CONCURRENT CHEMOTHERAPY AND IMMUNOTHERAPY

[01] This invention was made with support from the U.S. government under Grant No. R01 CA097222 from the National Institutes of Health. The U.S. government therefore retains certain rights to this invention.

[02] This application claims the benefit of U.S. provisional application 60/732,741 filed November 2, 2005, the entire contents of which are expressly incorporated herein.

TECHNICAL FIELD OF THE INVENTION

[03] This invention is related to the area of cancer immunotherapy. In particular, it relates to enhancing response to tumor vaccines.

BACKGROUND OF THE INVENTION

[04] Despite aggressive surgical resection, high-dose focused radiation therapy, and chemotherapy, patients diagnosed with GBM have a median survival of less than 15 months after diagnosis (Stupp et al., Optimal role of temozolomide in the treatment of malignant gliomas. Curr Neurol Neurosci Rep. 2005 May;5(3):198-206.). Failure of therapy can be attributed, at least in part, to a relatively narrow therapeutic index so that attempts at dose escalation results in dose-limiting systemic or neurological toxicity. The use of immunotherapy has held promise for the potential treatment of these tumors but until recently, few have demonstrated clinical efficacy. Several clinical trials, with selected patients, involving vaccination of glioma patients with dendritic cells (DCs) and either acid-eluted peptides (Ashkenazi et al., A selective impairment of the IL-2 system in lymphocytes of patients with glioblastomas: increased level of soluble IL-2R and reduced protein tyrosine phosphorylation. Neuroimmunomodulation.

1997; Kolenko et al., Tumor-induced suppression of T lymphocyte proliferation coincides with inhibition of Jak3 expression and IL-2 receptor signaling: role of soluble products from human renal cell carcinomas. J Immunol. 1997 Sep 15;159(6):3057-67; Liau et al., Dendritic cell vaccination in glioblastoma patients induces systemic and intracranial T-cell responses modulated by the local central nervous system tumor microenvironment. Clin Cancer Res. 2005 Aug 1;11(15):5515-25) or an antigen-specific peptide (Heimberger AB, Archer GE, et al., Dendritic cells pulsed with a tumor-specific peptide induce long-lasting immunity and are effective against murine intracerebral melanoma. Neurosurgery. 2002 Jan;50(1):158-64; discussion 164-6) have demonstrated promise by increasing median survival time to a range of 20-31 months. Furthermore, in a recently completed phase II clinical trial utilizing an antigen-specific immunotherapeutic approach, time to progression (TTP) in GBM patients was delayed to 15 months, which is in marked contrast to the standard of care consisting of radiotherapy and temozolomide that had a TTP of 7 months (Stupp et al., 2005, supra), and median survival was 29 months (Heimberger et al, J Transl Med. 2005 Oct 19;3:38 The natural history of EGFR and EGFRvIII in glioblastoma patients.). Cumulatively, these immunotherapy trials suggest that despite the inherent immunosuppression of malignant glioma patients, efficacious immune responses can be generated. However, there is reluctance to not treat GBM patients with some form of chemotherapy given the recently established standard of care and the overall poor prognosis.

[05] There is a continuing need in the art to develop better methods for treating tumors in general and glioblastomas in particular.

SUMMARY OF THE INVENTION

[05a] In a first aspect, there is provided the use of a treatment effective amount of an EGFRvIII peptide in the manufacture of a medicament for treating a tumor in a subject, wherein said medicament is to be administered with a treatment effective amount of a chemotherapeutic agent which induces lymphopenia, said chemotherapeutic agent is temozolomide or a pharmaceutically acceptable salt thereof.

[05b] In a second aspect, there is provided the use of a treatment effective amount of an EGFRvIII peptide, the EGFRvIII peptide conjugated to KLH, in the manufacture of a medicament for treating tumor in a subject, wherein said medicament is to be administered concurrently with an effective amount of GM-CSF as an adjuvant and wherein said medicament is to be administered with an effective amount of temozolomide or a pharmaceutically acceptable salt thereof.

[05c] In a third aspect, there is provided the use of a treatment effective amount of an EGFRvIII peptide in the manufacture of a medicament for treating a tumor in a subject, wherein said medicament is to be administered with a treatment effective amount of temozolomide or a pharmaceutically acceptable salt thereof.

