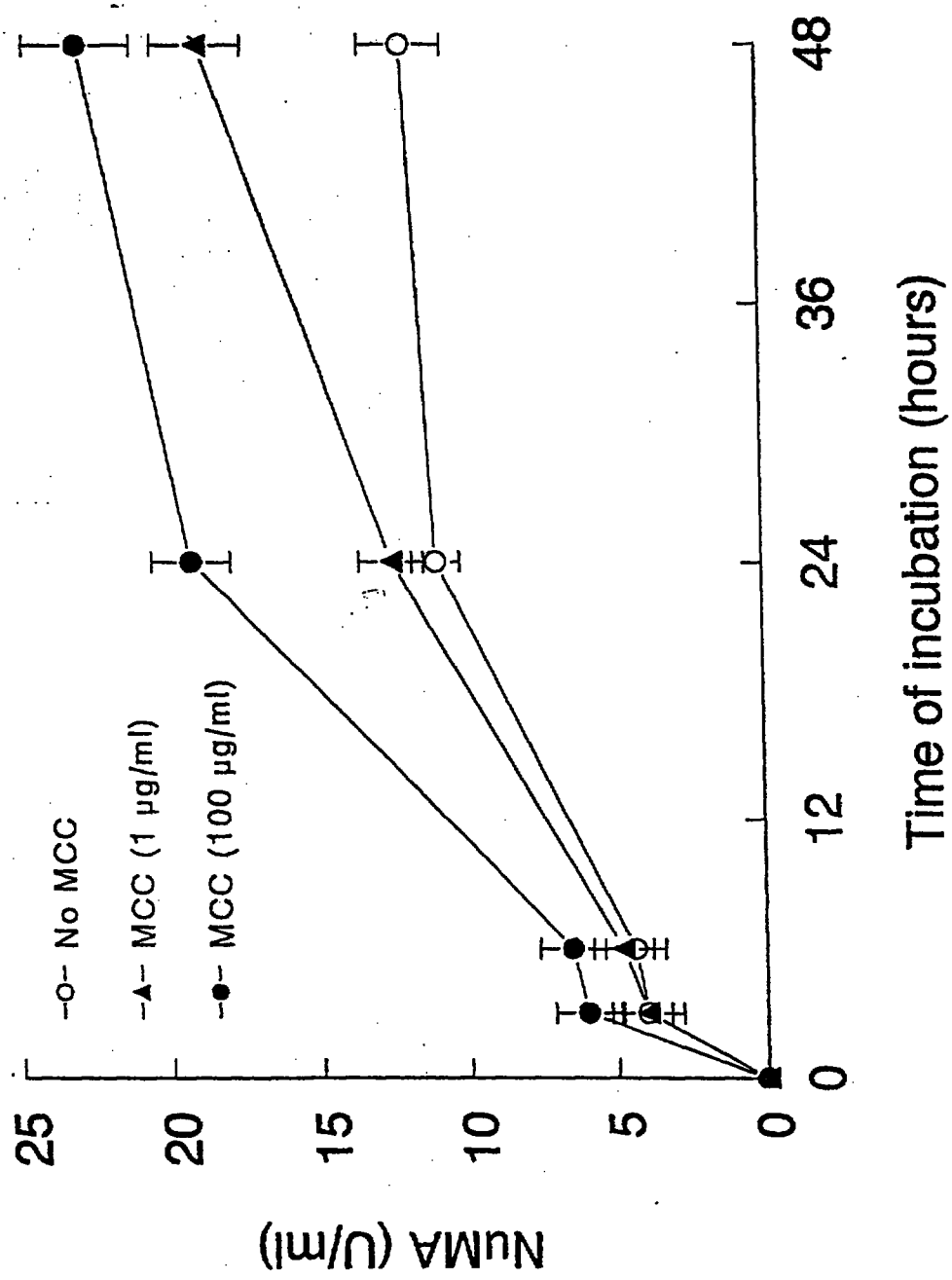

NuMA (U/ml)
25
20
15
10
5
0
0
12
24
36
48
Time of incubation (hours)
No MCC
MCC (1 µg/ml)
MCC (100 µg/ml)

