HAPTEN-MODIFIED TUMOR CELL MEMBRANES, AND METHODS OF MAKING AND USING HAPTEN-MODIFIED TUMOR CELL MEMBRANES

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Background of the Invention

It was theorized in the 1960's that tumor cells bear specific antigens (TSA) which are not present on normal cells and that the immune response to these antigens might enable an individual to reject a tumor. It was later suggested that the immune response to TSA could be increased by introducing new immunological determinants on cells. Mitchison, *Transplant. Proc.*, **1970**, *2*, 92. Such a "helper determinant", which can be a hapten, a protein, a viral coat antigen, a transplantation antigen, or a xenogenous cell antigen, could be introduced into a population of tumor cells. The cells would then be injected into an individual who would be expected to be tolerant to the growth of unmodified tumor cells. Clinically, the hope was that an immunologic reaction would occur against the helper determinants, as a consequence of which the reaction to the accompanying TSA is increased, and tumor cells which would otherwise be tolerated are destroyed. Mitchison, *supra*, also suggests several modes of action of the helper determinants including 1) that the unmodified cells are merely attenuated, in the sense that their growth rate is slowed down or their susceptibility to immunologic attack increased; 2) that helper determinants merely provide points of attack and so enable the modified cells to be killed by an immune response not directed

against TSA; 3) that the helper determinants have an adjuvant action such as binding to an antibody or promoting localization of the cells in the right part of the body for immunization, in particular, in lymph nodes.

Fujiwara et al., *J. Immunol.*, 1984, *132*, 1571 showed that certain haptenized tumor cells, i.e., tumor cells conjugated with the hapten trinitrophenyl (TNP), could induce systemic immunity against unmodified tumor cells in a murine system, provided that the mice were first sensitized to the hapten in the absence of hapten-specific suppressor T cells. Spleen cells from the treated mice completely and specifically prevented the growth of tumors in untreated recipient animals. Flood et al., *J. Immunol.*, 1987, *138*, 3573 showed that mice immunized with a TNP-conjugated, ultraviolet light-induced "regressor" tumor were able to reject a TNP-conjugated "progressor" tumor that was otherwise non-immunologic. Moreover, these mice were subsequently resistant to challenge with unconjugated "progressor" tumor. In another experimental system, Fujiwara et al., *J. Immunol.*, 1984, *133*, 510 demonstrated that mice sensitized to trinitrochlorobenzene (TNCB) after cyclophosphamide pretreatment could be cured of large (10mm) tumors by *in situ* haptenization of tumor cells; subsequently, these animals were specifically resistant to challenge with unconjugated tumor cells.

The teachings of Fujiwara et al. differ from the present invention for several reasons including the following: A. The cells used in Fujiwara's composition are derived from induced transplantable murine tumors - not from spontaneous human tumors; B. Fujiwara's composition is used in immunoprophylaxis - the present invention uses immunotherapy; C. Fujiwara's composition is administered as a local therapy - the composition of the present invention is administered by systemic inoculation; D. Fujiwara's composition did not result in tumor regression - the composition of the present invention results in regression and/or prolonged survival for at least a substantial portion of the patients treated; and E. Fujiwara administers tumor cells - the present invention teaches administration of tumor cell membranes.

The existence of T cells which cross-react with unmodified tissues has recently been demonstrated. Weltzien and coworkers have shown that class I MHC-restricted T cell clones generated from mice immunized with TNP-modified syngeneic lymphocytes respond to MHC-associated, TNP-modified "self" peptides. Ortmann, B., et al., *J. Immunol.*, **1992**, *148*, 1445. In addition, it has been established that immunization of mice with TNP-modified lymphocytes results in the development of splenic T cells that exhibit secondary proliferative and cytotoxic responses to TNP-modified cells *in vitro*. Shearer, G. M. *Eur. J. Immunol.*, **1974**, *4*, 527. The potential of lymphocytes elicited by immunization with DNP- or TNP-modified autologous cells to respond to unmodified autologous cells is of considerable interest because it may be relevant to two clinical problems: 1) drug-induced autoimmune disease, and 2) cancer immunotherapy. In regard to the former, it has been suggested that ingested drugs act as haptens, which combine with normal tissue protein forming immunogenic complexes that are recognized by T cells. Tsutsui, H., et al., *J. Immunol.*, **1992**, *149*, 706. Subsequently, autoimmune disease, e.g., systemic lupus erythematosus, can develop and continue even after withdrawal of absence of the offending drug. This would imply the eventual generation of T lymphocytes that cross-react with unmodified tissues.

The common denominator of these experiments is sensitization with hapten in a milieu in which suppressor cells are not induced. Spleen cells from cyclophosphamide pretreated, TNCB-sensitized mice exhibited radioresistant "amplified helper function" i.e., they specifically augmented the *in vitro* generation of anti-TNP cytotoxicity. Moreover, once these amplified helpers had been activated by *in vitro* exposure to TNP-conjugated autologous lymphocytes, they were able to augment cytotoxicity to unrelated antigens as well, including tumor antigens (Fujiwara et al., 1984). Flood et al., (1987), *supra*, showed that this amplified helper activity was mediated by T cells with the phenotype $Lyt1^+$, $Lyt2^-$, $L3T4^+$, $I\text{-}J^+$ and suggests that these cells were contrasuppressor cells, a new class of immunoregulatory T cell.

Immunotherapy of patients with melanoma had shown that administration of cyclophosphamide, at high dose (1000 mg/M^2) or low dose (300 mg/M^2), three days before sensitization with the primary antigen keyhole limpet hemocyanin markedly augments the acquisition of delayed type hypersensitivity to that antigen (Berd et al., *Cancer Res.*, **1982**, *42*, 4862; *Cancer Res.*, **1984**, *44*, 1275). Low dose cyclophosphamide pretreatment allows patients with metastatic melanoma to develop delayed type hypersensitivity to autologous melanoma cells in response to injection with autologous melanoma vaccine (Berd et al., *Cancer Res.*, **1986**, *46*, 2572; *Cancer Invest.*, **1988**, *6*, 335). Cyclophosphamide administration results in reduction of peripheral blood lymphocyte non-specific T suppressor function (Berd et al., *Cancer Res.*, **1984**, *44*, 5439; *Cancer Res.*, **1987**, *47*, 3317), possibly by depleting CD4+, CD45R+ suppressor inducer T cells (Berd et al., *Cancer Res.*, **1988**, *48*, 1671). The anti-tumor effects of this immunotherapy regimen appear to be limited by the excessively long interval between the initiation of vaccine administration and the development of delayed type hypersensitivity to the tumor cells (Berd et al., *Proc. Amer. Assoc. Cancer Res.*, **1988**, *29*, 408 (#1626)). Therefore, there remains a need to increase the therapeutic efficiency of such a vaccine to make it more immunogenic.

Most tumor immunologists now agree that infiltration of T lymphocytes, white cells responsible for tumor immunity, into the tumor mass is a prerequisite for tumor destruction by the immune system. Consequently, a good deal of attention has been focused on what has become known as "TIL" therapy, as pioneered by Dr. Stephen Rosenberg at NCI. Dr. Rosenberg and others have extracted from human cancer metastases the few T lymphocytes that are naturally present and greatly expanded their numbers by culturing them *in vitro* with Interleukin 2 (IL2), a growth factor for T lymphocytes. Topalian et al., *J. Clin. Oncol.*, **1988**, *6*, 839. However this therapy has not been very effective because the injected T cells are limited in their ability to "home" to the tumor site.

The ability of high concentrations of IL2 to induce lymphocytes to become non-specifically cytotoxic killer cells has been exploited therapeutically in a number of

studies (Lotze et al., *J. Biol. Response*, **1982**, *3*, 475; West et al., *New Engl. J. Med.*, **1987**, *316*, 898). However, this approach has limitations due to the severe toxicity of high dose intravenous IL2. Less attention has been given to the observation that much lower concentrations of IL2 can act as an immunological adjuvant by inducing the expansion of antigen-activated T cells (Talmadge et al., *Cancer Res.*, **1987**, *47*, 5725; Meuer et al., *Lancet*, **1989**, *1*, 15). Therefore, there remains a need to understand and attempt to exploit the use of IL2 as an immunological adjuvant.

Human melanomas are believed to express unique surface antigens recognizable by T lymphocytes. Old, L. J., *Cancer Res.*, **1981**, *41*, 361; Van der Bruggen, P., et al., *Science*, **1991**, *254*, 1643; Mukherji, B., et al., *J. Immunol.*, **1986**, *136*, 1888; and Anichini, A., et al., *J. Immunol.*, **1989**, *142*, 3692. However, immunotherapeutic approaches prior to work done by the present inventor had been limited by the difficulty of inducing an effective T cell-mediated response to such antigens *in vivo*.