[06] A method is provided for treating a tumor in a subject. A treatment-effective amount of an EGFRvIII peptide and a treatment-effective amount of a chemotherapeutic agent which induces lymphopenia are administered to the subject.

[07] According to another embodiment a method is provided for treating a tumor in a subject. A treatment-effective amount of an EGFRvIII peptide conjugated to KLH is administered to the subject with the tumor. Granulocyte/macrophage colony stimulating factor (GM-CSF) is also administered as an adjuvant in an effective amount concurrently with the EGFRvIII peptide. A treatment-effective amount of an alkylating agent is also administered to the subject.

[08] According to still another embodiment, a method is provided for treating a tumor in a subject. A treatment-effective amount of an anti-tumor vaccine and a treatment-effective amount of temozolomide or a pharmaceutically acceptable salt thereof are administered to the subject.

[09] According to still another embodiment, a method is provided for treating a tumor in a subject. A treatment-effective amount of an anti-tumor vaccine and a treatment-effective amount of a chemotherapeutic agent which induces lymphopenia are administered to the subject.

[10] These and other embodiments which will be apparent to those of skill in the art upon reading the specification provide the art with additional methods for treating treatment-refractory tumors.

DETAILED DESCRIPTION OF THE INVENTION

[11] The concurrent administration of chemotherapy and immunotherapy has been considered a contraindication because of the concern that the chemotherapy-induced lymphopenia would ablate therapeutic efficacy of immunotherapy. Temozolomide has been shown to be an effective chemotherapeutic for patients with malignant gliomas and to deprive patients with glioblastoma (GBM) patients of this agent in order to treat with immunotherapy is controversial. Despite conventional dogma, the inventors demonstrate that both chemotherapy and immunotherapy can be delivered concurrently without negating the effects of immunotherapy. In fact, the temozolomide induced lymphopenia may actually be synergistic with a peptide vaccine. Although applicants do not wish to be bound by an particular theory regarding mechanism of action, the observed synergy may be secondary to inhibition of Tregs or the failure to recover of Tregs, which permits an increase of effector cytotoxic $CD8^+$ T cells. Other mechanisms may also be involved.

[12] "EGFRvIII" or "Epidermal Growth Factor Receptor mutation III" is a known mutant form of the Epidermal Growth Factor Receptor. See, e.g., U.S. Patent No. 6,503,503.; see also US Patents Nos. 6,900,221; 6,673,602; 6,479,286; and 6,129,915. The mutation which causes the production of the vIII protein is typically characterized by a consistent and tumor-specific in-frame deletion of 801 base pairs from the extracellular domain that splits a codon and produces a novel glycine at the fusion junction.

[13] "EGFRvIII peptide", as used herein refers to a peptide of suitable length, e.g., at least 10 or 12 amino acids, and up to 16, 20 or 30 amino acids, or more, which spans the mutated splice junction of the corresponding EGFRvIII protein. Examples include but are not limited to: H-LEEKKGNYVVTDHS-OH, or "PEP-3." The EGFRvIII peptide

may be from (or correspond in sequence to) the EGFRvIII of any mammalian species, but is preferably human. Particular wild-type sequences of EGFR are shown in SEQ ID NO: 6 to 9.

[14] "Carrier protein" as used herein refers to a protein which does not possess high homology to a protein found in the species that is receiving a composition of the invention and elicits an immune response. A protein possesses high homology if it is at least 75% identical, more preferably at least 85% identical or at least 90% identical to a protein as determined by any known mathematical algorithm utilized for the comparison of two amino acid sequences (see, e.g., Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87: 2264-2268; Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. USA 90: 5873-5877; Torellis and Robotti, 1994, Comput. Appl. Biosci. 10: 3-5; and Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. 85: 2444-8). Preferably, the percent identity of two amino acid sequences is determined by BLAST protein searches with the XBLAST program, score=50, word length=3. Examples of heterologous carrier proteins include, but are not limited to, KLH, PhoE, mLT, TraT, or gD from BhV-1 virus. See, e.g., US Patent No. 6,887,472. Such carrier proteins may be conjugated or linked to the tumor antigen directly or by an intervening linker segment such as a chain of one or more (e.g., 2, 4, 6) intervening amino acids (e.g., an intervening CYS residue) in accordance with known techniques.