FIGURE 12B

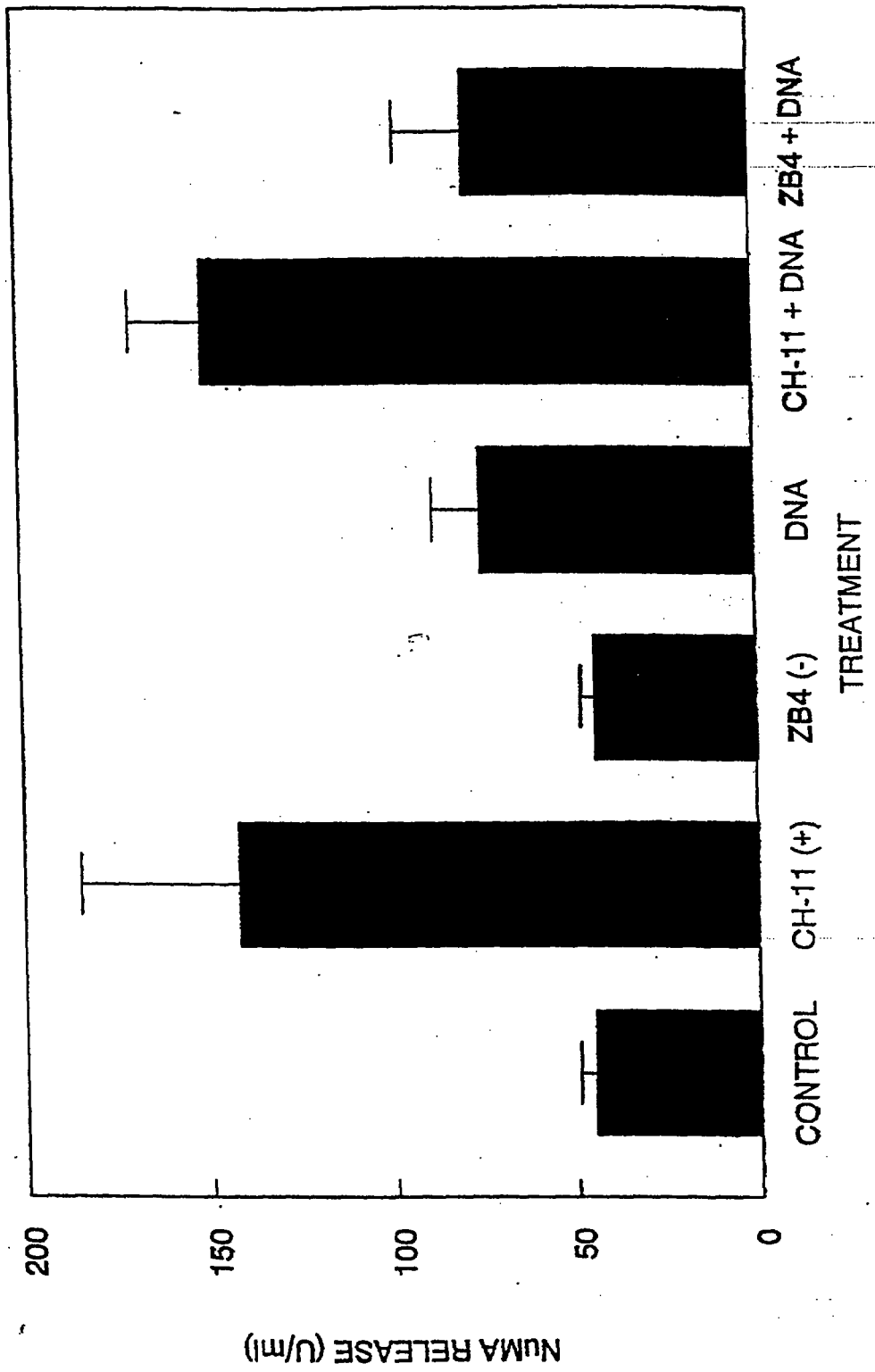

NuMA RELEASE (U/ml)
200
150
100
50
0
CONTROL
CH-11 (+)
ZB4 (-)
DNA
CH-11 + DNA
ZB4 + DNA
TREATMENT

FIGURE 13

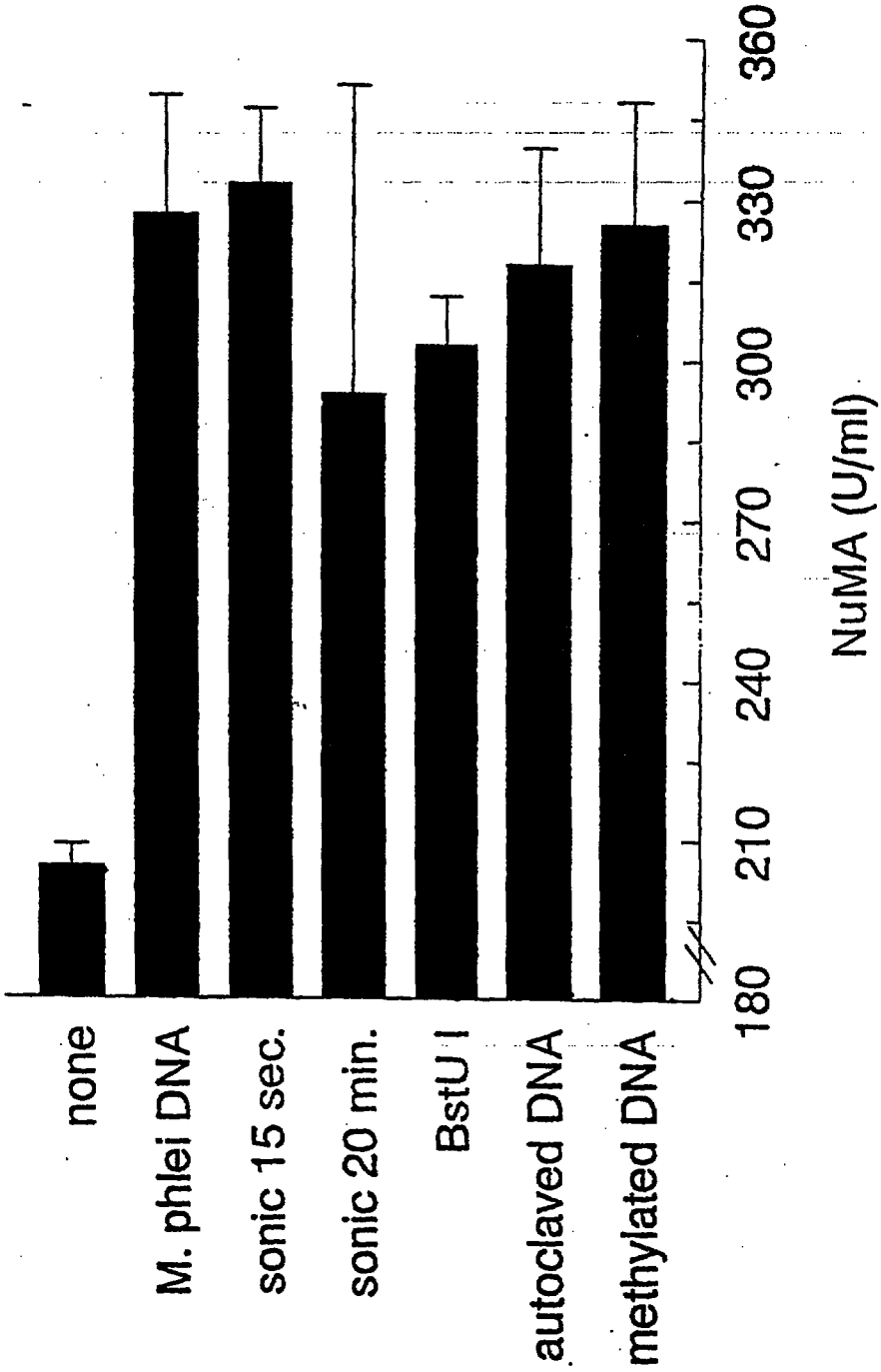

360
330
300
270
240
210
180
NuMA (U/ml)
none
M. phlei DNA
sonic 15 sec.
sonic 20 min.
BstU I
autoclaved DNA
methylated DNA