There are several models proposed to explain what appears to be tolerance to human tumor-associated antigens. They include:

1) Tumor antigen-specific suppressor cells that down-regulated incipient anti-tumor responses. Mukherji, et al., *supra*; Berendt, M. J. and R. J. North., *J. Exp. Med.*, **1980**, *151*, 69.

2) Failure of human tumor cells to elicit T helper cells or to provide costimulatory signals to those T cells. Fearon, E. R., et al., *Cell*, **1990**, *60*, 397; Townsend, S. E. and J. P. Allison, *Science*, **1993**, *259*, 368; and

3) Reduced surface expression of major histocompatibility products on tumor cells which limits their recognition by T cells. Ruiter, D. J., *Seminars in Cancer Biology*, **1991**, *2*, 35. None of these hypotheses has yet been corroborated in a clinical system.

Regardless of whether such explanations are true or not, there is a continuing need for more effective treatment of various malignancies.

In regard to acute myelogenous leukemia (AML), the treatment for AML is divided into one or two initial induction phases and several courses of post-remission,

also known as consolidation, chemotherapy. Initial induction chemotherapy may induce a complete response in 55 to 88% of the patients, depending on the protocol used. However, the vast majority of these patients relapse, and the long-term (5 year +) survival of AML patients is only 20-30%. The addition of high-dose chemotherapy and bone marrow transplantation (BMT) to this therapeutic regime during the first remission can bring about modest improvements in result. For example, patients undergoing allogeneic BMT are afforded a 5 to 10% increase in the 5 year survival. However, the strict eligibility criteria for BMT (*e.g.*, age, availability of an HLA-matched donor) severely limit the number of patients who can be treated. Once AML patients relapse, there is only a 30% chance of achieving a second remission, and very few of these patients remain disease-free in the long run. Treatment modalities on relapse include similar protocols to those used in achieving the first remission (induction therapy followed by several courses of consolidation chemotherapy), although high dose of a single agent and BMT can also be used (Keating et al).

Experience with bone marrow transplantation has suggested that immunological rejection may play a role in the control of the disease. Graft-versus-host disease (GVHD) and relapse are the two main causes of death of patients treated with BMT. The risk of relapse decreases if mild GVHD occurs (Horowitz et al). Therefore it has been hypothesized that grafted lymphocytes are able to immunologically reject host leukemia cells (graft-versus-leukemia reaction, GVL). This GVL reaction could be mediated by a T-cell response against specific leukemia cell antigens, although immunogenic human leukemia antigens have not yet been demonstrated (the same is true for melanoma). It is known that human AML cells strongly express both class I and class II major histocompatibility complex (MHC) antigens (Ashman et al; Andreasen et al) which are prerequisites for the induction of CD8- and CD4-mediated T cell responses, respectively. However, induction of a T cell response targeted to leukemia cells has not been successful.

Several immunological approaches have been used for the treatment of acute leukemia (Foon et al; Caron and Scheinberg). These approaches are divided into

non-specific, such as *Bacillus Calmette Guerin (BCG)*, interleukin-2, levamisole, methanol-extraction residue of tubercle bacillus, and specific, such as monoclonal antibodies and vaccines (harvested leukemia cells, cell free extracts and cultured cells). The majority of these studies have been performed in patients already in remission, in which immunotherapy would have to be successful in controlling residual disease.

In the late 1960's and early 1970's, the research group of R. Powles at St. Barthlomew's Hospital in England conducted a series of studies of vaccine treatment of AML patients after chemotherapy-induced remission (Powles, 1974; Powles *et al*, 1977). They used allogeneic AML cells with *BCG* as an adjuvant. Several trials were performed, all with small sample sizes (N=10-15). There was some prolongation of survival using a combination of chemotherapy and immunotherapy compared to chemotherapy alone, but no prolongation of relapse-free survival. No serious toxicity was observed; autoimmunity (*e.g.*, toxicity to normal bone marrow) was not seen. In retrospect, there were a number of technical problems with these trials: 1) allogeneic, rather than autologous, leukemia cells were used; 2) the dose of leukemia cells in the vaccine was excessive (up to 10^9 cells/dose); 3) the *BCG* dose was very high and *BCG* administration was separated by time and location from the leukemia cell vaccine; and 4) the vaccine was administered while the patients were receiving cytotoxic drugs (maintenance or consolidation chemotherapy).

The immunochemical basis of the limited success of the above-mentioned treatments remains speculative, but several hypotheses are being tested. Kim and Jang (1992) have suggested that the lack of T cell response to a particular epitope may not be due to absence of a T cell repertoire, but rather to difficulty in generating the particular epitope. Martin et al (1993) have explained their results by hypothesizing the existence of autoreactive T cells that escape thymic selection because of low affinity for "self" peptides.

Conventional attempts to treat human cancer have been unsuccessful. Administration of compositions, exemplified by those set forth above, failed to reliably

induce the development of cell-mediated immunity as indicated by delayed-type hypersensitivity (DTH), T cell infiltration, and inflammatory immune response.

Accordingly, despite the number of attempts based on various theories proposed for the immunological effects reported in the treatments of cancer, there remains a need for a composition which, upon administration to a mammal, is capable of eliciting T lymphocytes that infiltrate a tumor, eliciting an inflammatory immune response to a tumor, and eliciting a delayed-type hypersensitivity response to a tumor. Applicants have now surprisingly discovered that using membranes isolated from either syngeneic or allogeneic tumor cells have these desired properties.

Summary of the Invention

The present invention is directed to an isolated tumor cell membrane, a composition containing such membrane, methods for isolating and preparing the tumor cell membrane and compositions containing such membrane, and their use *in vitro* and for treating cancer. The tumor cell membrane, which may be hapten modified, is preferably a tumor cell plasma membrane that may be syngeneic or allogeneic. The syngeneic tumor cell membrane may be autologous. The cancer to be treated includes carcinomas and non-solid tumors, including leukemia (such as acute myelogenous leukemia), lymphoma, multiple myeloma, ovarian, colon, rectal, colorectal, melanoma, breast, lung, kidney, and prostate cancer.

In one aspect, the present invention relates to an isolated mammalian, preferably human, tumor cell membrane modified with a hapten. The hapten may be selected from the group consisting of dinitrophenyl, trinitrophenyl, N-iodoacetyl-N'-(5-sulfonic 1-naphthyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid benzene isothiocyanate, trinitrobenzenesulfonic acid, phosphorylcholine, sulfanilic acid, arsanilic acid and dinitrobenzene-S-mustard and combinations thereof.

In another aspect, the present invention is directed to a composition comprising a hapten modified mammalian tumor cell membrane, alone or in combination with a hapten modified mammalian tumor cell.

In yet another aspect, the invention provides for a vaccine composition comprising a therapeutically effective amount of a mammalian, preferably human, tumor cell membrane for administration to a mammal which suffers from a malignant tumor of the same type as the tumor cell membrane.

In another aspect of the invention, the composition contains an adjuvant, such as, for example, *Bacille Calmette-Guerin*, QS-21, detoxified endotoxin and cytokines such as interleukin-2, interleukin-4, gamma interferon (IFN-γ), interleukin-12, interleukin-15 and GM-CSF.

The membrane and the composition of the present invention, have (when administered to a mammal, preferably a human, suffering from a malignant tumor of the same type as the tumor cell from which the membrane was isolated) at least one of the following properties: (i) eliciting T lymphocytes that infiltrate the tumor of a treated mammal, (ii) eliciting an inflammatory immune response against the tumor of the mammal, and (iii) eliciting a delayed-type hypersensitivity response to the tumor of the mammal. The membrane and the composition of the invention also have the property of stimulating T cells *in vitro*.

In yet another aspect, the present invention is directed to a method of treating cancer comprising administering to a mammal, preferably a human, a composition comprising a therapeutically effective amount of a hapten modified human tumor cell membrane wherein said mammal suffers from a malignant tumor of the same type as said tumor cell membrane.

Yet further, the present invention relates to a method of eliciting T lymphocytes that have a property of infiltrating said tumor of said mammal, preferably a human, and, optionally, measuring said T lymphocytes that infiltrate said tumor of said mammal. The invention further relates to a method of eliciting an inflammatory immune response to said tumor of said mammal and, optionally, measuring said

inflammatory immune response, or to a method of eliciting a delayed-type hypersensitivity response to said tumor of said mammal and, optionally, measuring said delayed-type hypersensitivity response. The invention also relates to a method of stimulating T cell *in vitro*.

In yet further aspect of the invention, the invention relates to a method of making a hapten-modified tumor cell membrane.

Detailed Description of the Invention

All patents, patent applications and references cited herein are hereby incorporated by reference. In case of inconsistencies, the present disclosure governs.

The present invention is directed to an isolated modified and un-modified tumor cell membrane, a novel composition containing such membrane, methods of isolating and making the membrane and compositions containing such membrane, and methods for using the membrane and compositions of the invention.