[15] "KLH" or "keyhole-limpet hemocyanin" is a known carrier protein to which another protein may be conjugated in accordance with known techniques. See, e.g., US Patent No. 6,911,204.

[16] "Adjuvant" as used herein refers to anyone of a diverse class of compounds that enhance the therapeutic efficacy of a vaccine which is administered concurrently with the adjuvant. In some embodiments the adjuvant is a hematopoietic growth factor such as GM- CSF.

Common examples of adjuvants include but are not limited to aluminium hydroxide, -phosphate or -oxide, oil-in-water or water-in-oil emulsion based on, for example a mineral oil, such as Bayol Fo or Marcol 52TM or a vegetable oil such as vitamin E acetate, saponins, BCG, *M. vaccae*, Tetanus toxoid, Diphtheria toxoid, *Bordetella pertussis*, interleukin 2, interleukin 12, interleukin 4, interleukin 7, Complete Freund's Adjuvant, Incomplete Freund's Adjuvant, and a nonspecific adjuvant. See, e.g., US Patent No. 6,699,483.

[17] "Hematopoietic growth factors" or "HGFs" are known. See, e.g., U.S. Patent No. 6,863,885. In general, HGFs are glycoprotein cytokines that regulate the proliferation and differentiation of hematopoietic progenitor cells. The hematopoietic growth factors intended to be used in the present invention can be selected from the group G-CSF (granulocyte colony stimulating factor), SCF (stem cell factor), GM-CSF (granulocyte macrophage colony stimulating factor), IL-1 (interleukin-1), IL-3, IL-6, IL-8, IL-ll, IL-12, LIF (leukemia inhibitory factor), FGF-beta (fibroblast growth factor beta), FLT3, or a combination thereof. These growth factors can be purchased (e.g., R&D Systems, Minneapolis, MN) or made following procedures set forth in the art generally and in publications describing the factors. Additionally, the hematopoietic growth factor can be a modified form of the factor or a fusion protein of hematopoietic growth factors selected from the group GCSF, SCF, GM-CSF, IL-I, IL-3, IL-6, IL-8, IL-11, IL-12, LIF, FGF-beta, and FLT3. HGFs include modified growth factors (e.g., muteins) and fusion proteins, which can be made according to methods known in the art. See, e.g. (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). Hematopoietic growth factors that stimulate macrophage function such as GM-CSF are particularly preferred. These can be used as adjuvants.

[18] "External beam radiotherapy" can be carried out by delivering a beam of high-energy x-rays to the location of the patient's tumor. The beam is generated outside the patient and is targeted at the tumor site. No radioactive sources are placed inside the patient's body. This can be used in conjunction with any other treatment step according to the invention.

[19] "Treat" as used herein refers to any type of treatment or prevention that imparts a benefit to a subject afflicted with a disease or at risk of developing the disease, including improvement in the condition of the subject (e.g., in one or more symptoms), delay in the progression of the disease, delay the onset of symptoms or slow the progression of symptoms, etc. As such, the term "treatment" also includes prophylactic treatment of the subject to prevent the onset of symptoms.

[20] As used herein, "treatment" and "prevention" are not meant to imply cure or complete ablatement of symptoms. Rather, these refer to any type of treatment that imparts a benefit to a patient afflicted with a disease, including improvement in the condition of the patient (e.g., in one or more symptoms), delay in the progression of the disease, etc.

[21] "Treatment-effective amount" as used herein means an amount of the antibody sufficient to produce a desirable effect upon a patient inflicted with cancer such as gliomblastoma, including improvement in the condition of the patient (e.g., in one or more symptoms), delay in the progression of the disease, etc.