FIGURE 14

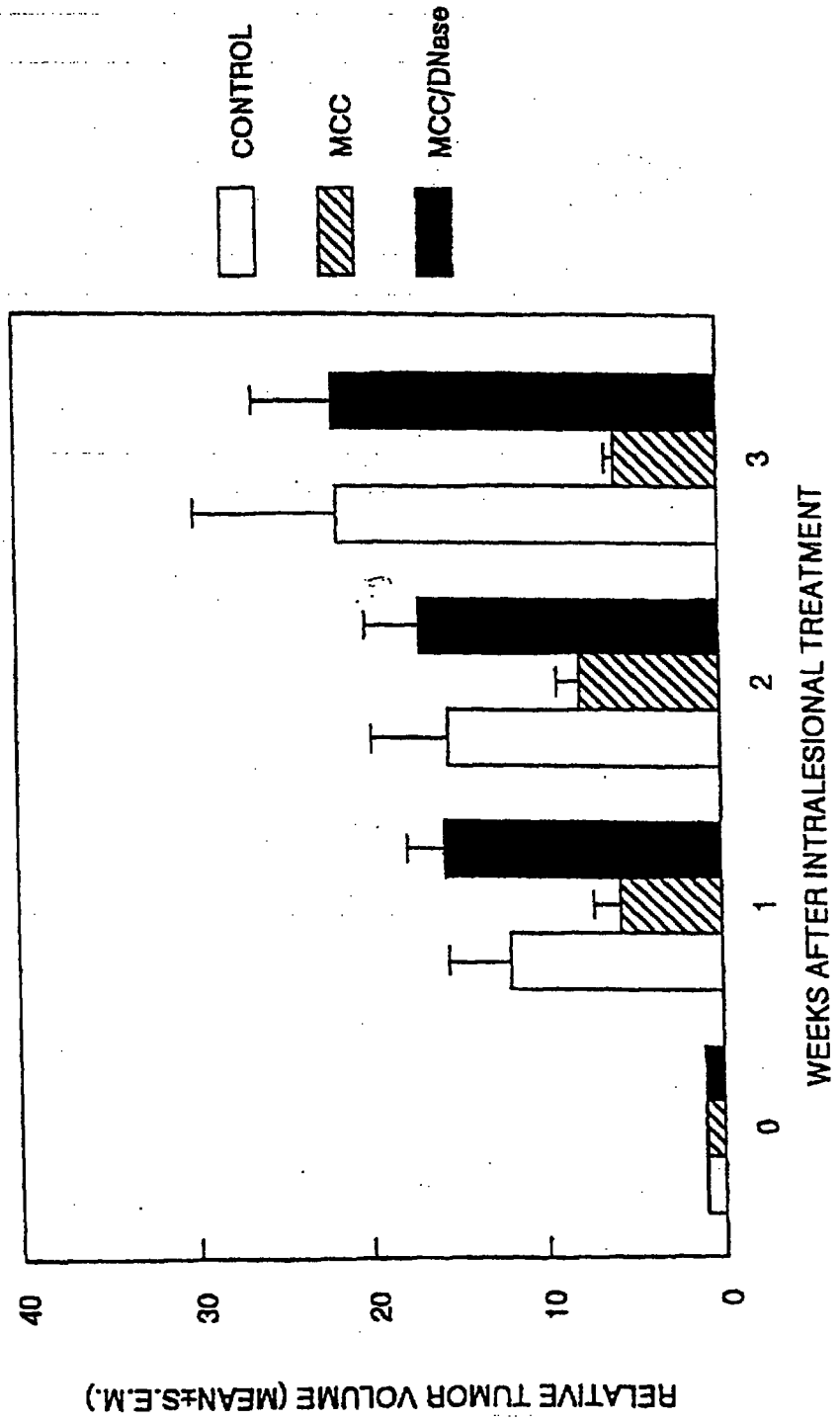

CONTROL
MCC
MCC/DNase
RELATIVE TUMOR VOLUME (MEAN±S.E.M.)
0
10
20
30
40
WEEKS AFTER INTRALESIONAL TREATMENT
0
1
2
3

FIGURE 15

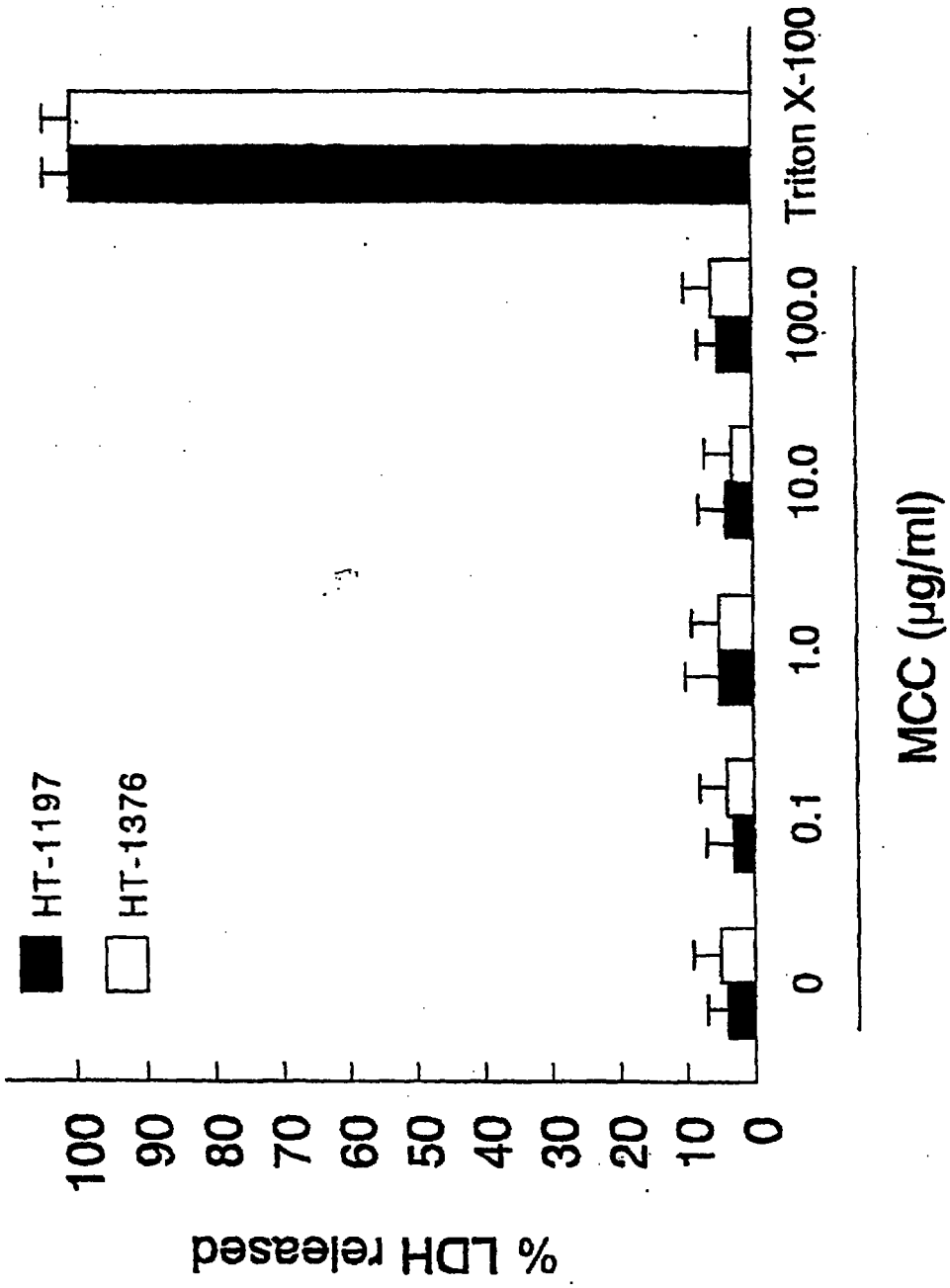

% LDH released
100
90
80
70
60
50
40
30
20
10
0
HT-1197
HT-1376
0
0.1
1.0
10.0
100.0
Triton X-100
MCC (µg/ml)