The membranes and compositions of the present invention may be used for treating cancer in a mammal, preferably a human, including metastatic and primary cancers, solid and non-solid cancers such as, for example, rectal, colorectal, melanoma, breast, lung, kidney, and prostate cancers. Stage I, II, III, or IV cancer may be treated with the isolated modified membranes, the compositions and methods of the present invention, preferably stages III and IV, even more preferably stage III. Mammals, particularly humans, having metastatic cancer of the foregoing type may be treated with the membranes, the compositions and methods of the present invention. In one embodiment, the present invention is used to treat domestic animals such as, for example, members of feline, canine, equine and bovine families.

The membranes and compositions of the invention may also be used for eliciting T lymphocytes that have a property of infiltrating a mammalian tumor, eliciting an inflammatory immune response to a mammalian tumor, eliciting a delayed-type hypersensitivity response to a mammalian tumor and/or stimulating T lymphocytes *in vitro*.

It will be understood that any disclosure in this specification with respect to use of isolated tumor cells equally applies to use of tumor cells membranes, or to a combination of tumor cells and tumor cell membranes.

TUMOR CELLS MEMBRANES AND COMPOSITIONS THEREOF

The isolated, modified tumor cell membranes of the present invention are prepared from mammalian, preferably human, tumor cells. In one embodiment of the invention, tumor cell membrane are isolated from a tumor of an animal from a feline, canine, equine or bovine family.

Included within the definition of a tumor cell for purposes of the present invention are whole and disrupted tumor cells. The tumor cells from which membranes are isolated may be live, attenuated, or killed cells. Tumor cells which do not grow and divide after administration into the subject such that they are substantially in a state of no growth can be used in the present invention. Such cells are preferred if they are administered to the patient alone or in combination with isolated tumor cell membranes. It is to be understood that "cells in a state of no growth" means live, attenuated or killed, whole or disrupted (or both whole and disrupted) cells that do not divide *in vivo*. Conventional methods of suspending cells in a state of no growth are known to skilled artisans and may be useful in the present invention. For example, cells may be irradiated prior to use such that they do not grow and divide. Tumor cells may be irradiated, for example at 2500 R, to prevent the cells from growing after administration. Alternatively, tumor cell membranes may also be isolated from tumor cells that can grow and divide *in vivo*. Preferably, in such a case, the tumor cell membrane preparation is not contaminated with tumor cells that are capable of dividing *in vivo*.

Tumor cell membranes are isolated from the tumor cells of the same type as the cancer to be treated. For example, membranes to be used for treating ovarian cancer are isolated from ovarian cancer cells. Preferably, the tumor cells originate from the same subject who is to be treated. The tumor cells are preferably syngeneic (e.g.

autologous), but may also be allogeneic to that subject. To be defined as "syngeneic," the tumor cell need not be completely (*i.e.*, 100 %) genetically identical to either the tumor cell or the non-tumor, somatic cell of the treated patient. Genetic identity of the MHC molecules between the tumor cell (from which membranes are isolated) and the patient is generally sufficient. Additionally, there may be genetic identity between a particular antigen on the tumor cell used as a membrane source and an antigen present on patient's tumor cells. Genetic identity may be determined according to the methods known in the art. A syngeneic tumor cell also means a cell that has been genetically altered (using for example recombinant DNA technology) to become genetically identical with respect to, for example, the particular MHC molecules of the patient and/or the particular antigen on the patient's cancer cells. Tumor cells from animals of the same species that differ genetically, such as allogeneic cells, may also be used for the preparation of tumor cell membranes of the invention. The tumor cells may be, and are not limited to, cells dissociated from biopsy specimens or from tissue culture. Membranes isolated from allogeneic cells and stem cells are also within the scope of the present invention.

Tumor cell membranes may include all cellular membranes, such as outer membrane, nuclear membranes, mitochondrial membranes, vacuole membranes, endoplasmic reticular membranes, golgi complex membranes, and lysosome membranes. In one embodiment of the invention greater than about 50% of the membranes are tumor cell plasma membranes. Preferably, greater than about 60% of the membranes consist of tumor cell plasma membranes, with greater than about 70% being more preferred, 80% being even more preferred, 90% being even more preferred, 95% being even more preferred, and 99% being most preferred.

Preferably, the isolated membranes are substantially free of nuclei and cells. For example, a membrane preparation is substantially free of nuclei or cells if it contains less than about 100 cells and/or nuclei in about 2×10^8 cell equivalents (c.e.) of membrane material. A cell equivalent is that amount of membrane isolated from the indicated

number of cells. An isolated tumor cell membrane which is substantially free of cells and/or nuclei may contain lymphocytes and/or lymphocyte membranes.

Preferably, the isolated tumor cell membranes are the outer cell membranes, *i.e.*, tumor cell plasma membranes. The membrane preparation of the invention may contain the entire outer membrane or a fraction thereof. An isolated membrane of the invention containing a fraction of the outer membrane contains at least an MHC molecule fraction and/or a heat shock protein fraction of the outer membrane. The size of membrane fragments is not critical.

Allogeneic tumor cell membranes may also be used in the methods of the present invention with syngeneic (*e.g.* autologous) antigen presenting cells. This approach permits immunization of a patient with tumor cell membranes originating from a source other than the patient's own tumor. Syngeneic antigen-presenting cells process allogeneic membranes such that the patient's cell-mediated immune system may respond to them.

The isolated tumor cell membranes as well as tumor cells may be modified, for example, with a hapten. Such modified tumor cell membranes (and tumor cells) have at least one of the following properties: (i) eliciting T lymphocytes that infiltrate the tumor of a treated mammal, (ii) eliciting an inflammatory immune response against the tumor of the mammal, and (iii) eliciting a delayed-type hypersensitivity response to the tumor of the mammal. Modified tumor cell membranes and cells also have the property of stimulating T cells *in vitro*.

A tumor cell membrane (modified or un-modified) as referred to in this specification includes any form in which such membrane preparation may be stored or administered such as, for example, a membrane resuspended in a diluent, a membrane pellet, or a frozen or a lyophilized membrane.

The membranes of the invention may be employed in the methods of the invention singly or in combination with other compounds, including and not limited to other compositions of the invention. Accordingly, tumor cells and tumor cell membranes may be used alone or co-administered. For purposes of the present invention, co-

administration includes administration together and consecutively. Further, the tumor cells and tumor cell membranes may be co-administered with other compounds including and not limited to cytokines such as interleukin-2, interleukin-4, gamma interferon (IFN-γ), interleukin-12, interleukin-15 and GM-CSF. The tumor cells and tumor cell membranes of the invention may also be used in conjunction with other cancer treatments including and not limited to chemotherapy, radiation, antibodies, and antisense oligonucleotides. However, it is the advantage of the present invention that it can be useful alone as a cancer treatment, such that the need for additional therapies is unnecessary.

A composition of the present invention may contain the isolated tumor cell membrane of the invention (modified or unmodified) and a pharmaceutically acceptable carrier or diluent, such as and not limited to Hanks solution, saline, phosphate-buffered saline, sucrose solution, and water. In general, the pharmaceutically-acceptable carrier is selected with regard to the intended route of administration and the standard pharmaceutical practice. The proportional ratio of active ingredient to carrier naturally depends on the chemical nature, solubility, and stability of the compositions, as well as the dosage contemplated and can be optimized using common knowledge in the art.

In one preferred embodiment of the invention, a composition of the invention is a vaccine composition containing an effective amount of isolated modified tumor cell membrane. For purposes of this disclosure, "an effective amount" is the amount necessary to achieve a desired result. For example, in a method for treating cancer, "an effective amount" means that amount of isolated modified tumor cell membranes that has the property of causing at least one of the following: (i) eliciting T lymphocytes that infiltrate tumor, (ii) eliciting an inflammatory response against the tumor, (iii) eliciting a delayed-type hypersensitivity response to a tumor, and (iv) tumor regression. Similarly, in a method for stimulating T cells *in vitro*, "an effective amount" is that amount of membranes that results in T cell stimulation.

The vaccine composition may contain, for example, at least 10^4 c.e. of isolated membranes per dose, preferably at least 10^5 c.e., and most preferably at least 10^6 c.e. A

dose is that amount of the vaccine composition that is administered in a single administration. In one embodiment, the vaccine composition contains from about 10^5 to about 2.5×10^7 c.e. membranes per dose, more preferably about 5×10^6 c.e. The amount of the tumor cells and tumor cell membranes of the invention to be used generally depends on such factors as the affinity of the compound for cancerous cells, the amount of cancerous cells present and the solubility of the composition. Dosages may be set with regard to weight, and clinical condition of the patient.

A vaccine composition of the invention may be packaged in a dosage form suitable for intradermal, intravenous, intraperitoneal, intramuscular, and subcutaneous administration. Alternatively, the dosage form may contain isolated tumor cell membranes to be reconstituted at the time of the administration with, for example, a suitable diluent.