[22] Subjects in need of treatment by the methods described herein include subjects afflicted with glioblastoma or astrocytoma, as well as subjects afflicted with other solid tumors or cancers such as lung, colon, breast, brain, liver, prostate, spleen, muscle, ovary, pancreas, head and neck, skin (including melanoma), etc. Subjects in need of treatment particularly include subjects afflicted with a tumor, such as a brain

tumor, that expresses EGFRvIII. The tumor may be a primary tumor, a metastatic tumor, or a recurrent tumor. Subjects to be treated by the methods of the invention particularly include subjects afflicted with a tumor expressing EGFRvIII, including gliomas, fibrosarcomas, osteosarcomas, melanoma, Wilms tumor, colon carcinoma, mammary and lung carcinomas, and squamous carcinomas. Subjects to be treated by the present invention most particularly include subjects afflicted with brain tumors or cancers, such as glioblastomas, particularly glioblastoma multiforme, and cystic astrocytoma.

[23] The present invention is primarily concerned with the treatment of human subjects, including male and female subjects and neonatal, infant, juvenile, adolescent, adult, and geriatric subjects, but the invention may also be carried out on animal subjects, particularly mammalian subjects such as mice, rats, dogs, cats, livestock and horses for veterinary purposes, and for drug screening and drug development purposes.

[24] The pharmaceutical compositions of the invention can be prepared in accordance with known techniques. Typically, the active agents are included in a pharmaceutically acceptable carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.9% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

[25] The compositions and methods of the invention may include the administration of one or more co-adjuvants. Suitable co- adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-inwater emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (PCT Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 formulated into submicron particles, (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) RibiTM adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT.) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL+CWS (DetoxTM) (for a further discussion of suitable submicron oil-in-water emulsions for use herein, see PCT Publication No. WO 99/30739, published Jun. 24, 1999); (3) saponin adjuvants, such as StimulonTM (Cambridge Bioscience, Worcester, MA) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freunds Adjuvant (CF A) and Incomplete Freunds Adjuvant (IF A); (5) cytokines, such as interleukins (IL-l, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an E. coli heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-SI09 (where serine is substituted for the wild-type amino acid at position 109), adjuvants derived from the CpG family of molecules, CpG dinucleotides and synthetic oligonucleotides which comprise CpG

motifs (see, e.g., Krieg et al., Nature, 374:546 (1995) and Davis et al., J. Immunol., 160:870-876 (1998)) and PT-K9/GI29 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., PCT Publication Nos. W093/13202 and W092/19265); (7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. See, e.g., U.S. Patent No. 6,534,064; and (8) other ligands for Toll-like receptors in addition to CpG and RIBI adjuvants, such as bacterial flagellin (an effective adjuvant for CD4+ T cells; see IJ Immunol. 169: 3914-9 (Oct. 2002).

[26] The active agents may be administered by any medically appropriate procedure, e.g., normal intravenous or intra-arterial administration, injection into the cerebrospinal fluid). In certain cases, intradermal, intracavity, intrathecal or direct administration to the tumor or to an artery supplying the tumor is advantageous. Where the tumor or a portion thereof has been previously surgically removed the treatment agents may be administered into the site of the tumor (and particularly into an enclosed cavity or "resection cavity" at the site of the tumor) by direct injection or through a pre-implanted reservoir.