FIGURE 16

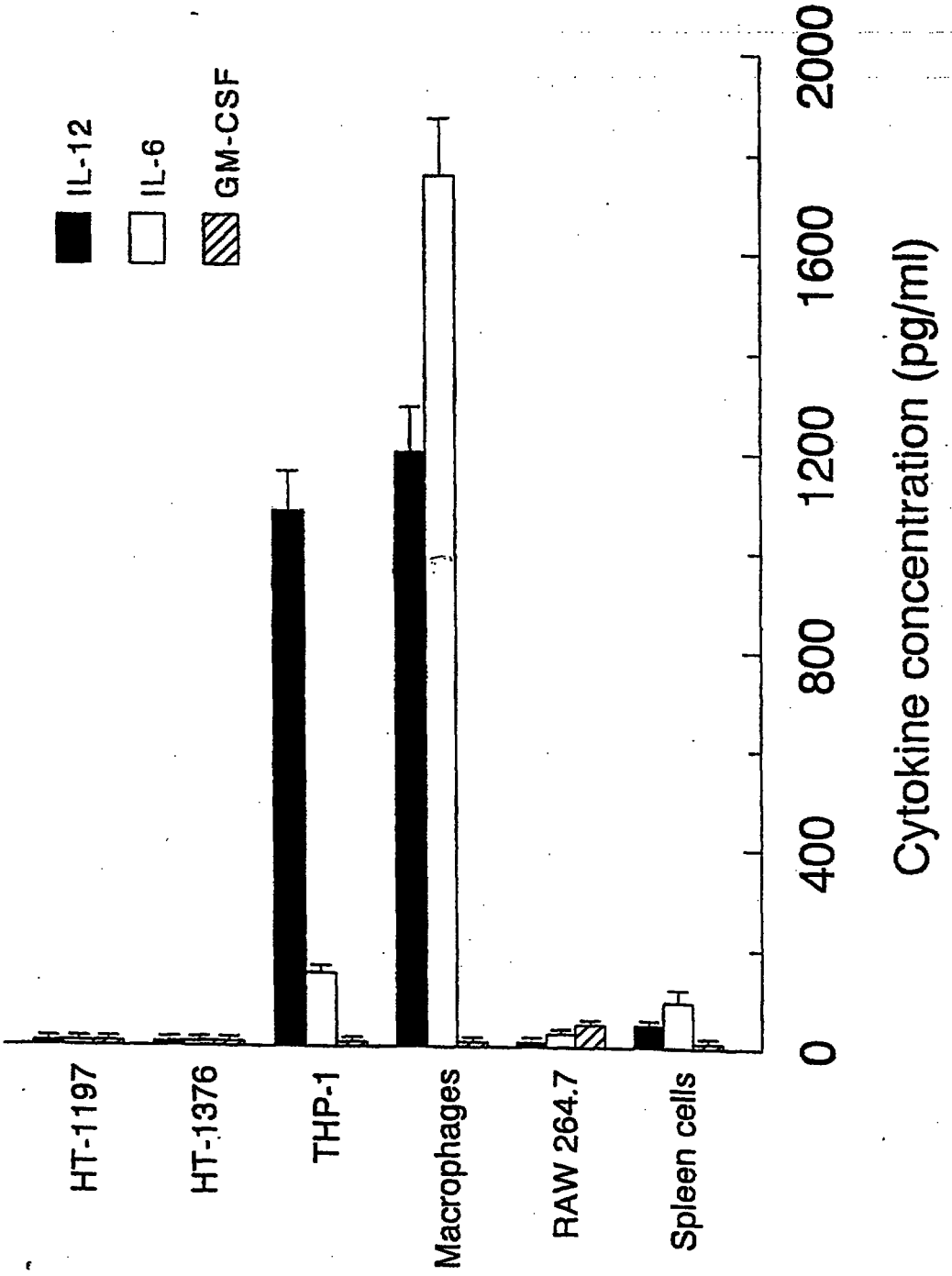

IL-12
IL-6
GM-CSF
Cytokine concentration (pg/ml)
0
400
800
1200
1600
2000
HT-1197
HT-1376
THP-1
Macrophages
RAW 264.7
Spleen cells

FIGURE 17

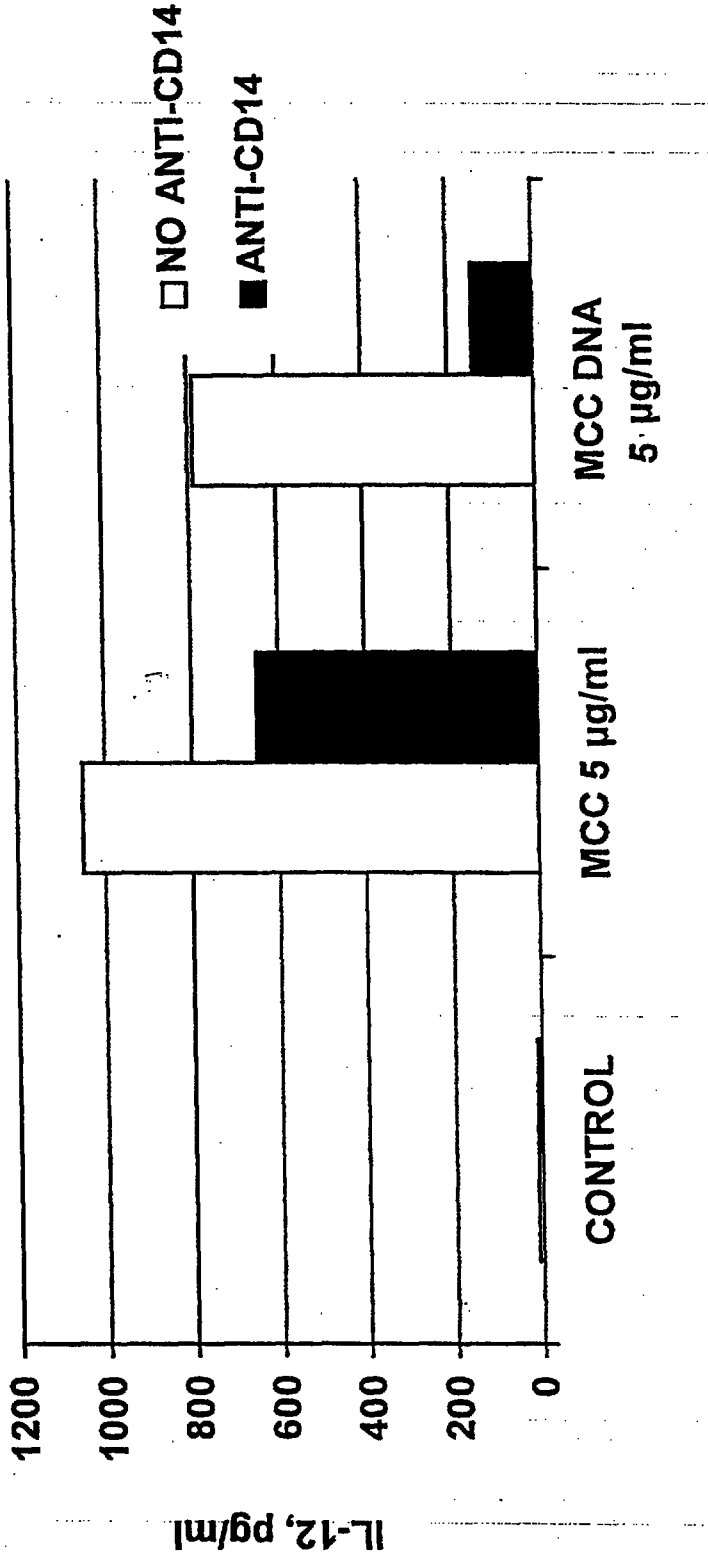

NO ANTI-CD14
ANTI-CD14
MCC DNA 5 µg/ml
MCC 5 µg/ml
CONTROL
1200
1000
800
600
400
200
0
IL-12, pg/ml