HAPTEN

The tumor cells and tumor cell membranes of the present invention may be used as modified, unmodified, or a combination of modified and unmodified tumor cells and tumor cell membranes. For purposes of the present invention, modified includes and is not limited to modification with a hapten. Any small molecule that does not alone induce an immune response (but that enhances immune response against another molecule to which it is conjugated or otherwise attached) may function as a hapten. Generally, the molecule used should have less than about 1,000 mw.

A variety of haptens are known in the art such as for example: TNP (Kempkes *et al.*, *J. Immunol.* **1991** *147*:2467); phosphorylcholine (Jang *et al.*, *Eur. J. Immunol.* **1991** *21*:1303); nickel (Pistoor *et al.*, *J. Invest. Dermatol.* **1995** *105*:92); arsenate (Nalefski and Rao, *J. Immunol.* **1993** *150*:3806).

Generally, haptens suitable for use in the present invention have the property of binding to a hydrophilic amino acid (such as for example lysine). Hapten can be conjugated to a cell via ε-amino groups of lysine or -COOH groups. Additionally, hapten that can bind to hydrophobic amino acids such as tyrosine and histidine *via* diaza

coupling can also be used. Examples of haptens suitable for use in the present invention are: dinitrophenyl, trinitrophenyl, N-iodoacetyl-N'-(5-sulfonic 1-naphthyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid benzene isothiocyanate, trinitrobenzenesulfonic acid, phosphorylcholine, sulfanilic acid, arsanilic acid, dinitrobenzene-S-mustard (Nahas and Leskowitz, *Cellular Immunol.* **1980** *54*:241) and combinations thereof. Once armed with the present disclosure, skilled artisans, would be able to choose haptens for use in the present invention. For example, haptens can be routinely tested using a delayed type hypersensitivity (DTH) test.

ADJUVANT

In one preferred embodiment, the tumor cell or tumor cell membrane is administered with an immunological adjuvant. The adjuvant has the property of augmenting an immune response to hapten modified tumor cells and membranes. Representative examples of adjuvants are *Bacille Calmette-Guerin, BCG,* or the synthetic adjuvant, QS-21 comprising a homogeneous saponin purified from the bark of *Quillaja saponaria, Corynebacterium parvum,* McCune *et al., Cancer* **1979** *43*:1619, saponins in general, detoxified endotoxin and cytokines such as interleukin-2, interleukin-4, gamma interferon (IFN-γ), interleukin-12, interleukin-15, GM-CSF and combinations thereof.

It will be understood that the adjuvant may be subject to optimization. In other words, the skilled artisan may use routine experimentation to determine the most optimal adjuvant to use.

METHODS OF MAKING TUMOR CELL MEMBRANES OF THE INVENTION

The tumor cells for use in the present invention may be prepared as follows. Tumors are processed as described by Berd et al. (1986), *supra,* Sato, *et al.* (1997), U.S. Patent No. 5,290,551, and applications U.S. Serial Nos. 08/203,004, 08/479,016, 08/899,905, 08/942,794, or corresponding PCT application

PCT/US96/09511, each of which is incorporated herein by reference in its entirety. Briefly, the cells are extracted by dissociation, such as by enzymatic dissociation with collagenase and DNase, by mechanical dissociation in a blender, by teasing with tweezers, using mortar and pestle, cutting into small pieces using a scalpel blade, and the like. With respect to liquid tumors, blood or bone marrow samples may be collected and tumor cells isolated by density gradient centrifugation.

Tumor cell membranes are prepared from tumor cells by disrupting the cells using, for example, hypotonic shock, mechanical dissociation and enzymatic dissociation, and separating various cell components by centrifugation. Briefly, the following steps may be used: lysing tumor cells, removing nuclei from the lysed tumor cells to obtain nuclei-free tumor cells, obtaining substantially pure membranes free from cells and nuclei, and subjecting the tumor cell membranes to a hapten to obtain hapten-modified tumor cell membranes. Membrane isolation may be conducted in accordance with the methods of Heike *et al.*

In one embodiment of the invention, intact cells and nuclei may be removed by consecutive centrifugation until membranes are substantially free of nuclei and cells, as determined microscopically. For example, lysed cells may be centrifuged at low speed, such as for example, at about 500-2,000 *g* for about five minutes. The separation procedure is such that less than about 100 cells and/or nuclei remain in about 2 x 10^8 cell equivalents (c.e.) of membrane material. The postnuclear supernatant containing membranes may be pelleted by ultracentrifugation at about 100,000 *g* for about 90 minutes, for example. The pellet contains total membranes. Membranes may be resuspended, for example, in about 8% sucrose, 5 mM Tris, pH 7.6 and frozen at about -80°C until use. Any diluent may be used, preferably one that acts as a stabilizer. To determine the quality of membrane preparation (about 6 x 10^7 c.e. membranes) may be regularly cultured. Cell colonies should not develop and cells or nuclei should not be detected by light microscopy.

Modification of the prepared cells or membranes with DNP or another hapten may be performed by known methods, e.g. by the method of Miller and Claman, *J.*

Immunol., **1976**, *117*, 1519, incorporated herein by reference in its entirety, which involves a 30 minute incubation of tumor cells or membranes with a hapten under sterile conditions, followed by washing with sterile saline. The hapten-modification may be confirmed by flow cytometry using a monoclonal anti-hapten antibody.

The dissociated cells or isolated membranes may be used fresh or stored frozen, such as in a controlled rate freezer or in liquid nitrogen until needed. The cells and membranes are ready for use upon thawing. Preferably, the cells or membranes are thawed shortly before they are to be administered to a patient. For example, on the day that a patient is to be skin tested or treated, the cells or membranes may be thawed. Optionally, the cells or membranes may be washed, and optionally irradiated to 2500 R. They may be washed again and then suspended in Hanks balanced salt solution without phenol red.

Allogeneic tumor cell membranes may be prepared as described above. However, prior to administration to a subject they are co-incubated with syngeneic (*e.g.* autologous) antigen presenting cells. Syngeneic antigen-presenting cells process allogeneic membranes such that the patient's cell-mediated immune system may respond to them. This approach permits immunization of a patient with tumor cell membranes originating from a source other than the patient's own tumor. Allogeneic tumor cell membranes are incubated with antigen-presenting cells for a time period varying from about several hours to about several days. The membrane-pulsed antigen presenting cells are then washed and injected into the patient.

Antigen-presenting cells may be prepared in a number of ways including for example the methods of Grabbe et al., 1995 and Siena et al., 1995. Briefly, blood is obtained, for example by venipuncture, from the patient to be immunized. Alternatively, bone marrow may be obtained. Alternatively, blood leukocytes may be obtained by leukapheresis. From any of these sources, mononuclear leukocytes are isolated by gradient centrifugation. The leukocytes may be further purified by positive selection with a monoclonal antibody to the antigen, CD34.

The purified leukocytes are cultured and expanded in tissue culture medium (for example, RPMI-1640 supplemented with serum, such as fetal calf serum, pooled human serum, or autologous serum). Alternatively, serum-free medium may be used. To stimulate the growth of antigen-presenting cells, cytokines may be added to the culture medium. Cytokines include and are not limited to granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin 4 (IL4), TNF (tumor necrosis factor), interleukin 3 (IL3), FLT3 ligand and granulocyte colony stimulating factor (G-CSF).

The antigen-presenting cells isolated and expanded in culture may be characterized as dendritic cells, monocytes, macrophages, and Langerhans cells, for example.

METHODS OF USING TUMOR CELL MEMBRANES AND COMPOSITIONS

Method for Treating Cancer The present invention relates to a method of treating a mammal, preferably a human, diagnosed with or suspected of having cancer by administering a pharmaceutically acceptable amount of a hapten modified tumor cell membrane, hapten modified tumor cell, or a combination thereof. The membranes and/or cells may be mixed with an immunological adjuvant and/or a pharmaceutically acceptable carrier. A pharmaceutically acceptable amount of a low-dose cyclophosphamide or another low-dose chemotherapy may be administered preceding the administration of the composition. The haptenized composition may optionally be followed by administration of a pharmaceutically acceptable amount of a non-haptenized tumor cell or tumor cell membrane. A non-haptenized composition may also be administered in accordance with the methods of the present invention.

Any malignant tumor may be treated according to the present invention including metastatic and primary cancers and solid and non-solid tumors. Solid tumor include carcinomas, and non-solid tumors include hematologic malignancies. Carcinomas include and are not limited to adenocarcinomas and epithelial carcinomas. Hematologic malignancies include leukemias, lymphomas, and multiple myelomas. The following are non-limiting examples of the cancers treatable with isolated modified

tumor cell membranes according to the methods of the present invention: ovarian, including advanced ovarian, leukemia, including and not limited to acute myelogenous leukemia, colon, including colon metastasized to liver, rectal, colorectal, melanoma, breast, lung, kidney, and prostate cancers. The ovarian cancers may be adenocarcinomas or epithelial carcinomas. Colon and prostate cancers are adenocarcinomas. Leukemias may originate from myeloid bone marrow or lymph nodes. Leukemias may be acute, exhibited by maturation arrest at a primitive stage of development, and chronic, exhibited by excess accrual of mature lymphoid or myeloid cells. Stage I, II, III, or IV cancer may be treated according to the present invention, preferably stages III and IV, even more preferably stage III.