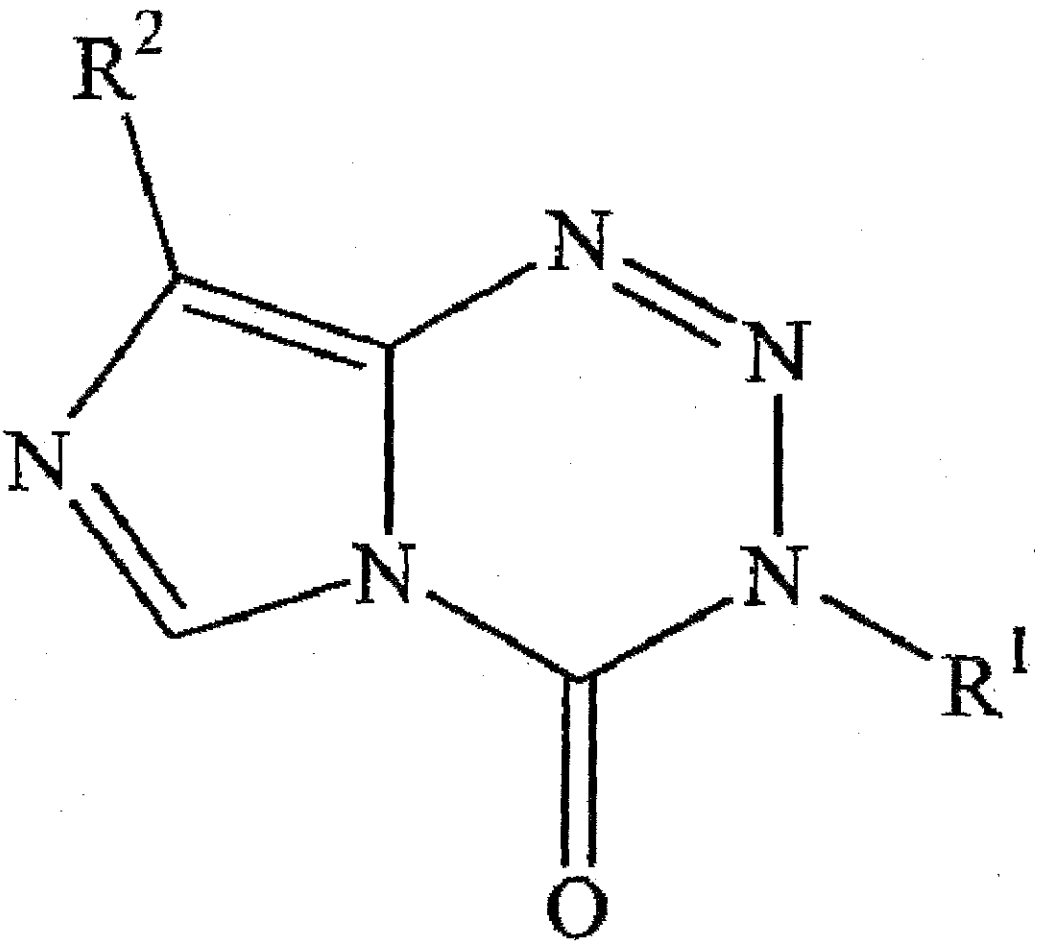
[27] Dosage of the active agents will depend on, among other things, the condition of the subject, the particular category or type of cancer being treated, the route of administration, the nature of the therapeutic agent employed, and the sensitivity of the tumor to the particular therapeutic agent.

[28] In general, the dose of the tumor antigen or vaccine, such as EGFRvIII, including any carrier protein or peptide conjugated thereto, will be from 10, 100 or 500 μg up to 2 or 3 mg per subject, for each dose. Doses may be given on a single occasion, optionally including follow-up or "booster" doses (e.g., one, two or three follow up or "booster" dosages given at intervals of from one to three weeks). Note that doses

can be divided, such as administering to different injection sites, to reduce side effects such as local responses, if desired. Where the formulation contains both tumor antigen bound (or "conjugated") to the carrier protein and tumor antigen free of the carrier protein, the calculated dosage can include both the amount of both bound and free tumor antigen and carrier protein.

[29] In general, the dose of the adjuvant such as GM -CSF will also be from 10 or 20 μg up to 500 μg, or 1 or 2 mg per subject, administered on the same schedule or different schedule from the dose of the tumor antigen. When administered on the same schedule the adjuvant may be administered in the same carrier as the tumor antigen. When not combined in the same carrier, the dose of adjuvant need only be administered sufficiently close in time to the dose of tumor antigen to enhance the efficacy thereof (e.g., within one or two hours; on the same day; etc.).

[30] Alkylating agents useful for carrying out the present invention include (but are not limited to) 1,3-bis(2-chloroethyl)-1- nitrosourea (BCNU) and tetrazine derivatives, particularly [3H]imidazo[5,1-d] 1,2,3,5-tetrazin-4one derivatives such as temozolomide and analogs thereof (including pharmaceutically acceptable salts and pro drugs thereof). Such compounds are known. See, e.g., U.S. Patent Nos. 6,096,724; 6,844,434; and 5,260,291. Examples of alkylating agents useful for carrying out the present invention include [3H]imidazo[5,1-d]-1,2,3,5-tetrazin-4-ones alkylating agents, particularly those of the general formula:



[31] wherein R^1 represents a hydrogen atom, or a straight- or branched-chain alkyl, alkenyl or alkynyl group containing up to 6 carbon atoms, each such group being unsubstituted or substituted by from one to three substituents selected from halogen (i.e. bromine, iodine or, preferably, cblorine or fluorine) atoms, straight- or branched-chain alkoxy, (e.g. methoxy), alkylthio, alkylsullihinyl and alkylsulphonyl groups containing up to 4 carbon atoms, and optionally substituted phenyl groups, or R^1 represents a cycloalkyl group, and R^2 represents a carbamoyl group which may carryon the nitrogen atom one or two groups selected from straight- and branched-chain alkyl and alkenyl groups, each containing up to 4 carbon atoms, and cycloalkyl groups, *e.g.*, a methylcarbamoyl or dimethylcarbamoyl group. When the symbol R^1 represents an alkyl, alkenyl or alkynyl group substituted by two or three halogen atoms, the aforesaid halogen atoms may be the same or different. When the symbol R^1

represents an alkyl, alkenyl or alkynyl group substituted by one, two or three optionally substituted phenyl groups the optional substituents on the phenyl radical(s) may be selected from, for example, alkoxy and alkyl groups containing up to 4 carbon atoms (e.g. methoxy and/or methyl group(s)) and the nitro group; the symbol R^1 may represent, for example, a benzyl or p-methoxybenzyl group. Cycloalkyl groups within the definitions of symbols R^1 and R^2 contain 3 to 8, preferably 6, carbon atoms. The compounds may be provided as salts or prodrugs, particularly alkali metal salts when R^1 is H. *See, e.g.,* U.S. Patent No. 5,260,291.

[32] Temozolomide, in oral dosage form as 5 mg, 20 mg, 100 mg, and 250 mg capsules, is commercially available as TEMODAR™ from Schering Corporation, Kenilworth NJ 07033 USA.

[33] Alkylating agents may be prepared in pharmaceutically acceptable formulations in like manner as described above, in the same or different formulation that contains the tumor vaccine, e.g., EGFRvIII peptide.

[34] In a preferred embodiment, the alkylating agent is administered in a cycle of daily doses for 3, 4, 5, 6 or 7 consecutive days. A suitable daily dose may be from 50, 100 or 150 mg/m^2/dose, up to 200, 250 or 300 mg/m^2/dose. This cycle may be repeated, e.g., every two, three, four or five weeks, for up to a total of 6, 8 or 10 cycles. The first dose in the first cycle of alkylating agent may be administered at any suitable point in time. In some embodiments the first dose of alkylating agent is administered up to two or four weeks before administration of the therapeutic antibody; in some embodiments the first dose of alkylating agent is administered at least two, four or six weeks following the administration of the therapeutic antibody. Additional schedules of administration may be included where additional therapeutic treatments such as external beam radiotherapy are also applied to the subject.

[35] Optionally, the subject may also receive external beam radiotherapy. For example, external beam radiotherapy may be utilized for brain tumors such as glioblastoma. External beam radiotherapy is known and can be carried out in accordance with known techniques. The beam can be generated by any suitable means, including medical linear accelerators and Cobalt 60 external beam units. The radiation source can be mounted in a gantry that rotates around the patient so that a target area within the patient is irradiated from different directions. Before irradiation the treatment is typically planned on a computer using algorithms that simulate the radiation beams and allow the medical personnel to design the beam therapy. Numerous variations of external beam therapy that can be used to carry out the present invention will be readily apparent to those skilled in the art. See, e.g., US Patent Nos. 6,882,702; 6,879,659; 6,865,253; 6,863,704; 6,826,254; 6,792,074; 6,714,620; and 5,528,650.

[36] External beam therapy is preferably administered in a series of sessions in accordance with known techniques, with the sessions preferably beginning two to four weeks after administration of the therapeutic antibody. For example, the external beam radiotherapy may be administered 3, 4, 5, 6 or 7 days a week, over a period of four, five, six or seven weeks, at a daily dose of 0.5 or 1 Gy, up to 2 or 3 Gy, until the total desired dose (e.g., 30 or 40 Gy, up to 50 or 60 Gy) is administered.