FIGURE 18

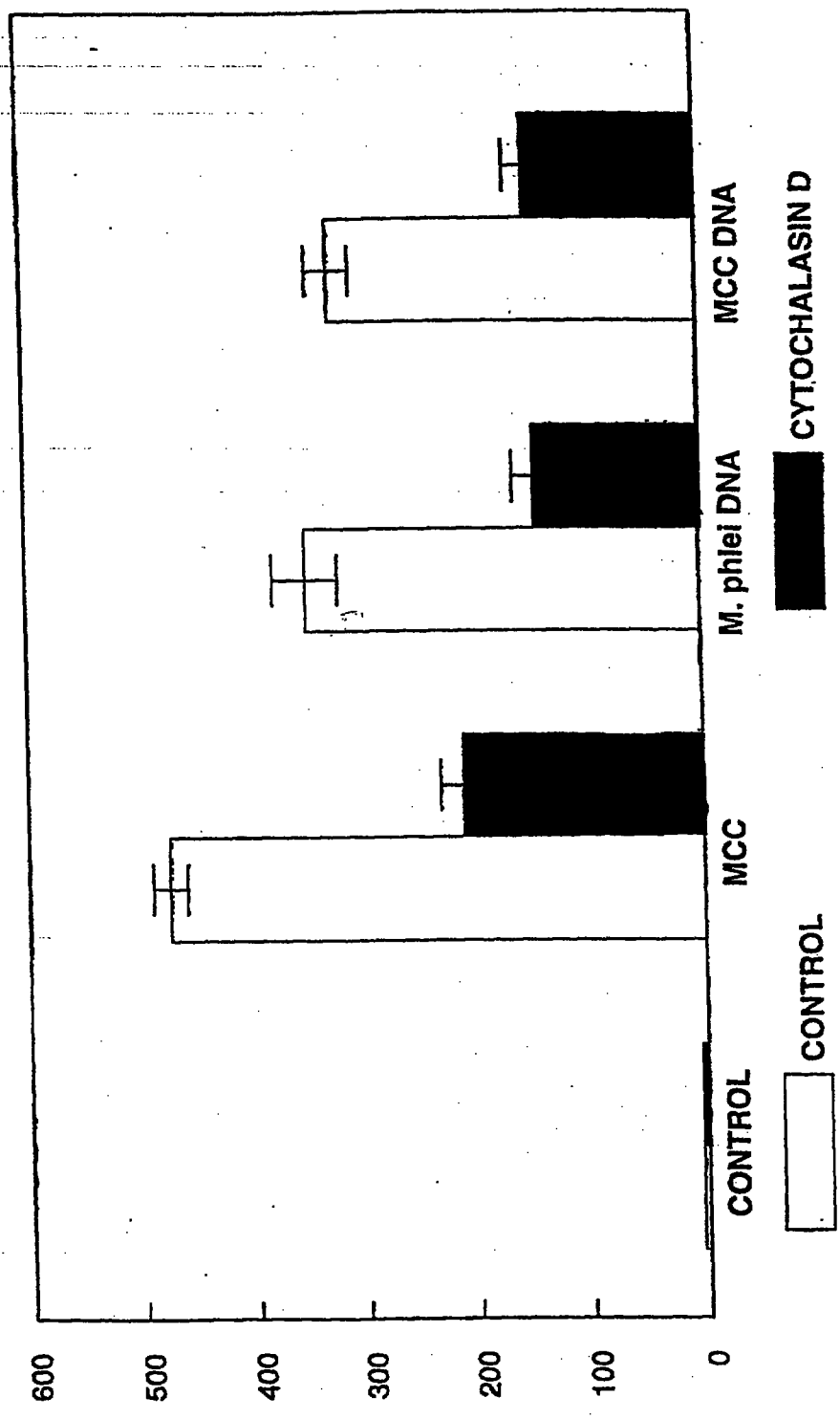

IL-12, pg/ml
600
500
400
300
200
100
0
CONTROL
MCC
M. phlei DNA
MCC DNA
CONTROL
CYTOCHALASIN D

FIGURE 19

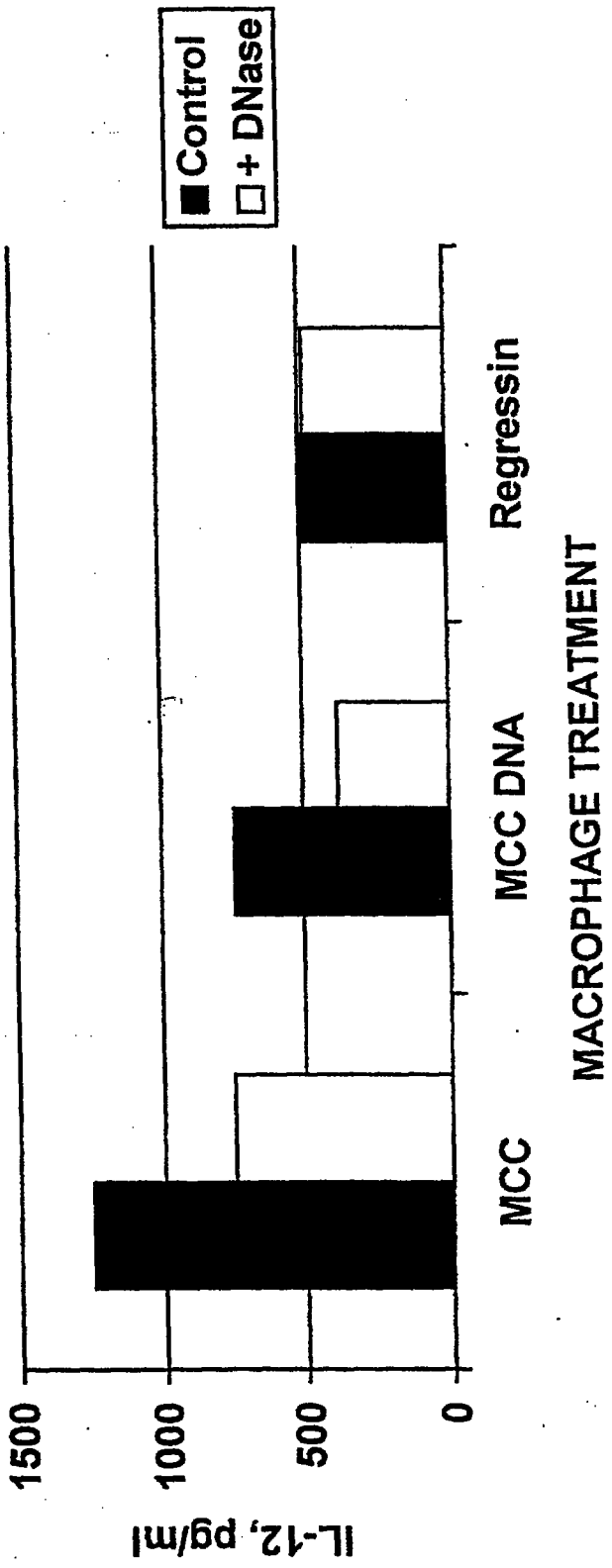

Control
+ DNase
Regressin
MCC DNA
MCC
MACROPHAGE TREATMENT
1500
1000
500
0
IL-12, pg/ml