Mammals, particularly humans, having metastatic cancer of the foregoing type may be treated with the membranes, the compositions and methods of the present invention. In one embodiment of the invention, domestic animals may be treated.

Prior to administration of the vaccine composition of the invention, the subject may be immunized to the hapten which is to be used to modify tumor cells and membranes by applying it to the skin. For example, dinitrofluorobenzene (DNFB) may be used. Subsequently (about two weeks later, for example), the subject may be injected with a tumor cell membrane composition. The composition may be administered (such as by reinjection) for a total of at least three and preferably at least six treatments. In one embodiment, the total number of administrations (including the initial administration) may be eight, and in another embodiment may be ten. The vaccination schedule may be designed by the attending physician to suit the particular subject's condition. The vaccine injections may be administered, for example, every 2 weeks, and preferably every week. A booster vaccine may be administered. Preferably, one or two booster vaccines are administered. The booster vaccine may be administered, for example, after about six months or about one year after the initial administration.

The immune response of the subject may be augmented with drugs. For example, cyclophosphamide (CY) may be administered prior to each administration.

The present invention may be used following conventional treatment for cancer, such as surgery. In case of solid tumors, such as ovarian cancer, the tumor may be optimally or sub-optimally debulked. Optimally debulked refers to excising the tumor so that only small tumor pieces remain in the treated subject.. Sub-optimally debulked refers to excising the tumor while large pieces remain in the subject. In the case of non-solid tumors, an appropriate blood or bone marrow sample may be collected, and cancer cells isolated by known techniques any reference. Excised tumors or collected tumor cells may be used to prepare tumor cell membranes as described above.

Tumor cell membranes may be administered by any suitable route, including inoculation and injection, for example, intradermal, intravenous, intraperitoneal, intramuscular, and subcutaneous. There may be multiple cites of administration per each vaccine treatment. For example, the vaccine composition may be administered by intradermal injection into at least two, and preferably three, contiguous sites per administration. In one embodiment of the invention, the vaccine composition is administered on the upper arms or legs.

The effectiveness of the vaccine may be improved by administering various biological response modifiers. These agents work by directly or indirectly stimulating the immune response. Biological response modifiers of the present invention include and are not limited to interleukin-12, interleukin-15 and gamma interferon. In one embodiment, IL12 is given following each vaccine injection. Administration of IL12 to patients with inflammatory responses may cause the T lymphocytes within the tumor mass to proliferate and become more active. The increased T cell numbers and functional capacity leads to immunological destruction and regression of the tumors.

Human cancer vaccines have been developed and tested by a number of workers. Although they can sometimes induce weak immunity to a patient's cancer, they rarely cause tumor regression or prolong survival. Evidence of an inflammatory response was surprisingly found with the vaccine of the present invention. Microscopically, infiltration of T lymphocytes is observed. Therefore, this approach, which increases the inflammatory response and the number of lymphocytes, is a significant advance in the

art. Therefore, the present invention also provided for methods of eliciting T cells that have at least one of the following properties: (i) eliciting T lymphocytes that infiltrate the tumor of a treated mammal, (ii) eliciting an inflammatory immune response against the tumor of the mammal, and (iii) eliciting a delayed-type hypersensitivity response to the tumor of the mammal when administered.

Method for Stimulating T Cells Isolated tumor cell membranes may be used to stimulate T cells *in vitro*. This assay can be used to assess, for example, whether a therapy using a particular tumor membrane is likely to be successful. T cell for use in this assay may be obtained according to the following method which is described in humans but may be applied to any mammalian subject.

T cells are generated by administering to a patient diagnosed with cancer of a certain type, a pharmaceutically acceptable amount of a composition comprising hapten-modified tumor cells, tumor cell membranes, or a combination thereof. The composition may optionally contain an immunological adjuvant and/or a pharmaceutically acceptable carrier. A pharmaceutically acceptable amount of a low-dose cyclophosphamide or another low-dose chemotherapy, such as and not limited to melphalan, about 5 to about 10mg/M^2, may optionally be administered preceding the administration of the first tumor cell composition. The haptenized composition may optionally be followed by administration of a pharmaceutically acceptable amount of a non-haptenized vaccine composition containing non-haptenized membranes, tumor cells or a combination thereof. A non-haptenized composition may be administered in accordance with the methods of the present invention.

Peripheral blood lymphocytes (PBL) may be obtained from patients who develop a strong delayed type hypersensitivity (DTH) reaction to hapten-modified autologous cells or membranes following administration thereof. The DTH reaction preferably has a diameter of about 10 mm, even more preferably of about greater than 10 mm. A T cell line may be established from PBL by repeated stimulation with hapten-modified cancer cells. The T cells can be isolated by known techniques, such as preparation of single cell suspension, filtration, depletion of monocytes and isolation of a subset expressing a

particular T cell receptor (TCR) type by causing that subset to expand in the presence of TCR-subtype specific antibody and/or in the presence of IL-2 and/or in the presence of a superantigen. The T cells of interest may be expanded *in vivo* since they are collected from infiltrates from or within the tumor which are already enriched in the T cells of interest.

The modified tumor cells and tumor cell membranes each have the property of stimulating T cells. "Stimulation" for purposes of the present invention refers to inducing proliferation of T cells as well as production of cytokines by T cells *in vitro*. Membranes and tumor cells each independently have the ability to stimulate T cells. Proliferation of T cells may be detected and measured by the uptake by T cells of modified nucleotides, such as and not limited to ^{3}H thymidine, $^{125}IUDR$ (iododeoxyuridine); and dyes such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) which stains live cells. In addition, production of cytokines such as and not limited to IFNγ, tumor necrosis factor (TNF), and IL-2 may be useful in exhibiting T cell proliferation. Production of cytokines may be detected and measured using tests well known in the art. Cytokine production should be above the background level, which is generally above 25 picograms/ml, and is preferably above 100 picograms/ml.

T cells are lymphocytes which mediate two types of immunologic functions, effector and regulatory, secrete proteins (lymphokines), and kill other cells (cytotoxicity). Effector functions include reactivity such as delayed type hypersensitivity, allograft rejection, tumor immunity, and graft-versus-host reactivity. Lymphokine production and cytotoxicity are demonstrated by T cell effector functions. Regulatory functions of T cells are represented by their ability to amplify cell-mediated cytotoxicity by other T cells and immunoglobulin production by B cells. The regulatory functions also require production of lymphokines. T cells produce gamma interferon (IFNγ) in response to an inducing stimulus including and not limited to mitogens, antigens, or lectins.

In one embodiment of the invention, a T cell line may be developed as follows. PBL (1×10^{6}) are mixed with autologous DNP-conjugated B lymphoblastoid cells (1×10^{5}) in 24 well flat bottom plates in lymphocyte culture medium. After 7 days of culture,

IL2 100 U/ml (Cetus Oncology, Emeryville, CA) is added. Expanding T cell cultures are maintained in medium containing IL2 and are split as needed to maintain a concentration of about 2 x 10^6 cells in a 22 mm diameter well. Every 14 days, the cultures are restimulated by adding autologous DNP-conjugated B lymphoblastoid cells. Phenotypes may be determined by flow cytometry with a panel of monoclonal antibodies (Becton-Dickinson, San Jose, CA). Separation of CD8+ and CD4+ T cells is accomplished by indirect panning in which T cells coated with anti-CD8 or anti-CD4 monoclonal antibodies are adhered to anti-immunoglobulin-coated dishes using standard techniques according to the methods of Wysocki, L. J. and V. L. Sato, *Proc. Natl. Acad. Sci. USA*, **1978**, *75*, 2844, incorporated herein by reference in its entirety; the adherent cells are isolated and expanded with DNP-modified stimulators, including and not limited to those set forth below, melanoma cells and β lymphoblastoid cells; and IL2.

Phenotypically homogeneous subpopulations of T cells are obtained, for example, by culturing at limiting dilution in round-bottom microtiter wells in lymphocyte culture medium containing 2 x 10^5 irradiated allogeneic feeder cells, 200 U/ml IL2, and phytohemagglutinin. Wells with growing lymphocyte colonies are screened for ability to proliferate in response to DNP-modified B lymphoblastoid cells. Positive wells are expanded in IL2 and restimulated with autologous DNP-conjugated B lymphoblastoid cells every 14 days.