FIGURE 20

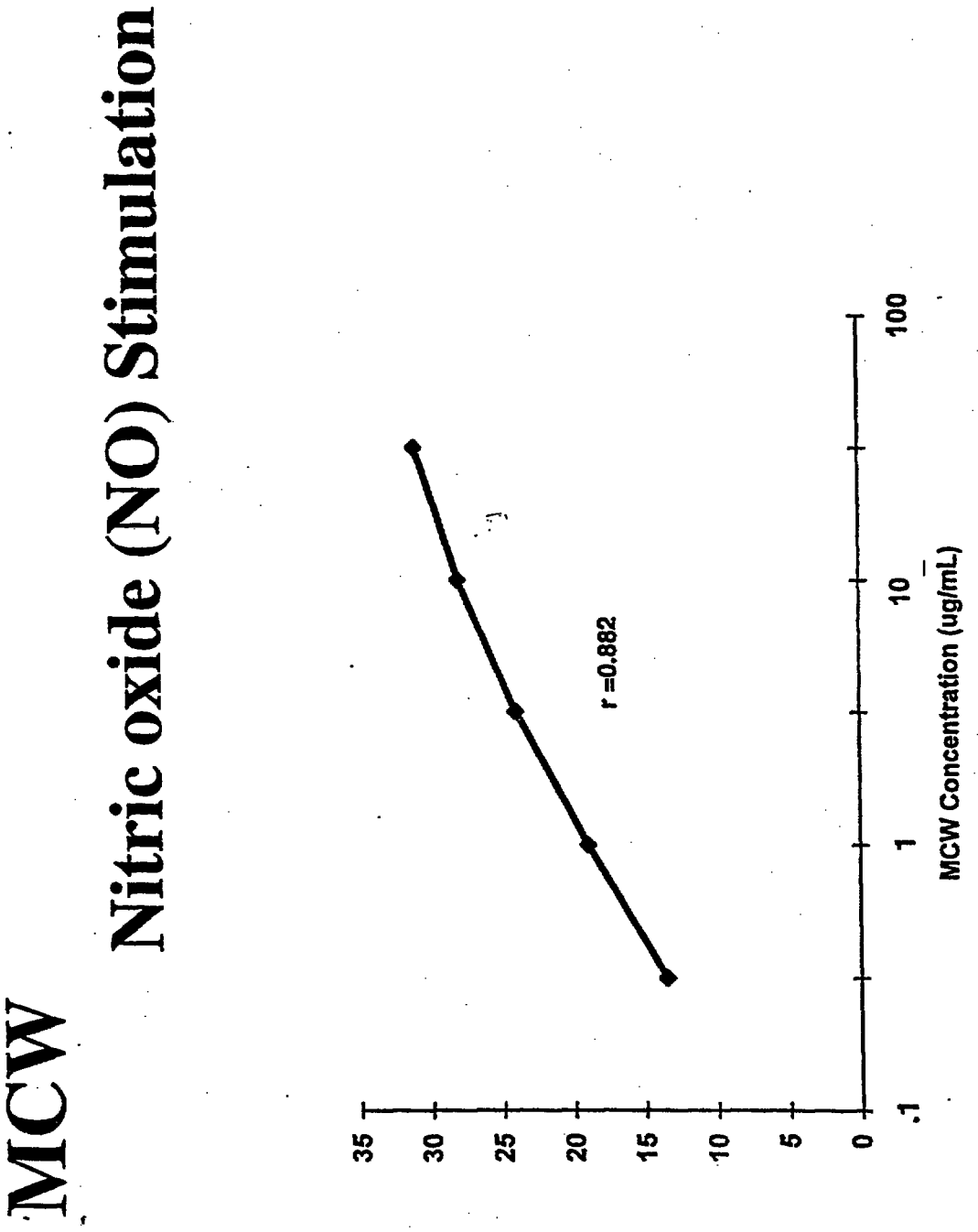

MCW
Nitric oxide (NO) Stimulation
r =0.882
35
30
25
20
15
10
5
0
.1
1
10
100
MCW Concentration (ug/mL)

FIGURE 21

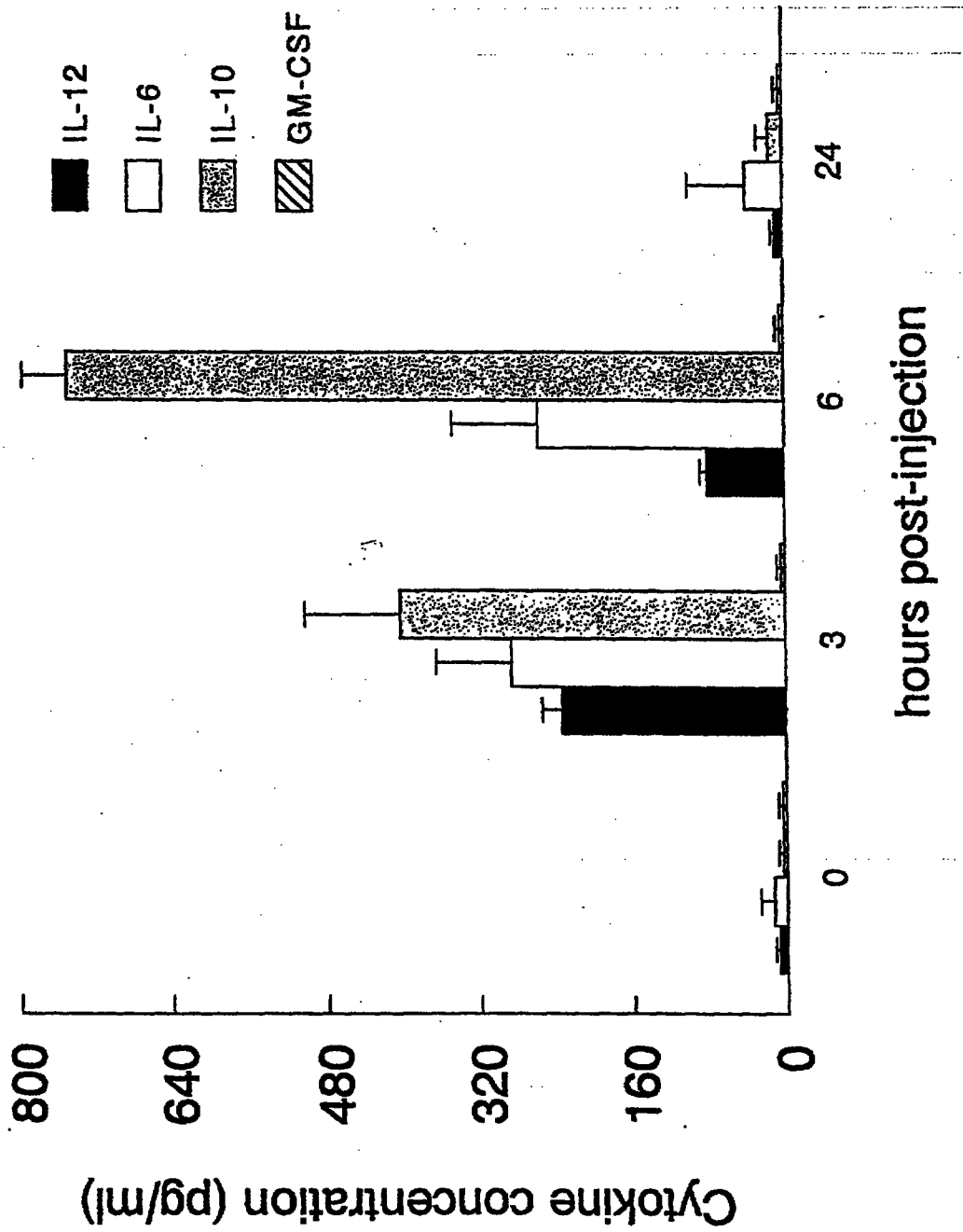

IL-12
IL-6
IL-10
GM-CSF
0
3
6
24
hours post-injection
0
160
320
480
640
800
Cytokine concentration (pg/ml)

FIGURE 22

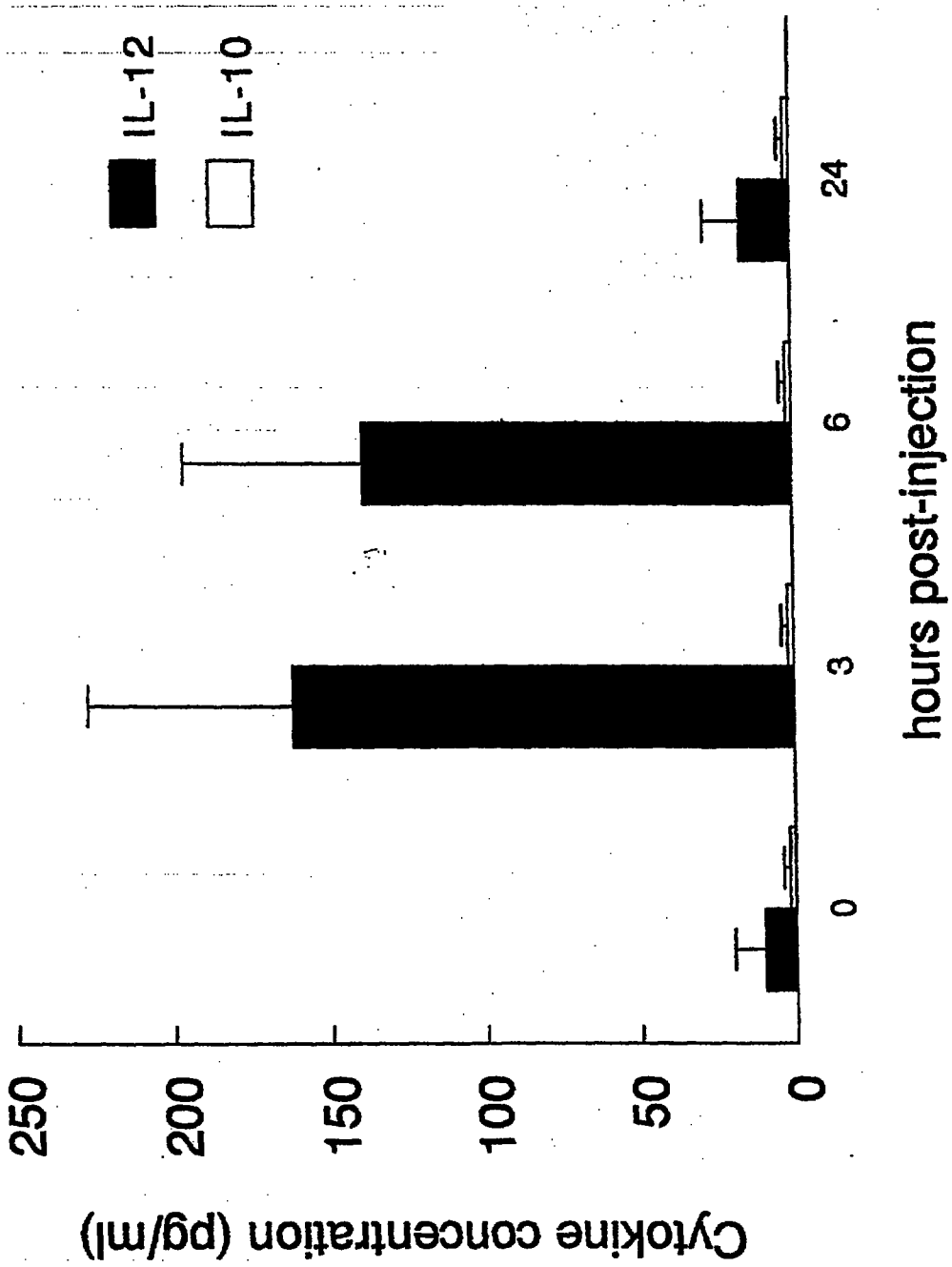

IL-12
IL-10
Cytokine concentration (pg/ml)
250
200
150
100
50
0
0
3
6
24
hours post-injection

FIGURE 23

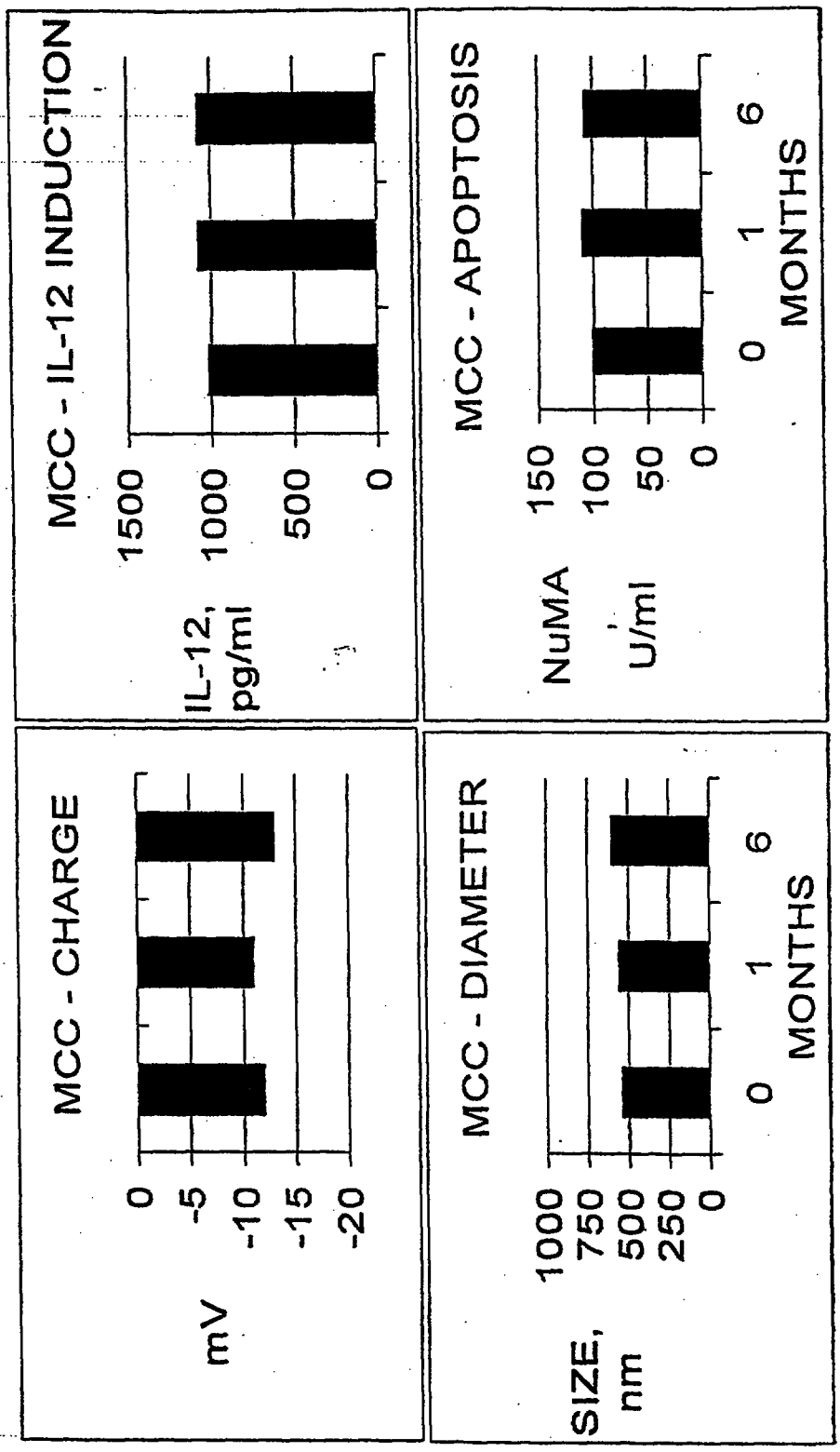

MCC - IL-12 INDUCTION
1500
1000
500
0
IL-12, pg/ml
MCC - APOPTOSIS
150
100
50
0
NuMA U/ml
0
1
6
MONTHS
MCC - CHARGE
0
-5
-10
-15
-20
mV
MCC - DIAMETER
1000
750
500
250
0
SIZE, nm
0
1
6
MONTHS

FIGURE 24

# CLAIMS

We claim:

1. A composition comprising:
   a. *Mycobacterium phlei*-DNA (M-DNA), and
   b. a first pharmaceutically acceptable carrier,
wherein the M-DNA has anti-cancer activity.

2. The composition of claim 1, wherein the anti-cancer activity of M-DNA is inhibition of proliferation of cancer cells.

3. The composition of claim 1, wherein the anti-cancer activity of M-DNA is induction of apoptosis in cancer cells.

4. The composition of claim 3, wherein the induction of apoptosis in cancer cells is independent of a factor selected from the group consisting of Fas, p53/p21, and drug resistance.

5. The composition of claim 3, wherein the induction of apoptosis in cancer cells is induction of caspase-3 activity in the cancer cells.

6. The composition of claim 1, wherein the anti-cancer activity of M-DNA is stimulation of cytokine production by cells of the immune system.

7. The composition of claim 6, wherein the cells of the immune system are selected from the group consisting of macrophages and monocytes.