Peripheral blood lymphocytes (PBL) may be tested as responder cells. They are suspended in lymphocyte culture medium (RPMI-1640, 10% pooled human AB^+ serum, insulin-transferrin-selenite media supplement (Sigma Chemical Co.) 2 mM L-glutamine, 1% non-essential amino acids, 25 mM HEPES buffer, penicillin + streptomycin) and added to 96-well, round bottom microtiter plates at 1 x 10^5 cells/well. Stimulator cells, including: 1) autologous or allogeneic PBL, 2) autologous or allogeneic B lymphoblastoid lines made by transfection with Epstein-Barr virus, 3) autologous cultured melanoma cells; inactivated by irradiation (5000 R), are also added. In most experiments, the responder:stimulator ratio is preferably 1:1. The plates are incubated in a CO_2 incubator at 37°C for 5 days; then the wells pulsed with ^{125}I-labeled IUDR (ICN

Radiochemical, Costa Mesa, CA) for 6 hours, harvested with an automatic harvesting device, and counted in a gamma counter. The mean of triplicate wells is calculated. Cultured T cells are also tested for a lymphoproliferative response in accordance with the above methods.

PBL, obtained and cryopreserved from patients at the time of maximum DTH reactivity to DNP-modified autologous cells, are thawed and tested for *in vitro* proliferative responses. DNFB application alone does not result in detectable numbers of circulating responding cells. Reactive PBL are expected to be detected after two injections of DNP-vaccine (day 63) and will continue to be detected throughout the period of vaccine treatment, based on prior experience with DNP-modified melanoma cells.

To test for cytokine production, T cells may be added to round bottom microtiter plates at about 1 x 10^5 cells/well. An equal number of stimulators (DNP-modified autologous B lymphoblastoid cells) is added, and supernatants are collected after 18 hours incubation. Commercially available ELISA kits are used to measure gamma interferon (Endogen, Boston, MA; sensitivity = 5 pg/ml).

To determine the MHC-dependence of the response, stimulator cells may be pre-incubated with monoclonal antibodies to MHC class I (W6/32) or MHC class II (L243) at a concentration of 10 µg/ml for one hour before adding responder cells. Non-specific mouse immunoglobulin at the same concentration may be tested as a negative control.

DNP-reactive CD8+ T cells obtained by panning of the bulk population are able to be maintained in long-term (> 3 months) culture in IL2-containing medium by repeated stimulation with DNP-modified autologous B lymphoblastoid cells; they retained the stable phenotype, CD3+, CD8+. Two lines of evidence suggest that their response will be MHC class I restricted: 1) Gamma interferon production will be blocked by pre-incubation of stimulator cells with anti-class I framework antibody, but not by anti-class II antibody, 2) The T cells will be able to respond to allogeneic DNP-modified stimulators that are matched at one or both HLA-A loci, but not to stimulators that are HLA-A mismatched.

To test for gamma interferon production by T cells, lymphocytes from a patient's blood may be obtained. About 1,000,000 lymphocytes are mixed with DNP modified autologous melanoma cell membranes to stimulate T cells. Every seven days, 100 U/ml of interleukin-2 may be added. The T cells are expanded by passage. The T cells are then restimulated by the DNP modified autologous melanoma cell membranes. An enriched population of T cells results which are responsive to the DNP modified autologous melanoma cells. Stimulation is determined by the amount of gamma interferon production by the T cells. Generally the production of gamma interferon at greater than 15 picograms/ml is considered significant.

The invention is further illustrated by means of the following Examples which is meant to be an illustration only and is not intended to limit the present invention to these specific embodiments.

Example 1 ***In vitro*** **Stimulation of T Cells by Isolated Melanoma Membranes**

Establishment of T Cell Line Peripheral blood lymphocytes (PBL) were obtained from a patient who developed a strong delayed type hypersensitivity (DTH) reaction to DNP-modified autologous melanoma cells following DNP-vaccine administration according to the present method. PBL were separated from blood by density gradient centrifugation, suspended in freezing medium, such as RPMI-1640 with 2.5% human albumin and DMSO, frozen in a control-rate freezer, and stored in liquid nitrogen until use (Sato *et al.*, 1995).

A T cell line was established from these PBL by repeated stimulation with DNP-modified autologous melanoma cells (DNP-Mel) and maintained with recombinant interleukin 2 (IL-2) (Sato *et al.*, 1995).

Melanoma Cells Melanoma cells were enzymatically extracted as described above from metastatic masses surgically removed from the same patient and cryopreserved by a previously described method (Sato *et al.*, 1997). An autologous melanoma cell line was established from the melanoma cell suspension. Briefly, melanoma cells were enzymatically dissociated from metastatic masses and suspended

in tissue culture medium (RPMI-1640 with fetal calf serum or human serum) and added to tissue culture plates. After several days, non-adherent tumor cells were removed and fresh medium was added. After several weeks, adherent melanoma cells began to rapidly proliferate. When the cells grew to confluence on the culture plate, they were split by removing the cells with EDTA and adding to fresh tissue culture plates.

The melanoma cell line cells were modified with DNP by the method of Miller and Claman. This involves a 30 minute incubation of tumor cells with dinitrofluorobenzene (DNFB, Sigma Chemical Co.) under sterile conditions, followed by washing out of excess DNFB with Hanks solution. The DNP-modification was confirmed by flow cytometry with a mouse monoclonal anti-DNP antibody (SPE-7; Sigma Immunochemicals, St. Louis, MO) (100 % of the cells were shown to be modified with DNP).

As an alternative procedure, cryopreserved melanoma cells were modified with DNP as described above without the intervening step of establishing a cell line.

Cell Membrane Extraction Cell membranes were extracted from DNP-modified melanoma cells (DNP-Mel) by the methods of Heike *et al.* Briefly, DNP-modified cells were lysed by hypotonic shock in 5 volumes of 30 mM sodium bicarbonate buffer, 1 mM phenyl methyl sulfonyl fluoride (PMSF), and by Dounce homogenization (10-20 strokes). Residual intact cells and nuclei were removed by consecutive centrifugation at 1,000 g for 5 minutes, until supernatant was free of nuclei and cells. Then, the membranes were pelleted by centrifugation at 100,000 g for 90 minutes. The total membranes in the pellet were resuspended in 8 % sucrose, 5 mM Tris, pH 7.6 at 10^7 cell equivalent units (*i.e.*, membranes extracted from 10^7 cells)/ml and frozen at -80° C until use.

As an alternate procedure, cell membranes were isolated from unmodified melanoma cells in an identical manner as described above. The membranes were suspended in Hanks solution without albumin at various cell equivalent concentrations (from 10^5 - 10^9 cell equivalents/ml). Then DNFB was added as described above. Then,

the membranes were pelleted by centrifugation at 100,000 g for 90 minutes and washed twice with saline.

Cytokine Production in Response to Membrane Preparation T cell responses induced by DNP-modified melanoma membranes were measured by IFN-gamma production. The T cells obtained from patients PBL were plated into a 96 round-bottom well plate at 10^5 cells/well in 100 µl culture medium (RAMI 1640 supplemented with 10 % human AB serum, 2 mM L-glutamate, 100 mg/ml/100 U/ml streptomycin/penicillin, 10 mM HEPES, 1 % non-essential amino acids). Various amounts (about 10^5 to about 10^8 cell equivalents) of cell membranes were added into each well and additional culture medium was added to make the total volume of each well to 250 µl.

Supernatants were collected for IFN-gamma assay after 18 hour incubation. The concentration of IFN-gamma in supernatants was measured by a commercially available ELISA kit (Endogen, Boston, MA; sensitivity = 5 pg/ml).

Significant IFN-gamma production by T cells (750 pg/ml) was detected after incubation with autologous DNP-Mel membranes. The production of IFN-gamma by T cells was related to the amount of coincubated DNP-Mel membranes. No significant response to unmodified Mel membranes was elicited. Two T cell sublines were developed by enriching for CD4+ and CD8+ T cells by the positive panning technique. Each subline responded to DNP-Mel membranes by IFN-gamma production. The response of the CD4+ T subline to DNP-Mel membranes was blocked by antibody to MHC class II, and the response of the CD8+ subline was blocked by antibody to MHC class I (73% and 80% blocking, respectively).

These results show that hapten-modified tumor cell membranes may be successfully used to vaccinate patients in need of tumor treatment.

Example 2: Treating Ovarian Stage III Cancer with Modified Tumor Cell Membranes

Patients may be initially treated according to standard medical practice (debulking surgery followed by chemotherapy). After the completion of chemotherapy, a six week

course of treatment with a vaccine containing ovarian cancer cell membranes modified with the hapten, dinitrophenyl (DNP) may be administered. Low dose cyclophosphamide may be administered prior to the first injection. After the completion of the course of treatment, patients may be tested for delayed type hypersensitivity to carcinoma cell membranes, both DNP-modified and unmodified. *In vitro* studies may be performed with cryopreserved lymphocytes extracted from metastatic tumors and/or separated from peripheral blood.