* C L M S *



8. The compositions of claim 6, wherein the cytokine is selected from the group consisting of IL-6, IL-10 and IL-12.

9. The composition of claim 1, wherein the anti-cancer activity of M-DNA is stimulation of reactive oxygen species production by cells of the immune system.

10. The compositions of claim 9, wherein the cells of the immune system are macrophages.

11. The composition of claim 1, wherein the first pharmaceutically acceptable carrier is selected from the group consisting of an aqueous carrier, a non-aqueous carrier and a *Mycobacterium phlei* (*M. phlei*) cell wall.

12. The composition of claim 11, wherein the first pharmaceutically acceptable carrier is *M. phlei* cell wall.

13. The composition of claim 1, further comprising a second pharmaceutically acceptable carrier, wherein the second pharmaceutically acceptable carrier is selected from the group consisting of an aqueous carrier and a non-aqueous carrier.

14. A composition comprising:

a. M-DNA,

b. *M. phlei* cell wall, wherein the M-DNA is preserved and complexed on the *M. phlei* cell wall (MCC) and,

c. a pharmaceutically acceptable carrier,

wherein the MCC has anti-cancer activity.

15. The composition of claim 14, wherein the anti-cancer activity of MCC is inhibition of proliferation of cancer cells.

16. The composition of claim 14, wherein the anti-cancer activity of MCC is induction of apoptosis in cancer cells.

17. The composition of claim 16, wherein the induction of apoptosis in cancer cells is independent of a factor selected from the group consisting of Fas, p53/p21, and drug resistance.

18. The composition of claim 14, wherein the induction of apoptosis in cancer cells is induction of caspase-3 activity in the cancer cells.

19. The composition of claim 1, wherein the anti-cancer activity of MCC is stimulation of cytokine production by cells of the immune system.

20. The composition of claim 19, wherein the cells of the immune system are selected from the group consisting of macrophages and monocytes.

21. The compositions of claim 19, wherein the cytokine is selected from the group consisting of IL-6, IL-10 and IL-12.

22. The composition of claim 14, wherein the anti-cancer activity of MCC is stimulation of reactive oxygen species production by cells of the immune system.

23. The composition of claim 22, wherein the cells of the immune system are macrophages.

24. A method of treating cancer in an animal, comprising administering to the animal in need of such treatment an amount of M-DNA and a first pharmaceutically acceptable carrier effective to treat the cancer in the animal.

25. The method of claim 24, wherein M-DNA inhibits proliferation of cancer cells in an animal to treat cancer in the animal.

26. The method of claim 24, wherein M-DNA induces apoptosis in cancers cells in an animal to treat cancer in the animal.

27. The method of claim 26, wherein the induction of apoptosis in cancer cells in an animal is independent of a factor selected from the group consisting of Fas, p53/p21 and drug resistance.

28. The method of claim 26, wherein the induction of apoptosis in cancer cells in an animal is induction of caspase 3 activity in cancer cells in the animal to treat cancer in the animal.

29. The method of claim 24, wherein M-DNA stimulates cytokine production by cells of the immune system in an animal to treat cancer in the animal.

30. The method of claim 29, wherein the cells of the immune system are selected from the group consisting of macrophages and monocytes.

31. The method of claim 30, wherein the cytokine is selected from the group consisting of IL-6, IL-10 and IL-12.

32. The method of claim 24, wherein M-DNA stimulates reactive oxygen species production by cells of the immune system in an animal to threat cancer in the animal.

33. The method of claim 32, wherein the cells of the immune system are macrophages.

34. The method of claim 24, wherein the first pharmaceutically acceptable carrier is selected from the group consisting of an aqueous carrier, a non-aqueous carrier and *M. phlei* cell wall.

35. The method of claim 34, wherein the first pharmaceutically acceptable carrier is *M. phlei* cell wall.

36. The method of claim 24, further comprising a second pharmaceutically acceptable carrier, wherein the second pharmaceutically acceptable carrier is selected from the group consisting of an aqueous carrier and a non aqueous carrier.

37. A method of treating cancer in an animal, comprising administering to the animal in need of such treatment an amount of a composition comprising M-DNA and *M. phlei* cell wall, wherein the M-DNA is preserved and complexed on the *M. phlei* cell wall (MCC), and a pharmaceutically acceptable carrier effective to treat the cancer in the animal.

38. The method of claim 37, wherein MCC inhibits proliferation of cancer cells in an animal to treat cancer in the animal.

39. The method of claim 37, wherein MCC induces apoptosis of cancers cells in an animal to treat cancer in the animal.

40. The method of claim 39, wherein the induction of apoptosis in cancer cells in an animal is independent of a factor selected from the group consisting of Fas, p53/p21 and drug resistance.

41. The method of claim 39, wherein the induction of apoptosis in cancer cells in an animal is induction of caspase 3 activity in cancer cells in the animal to treat cancer in the animal.

42. The method of claim 37, wherein MCC stimulates cytokine production by cells of the immune system in an animal to treat cancer in the animal.

43. The method of claim 42, wherein the cells of the immune system are selected from the group consisting of macrophages and monocytes.

44. The method of claim 43, wherein the cytokine is selected from the group consisting of IL-6, IL-10 and IL-12.

45. The method of claim 38, wherein MCC stimulates reactive oxygen species production by cells of the immune system in an animal to threat cancer in the animal.

46. The method of claim 45, wherein the cells of the immune system are macrophages.

47. The method of claim 37, wherein the pharmaceutically acceptable carrier is selected from the group consisting of an aqueous carrier and a non-aqueous carrier.

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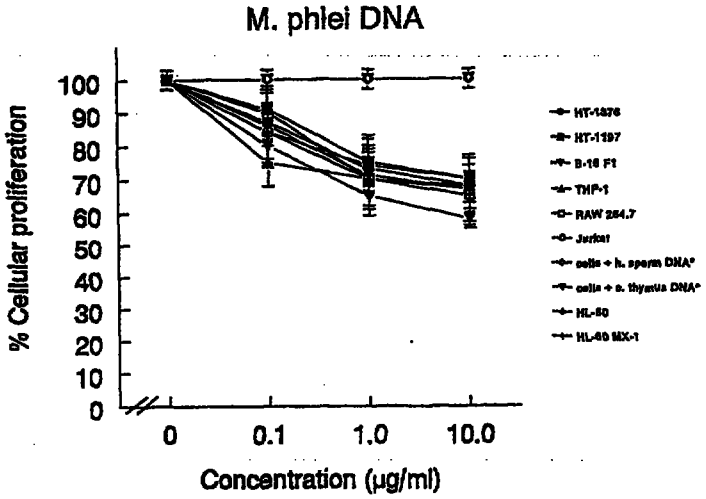
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1 appln.

2 Invs.

(54) Title: COMPOSITION AND METHOD FOR REGULATING CELL PROLIFERATION AND CELL DEATH




(57) Abstract

The present invention relates to a composition and method useful for regulating cell proliferation and cell death in a multicellular organism. The present invention particularly relates to a composition comprising a bacterial DNA (B-DNA) and a first pharmaceutically acceptable carrier, wherein the B-DNA induces a response in responsive cells of an animal. The present invention more particularly relates to a composition comprising a mycobacterial DNA (M-DNA) and a first pharmaceutically acceptable carrier, wherein the M-DNA inhibits proliferation of responsive cells of an animal, induces apoptosis in responsive cells of an animal, and stimulattes responsive cells of the immune system of an animal to produce bioactive molecules. Methods of making the M-DNA composition and methods of using the M-DNA composition also are disclosed.