Patients receiving surgical debulking or patients exhibiting tumor reduction by chemotherapy, for example, may be selected for treatment. The mass of tumor excised from each patient may be sufficient to obtain at least $100x10^6$ viable tumor cells. Such patient may receive chemotherapy, such as carboplatin and taxol, and are preferably clinically tumor-free following completion of chemotherapy (*i.e.*, normal physical examination and CT studies and serum CA-125 <35 IU/L).

Patients may be excluded from receiving the treatment of the present invention based upon: insufficient quantity of tumor cells for preparing a vaccine and skin-testing (< 100×10^6 cells), Karnovsky performance status less than 80, major field radiation therapy within the preceding 6 months, current administration of systemic corticosteroids, hematocrit <30% or WBC <3000, age <18, active autoimmune disease, active, serious infection, another active malignancy, evidence of infection with hepatitis B virus (circulating antigen) or with HIV (circulating antibody), or inability to provide informed consent.

Patients undergo surgical resection of the tumor and debulking of metastases. Patients who undergo either optimal or suboptimal debulking may be eligible. Tumor tissue may be delivered to the laboratory and processed to obtain membranes. The membranes may be cryopreserved and stored in liquid nitrogen.

Both syngeneic and allogeneic tumor cell membranes may be prepared and used as described in this specification.

Beginning within six (6) weeks after surgery, patients may begin chemotherapy, such as with carboplatin or cisplatin + taxol, according to the following dosage-schedule:

carboplatin AUC 7.5 or cisplatin 75 mg/M^2 - every 3 weeks, taxol 175 mg/M^2 i.v. over 3 hours - every 3 weeks. Six cycles of chemotherapy may be administered. Any other chemotherapy may be administered.

Approximately, four weeks after completion of chemotherapy, patients may undergo a metastatic evaluation to include computer tomography (CT) chest-abdomen-pelvis. Only patients with no evidence of recurrent carcinoma may be eligible for vaccine treatment. Patients with elevated serum level of CA125 may be eligible providing that CT studies are negative for recurrence. The tumor cell membrane therapy may be started at least 4 weeks after, and no more than 12 weeks after, the last administration of chemotherapy.

On day -7, patients may be skin-tested with: 1) autologous ovarian cancer cells or membranes modified with DNP, 2) diluent (Hanks balanced salt solution with 0.1% human albumen, and 3) PPD intermediate. DTH reactions may be measured on day -5. On day 0, patients may receive cyclophosphamide 300 mg/M^2 as a rapid i.v. infusion. Three days later they may be injected intradermally with a tumor cell membrane composition and this may be repeated weekly for six (6) weeks. Vaccines may consist of DNP-modified, ovarian cancer cell membranes mixed with *BCG*. Vaccines may be injected into the upper arm. If for some reason a left axillary lymph node dissection had been performed, the right arm may be used.

Two and a half weeks after the sixth vaccine, patients may undergo clinical evaluation, consisting of CBC, SMA-12, CA125, and chest x-ray. They may be tested for DTH to the following materials: autologous carcinoma cells, both DNP-modified and unmodified; autologous peripheral blood lymphocytes, both DNP-modified and unmodified; diluent; and PPD intermediate. Also, they may be tested for contact sensitivity to dinitrofluorobenzene (DNFB).

Patients who remain relapse-free may be given a seventh (booster) vaccine at the six month point (measured from beginning the vaccine program). For each patient at least one cryopreserved vial of tumor cell membranes may be saved for the six-month booster injection. If the number of cells available is anticipated to be insufficient for 6

weekly vaccines plus the six-month booster, then the initial course of weekly injections may be reduced to 5. Another booster vaccine may be administered in one year, but only if a sufficient number of cells is available. Just prior to the one-year booster, patients may be skin-tested with autologous tumor cells or membranes to determine whether their previous level of immunity has been maintained.

Example 3. Treating Melanoma Cancer with Modified Tumor Cell Membranes

Tumor masses may processed as previously described. Briefly, cells may be extracted by enzymatic dissociation with collagenase and DNase and by mechanical dissociation. Cell membranes may be isolated as described in this specification, and frozen in a controlled rate freezer, and stored in liquid nitrogen until needed. On the day that a patient is to be treated, the membranes may be thawed, washed, and resuspended in Hanks balanced salt solution without phenol red. Modification with DNP may be performed by the method of Miller and Claman (1976). This involves a 30 minute incubation of tumor cells with dinitrofluorobenzene (DNFB) under sterile conditions, followed by washing with sterile saline.

The vaccine composition may contain a minimum of $2.5x10^6$ c.e. trypan-blue-excluding melanoma cell membranes, and a maximum of $7.5x10^6$ c.e. melanoma cell membranes, suspended in 0.2 ml Hanks solution. Each vaccine treatment may consist of three injections into contiguous sites.

The freeze-dried material may be reconstituted with 1 ml sterile water or phosphate buffered saline, pH 7.2 (PBS). Appropriate dilutions may be made in sterile buffered saline. Then 0.1 ml may be drawn up and mixed with the vaccine just before injection. The first and second vaccines may be mixed with 0.1 ml of a 1:10 dilution of Tice *BCG* ("Tice-1"). *BCG* is a Tice strain (substrain of the Pasteur Institute strain) obtained from Organon Teknika Corporation (Durham, NC). The third and fourth vaccines may be mixed with 0.1 ml of a 1:100 dilution ("Tice-3"). The fifth and sixth and booster vaccines may be mixed with 0.1 ml of a 1:1000 dilution ("Tice-5"). The

ideal vaccine reaction is an inflammatory papule with no more than small (< 5mm) central ulceration.

Skin testing may be performed by the intradermal injection of 0.1 ml of test material on the forearm, and DTH is assessed at 48h by measuring the mean diameter of induration. The following materials may be tested: 1) $1x10^6$ autologous melanoma cell membranes cells unmodified and modified with DNP; both enzymatically-dissociated (TCE) and mechanically-dissociated (TCM) tumor cells may be used; 2) $3x10^6$ autologous peripheral blood lymphocytes unmodified and modified with DNP; 3) Hanks solution; and 4) PPD-intermediate strength. Also, contact sensitivity to DNFB may be tested by applying 200 μg DNFB to the skin of the ventral surface of the upper arm and examining the area for a circle of induration at 48 hours. The full battery of DTH tests may be performed following the six week course of vaccine administration. Pre-treatment DTH testing may be limited to DNP-modified melanoma cell membranes, PPD, and diluent. This strategy is designed to avoid: 1) sensitizing patients to DNP-modified lymphocytes and 2) tolerizing patients by injection of unmodified tumor cells.

All patients may have blood collected for separation and cryopreservation of lymphocytes and serum each time skin-testing is performed. Periodically, these may be tested for: response to autologous cancer cells, as measured by proliferation, cytokine release, and cytotoxicity.

Patients may be evaluated for metastatic disease before vaccine therapy begins. After the end of the first eight weeks of vaccine therapy, evaluations are performed every three months. Evaluations may continue through year two, every four months in year three, and every six months thereafter. Physical examination and routine bloodwork (CBC, SMA-12, and CA125) may be performed with each evaluation. CT of the chest-abdomen-pelvis may be performed prior to the administration of vaccine, at 6 months and 12 months (before vaccine boosters), and then as clinically indicated. Relapse-free and total survival may be measured. All patients may be followed for at least five years or until time of death.

Patients are expected to develop a local reaction to *BCG*, consisting of a draining, tender pustule that heals in 2-3 months leaving a smallpox vaccination-like scar. As patients develop sensitivity to *BCG*, the intensity of these reactions may increase. Anaphylaxis, other allergic phenomena, and auto-immunity have never been observed in haptenized-vaccine patients.

Reactions at the vaccine sites may be graded as follows: 0 - no symptoms; 1 - itching or discomfort, but no interference with arm movement or normal activity; 2 - discomfort causing interference with arm movement, but not requiring modification of normal activity; 3 - discomfort causing major interference with arm movement and requiring modification of normal activity; and 4 - discomfort causing inability to use the extremity for normal activity.

Cyclophosphamide may be reconstituted in sterile water and the proper dosage may be administered by rapid i.v. infusion. Typically, about one third of patients may experience nausea and about 10% may have vomiting after low dose cyclophosphamide. Leukopenia, alopecia, and cystitis do not occur at this dose. It is expected that this protocol be associated with a lower incidence of nausea and vomiting, since cyclophosphamide may be administered only once in association with the final vaccine inoculation.

Patients may be observed following injection of the vaccine. Patients experiencing unexpected symptoms or signs are instructed to contact the physician to be evaluated immediately. Fever that causes discomfort may be treated with acetaminophen. Nausea caused by low dose cyclophosphamide may be treated with oral prochlorperazine (Compazine). If severe local reactions (> 5 mm ulceration) occur at the vaccine site, subsequent doses of BCG may be reduced (see above).

Patients who are relapse-free at the 1 year evaluation may receive a final booster injection of vaccine. Then their condition may be followed without further treatment. Patients who develop metastases may be taken off study and treated as clinically indicated (usually surgery or chemotherapy).

An efficacy study to determine whether DNP-vaccine prolongs relapse-free and/or total survival in these patients may also be conducted. Survival parameters (Kaplan-Meier method) may be measured.

All Thomas Jefferson University, NIH, and FDA regulations regarding informed consent are be followed in regard to informed consent.

Our prior studies of DNP-modified autologous vaccine for melanoma using hapten-modified melanoma cells showed the following results: 100% of patients (N=60) developed a positive DTH response (≥ 5mm diameter of induration) to DNP-modified autologous tumor cells following treatment, and 85% developed a large positive response (≥ 10mm diameter of induration). Similar success is expected with a vaccine of DNP-modified autologous carcinoma membrane vaccine, *i.e.*, patients are expected to develop DTH to DNP-modified and to unmodified autologous carcinoma cell membranes.

Various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

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What is claimed is:

1. A composition comprising a hapten modified human tumor cell membrane.

2. The composition of claim 1 wherein said tumor cell membrane is isolated from a tumor cell selected from the group consisting of a carcinoma cell and a non-solid tumor cell.

3. The composition of claim 1 wherein said tumor cell membrane originates from a tumor selected from the group consisting of leukemia, lymphoma, multiple myeloma, ovarian, colon, rectal, colorectal, melanoma, breast, lung, kidney, and prostate cancer.

4. The composition of claim 3 wherein said leukemia is acute myelogenous leukemia.

5. The composition of claim 1 wherein said tumor cell membrane is a tumor cell membrane selected from the group consisting of a syngeneic tumor cell membrane and an allogeneic tumor cell membrane.

6. The composition of claim 5 wherein said syngeneic tumor cell membrane is autologous.

7. The composition of claim 1 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, N-iodoacetyl-N'-(5-sulfonic 1-naphthyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid benzene isothiocyanate, trinitrobenzenesulfonic acid, sulfanilic acid, arsanilic acid, dinitrobenzene-S-mustard and combinations thereof.

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8. The composition of claim 1 further comprising an adjuvant.

9. The composition of claim 8 wherein said adjuvant is selected from the group consisting of *Bacille Calmette-Guerin*, QS-21, detoxified endotoxin and a cytokine.

10. The composition of claim 1, having the property when administered to a human suffering from a malignant tumor of the same type as said tumor cell membrane, of eliciting T lymphocytes that infiltrate the tumor of said human.

11. The composition of claim 1, having the property when administered to a human suffering from a malignant tumor of the same type as said tumor cell membrane, of eliciting an inflammatory immune response against the tumor of said human.

12. The composition of claim 1, having the property when administered to a human suffering from a malignant tumor of the same type as said tumor cell membrane, of eliciting a delayed-type hypersensitivity response to the tumor of said human.

13. The composition of claim 1 wherein said tumor cell membrane is allogeneic, said composition further comprising an antigen presenting cell.

14. The composition of claim 1 wherein said tumor cell membrane is allogeneic and said antigen presenting cell is syngeneic.

15. The composition of claim 1 wherein said tumor cell membrane is allogeneic and said antigen presenting cell is autologous.

16. A method of treating cancer comprising administering to a human a composition comprising a therapeutically effective amount of a hapten modified human tumor cell membrane wherein said human suffers from a malignant tumor of the same type as said tumor cell membrane.

17. The method of claim 16 comprising eliciting T lymphocytes that infiltrate said tumor of said human and measuring said T lymphocytes that infiltrate said tumor of said human.

18. The method of claim 16 comprising eliciting an inflammatory immune response to said tumor of said human and measuring said inflammatory immune response.

19. The method of claim 16 comprising eliciting a delayed-type hypersensitivity response to said tumor of said human and measuring said delayed-type hypersensitivity response.

20. The method of claim 16 wherein said malignant tumor is selected from the group consisting of carcinomas and non-solid tumors.

21. The method of claim 16 wherein said malignant tumor is selected from the group consisting of leukemia, lymphoma, multiple myeloma, ovarian, colon, rectal, colorectal, melanoma, breast, lung, kidney, and prostate cancer.

22. The method of claim 21 wherein said leukemia is acute myelogenous leukemia.

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23. The method of claim 16 wherein said tumor cell membrane is a tumor cell membrane selected from the group consisting of a syngeneic tumor cell membrane and an allogeneic tumor cell membrane.

24. The method of claim 23 wherein said syngeneic tumor cell membrane is autologous.

25. The method of claim 16 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, N-iodoacetyl-N'-(5-sulfonic 1-naphthyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid benzene isothiocyanate, trinitrobenzenesulfonic acid, sulfanilic acid, arsanilic acid, dinitrobenzene-S-mustard and combinations thereof.

26. The method of claim 16 wherein said composition further comprises an adjuvant.

27. The method of claim 26 wherein said adjuvant is selected from the group consisting of *Bacille Calmette-Guerin*, QS-21, detoxified endotoxin and a cytokine.

28. The method of claim 16 wherein said tumor cell membrane is allogeneic, said composition further comprising an antigen presenting cell.

29. The method of claim 16 wherein said tumor cell membrane is allogeneic and said antigen presenting cell is syngeneic.

30. The method of claim 16 wherein said tumor cell membrane is allogeneic and said antigen presenting cell is autologous.

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31. A method of eliciting T lymphocytes that infiltrate a tumor of a mammal comprising administering to said mammal a therapeutically effective amount of a hapten modified mammalian tumor cell membrane wherein said mammal suffers from a malignant tumor of the same type as said tumor cell membrane.

32. The method of claim 31 wherein said mammal is a human or an animal selected from a canine, feline, bovine and equine families.

33. A method of eliciting an inflammatory immune response to a tumor of a mammal comprising administering to said mammal a therapeutically effective amount of a hapten modified mammalian tumor cell membrane, wherein said mammal suffers from a malignant tumor of the same type as said tumor cell membrane.

34. The method of claim 33 wherein said mammal is a human or an animal selected from a canine, feline, bovine and equine families.

35. A method of eliciting a delayed-type hypersensitivity response to a tumor of a mammal comprising administering to said mammal a therapeutically effective amount of a hapten modified mammalian tumor cell membrane, wherein said mammal suffers from a malignant tumor of the same type as said tumor cell.

36. The method of claim 35 wherein said mammal is a human or an animal selected from a canine, feline, bovine and equine families.

37. A method of preparing hapten modified tumor cell membranes comprising lysing tumor cells to obtain lysed tumor cells, removing nuclei from said lysed tumor cells to obtain nuclei-free tumor cells, obtaining substantially cell-free

tumor cell membranes from said nuclei-free tumor cells, and subjecting said tumor cell membranes to a hapten to obtain hapten-modified tumor cell membranes.

38. The method of claim 37 wherein said lysing is selected from the group consisting of hypotonic shock, mechanical dissociation and enzymatic dissociation.

39. The method of claim 37 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, N-iodoacetyl-N'-(5-sulfonic 1-naphthyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid benzene isothiocyanate, trinitrobenzenesulfonic acid, sulfanilic acid, arsanilic acid, dinitrobenzene-S-mustard and combinations thereof.

40. An isolated hapten modified mammalian tumor cell membrane.

41. The membrane of claim 40, wherein said mammal is a human or an animal selected from a canine, feline, bovine and equine families.

42. The membrane of claim 41, wherein said mammal is a human.

43. The membrane of claim 42, wherein said membrane is isolated from a tumor cell selected from the group consisting of carcinoma cell and non-solid tumor cell.

44. The membrane of claim 42, wherein said membrane originates from a tumor selected from the group consisting of leukemia, lymphoma, multiple myeloma, ovarian, colon, rectal, colorectal, melanoma, breast, lung, kidney, and prostate cancer.

45. The membrane of claim 44 wherein said leukemia is acute myelogenous leukemia.

46. The membrane of claim 40 selected from the group consisting of a syngeneic tumor cell membrane and an allogeneic tumor cell membrane.

47. The membrane of claim 46 wherein said syngeneic tumor cell membrane is autologous.

48. The membrane of claim 40 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, N-iodoacetyl-N'-(5-sulfonic 1-naphthyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid benzene isothiocyanate, trinitrobenzenesulfonic acid, sulfanilic acid, arsanilic acid, dinitrobenzene-S-mustard and combinations thereof.

49. The membrane of claim 40 having at least one of the following properties: (i) eliciting T lymphocytes that infiltrate a mammalian tumor, (ii) eliciting an inflammatory immune response against a mammalian tumor, (iii) eliciting a delayed-type hypersensitivity response to a mammalian tumor and (iv) stimulating T cells *in vitro*.

50. The membrane of claim 40 wherein said membrane is an outer cell membrane.



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51. The membrane of claim 40 wherein said membrane comprises a membrane fraction comprising an MHC molecule, a heat shock protein or a combination thereof.

52. A composition comprising the membrane of claim 40.

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