[37] The delivered dose may be to an area including a margin of normal tissue (e.g., ~1, 2 or 3 cm margin in all directions) around the tumor, or where the tumor or a portion thereof has previously been surgically removed, around the site of the tumor.

[38] Where external beam radiotherapy is employed, the patient may receive an additional schedule of chemotherapeutic agent administration, different from that described above, at a somewhat

lower dose, during the course of the radiotherapy. For example, the patient may receive daily doses of chemotherapeutic agent, e.g., alkylating agent in an amount of from 25 or 50 mg/m^2/dose up to 100 or 125 mg/m^2/dose daily during the course of the external beam therapy.

[39] Examples of tumor antigens which can be used as anti-tumor vaccines include but are not limited to Cyclin-dependent kinase 4; β-catenin ; Caspase-8; MAGE-1; MAGE-3; Tyrosinase; Surface Ig idiotype; Her-2/neu Receptor; MUC-1; HPV E6 and E7; CD5 Idiotype CAMPATH-1, CD20; Cell surface glycoprotein CEA, mucin-1; Cell surface carbohydrate Lewis^x; CA-125; Epidermal growth factor receptor; p185HER2; IL-2R; FAP-α; Tenascin; and metalloproteinases. EGFRvIII is exemplary of tumor-specific antigens. Cells which express these antigens can also be used as vaccines. Preferably the cells are killed prior to administration. The cells can be fractionated so that a fraction enriched for the tumor antigen is used as a vaccine. These antigens are merely exemplary and are not intended to be a comprehensive of the many useful antigens known in the art or which may be used.

[40] Multiple preclinical model systems have demonstrated that the depletion of immune cell subsets can abrogate the efficacy of several types of immunotherapeutic approaches (Heimberger et al., 2003) indicating that chemotherapy administered during the effector stages of immunotherapy may be deleterious to efficacy. However, this does not preclude utilizing these agents together when appropriately timed to minimize the aforementioned effects. Furthermore, although applicants do not wish to be bound by any particular theory regarding mechanism of action, the depletion of certain effector cells, such as Tregs, may be a highly desirable outcome of chemotherapy yielding greater immunotherapeutic efficacy or may promote a desirable cytokine profile for adequate tumor control.

[41] The above disclosure generally describes the present invention. All references disclosed herein are expressly incorporated by reference. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

[42] To test the hypothesis that chemotherapy and immunotherapy can be administered concurrently, we treated a patient with a newly diagnosed GBM using the standard of care, temozolomide, while also administering a peptide vaccine targeting the epidermal growth factor variant III (EGFRvIII) (Heimberger et al., 2006). The amplification of the epidermal growth factor receptor (EGFR) gene, which results in over expression of the EGFR, a transmembrane tyrosine kinase receptor (Ekstrand et al., 1991) is associated with the mutant EGFR gene, EGFRvIII (Wikstrand et al., 1997). Previous work has shown that EGFR amplification is evident in all GBMs expressing EGFRvIII (Heimberger et al., 2005) and GBMs lacking the amplified EGFR are not positive for EGFRvIII protein (Aldape et al., 2004).

[43] In May of 2005, a 51-year-old Caucasian man was evaluated following complaints of a three-week history of persistent morning headaches without associated nausea. A magnetic resonance (MR) image revealed a multi-lobular, irregularly enhancing lesion measuring 6.6 x 5.3 x 4.3 in the anterior aspect of the right temporal lobe. The sylvian fissure was bowed upward and there was 6 mm of midline shift. The patient underwent a gross total resection, with histology demonstrating a biphasic glioblastoma and malignant sarcoma. These components were confirmed by positive immunohistochemistry in the glioblastoma component with glial-fibrillary astrocytic protein (GFAP) and abundant

reticulin production in the sarcoma component. A trichrome stain confirmed the biphasic nature of the tumor. EGFR-528 and EGFRvIII antibody immunohistochemistry staining was positive (Heimberger et al., 2005), with the EGFRvIII staining demonstrating strong diffuse reactivity, while the EGFR-528 staining was more focal. PTEN was strongly positive and p53 reactivity was present in more than 30% of tumor nuclei. The methylguanine-DNA methyltransferase (MGMT) DNA-repair gene was methylated (Hegi et al., 2005).

[44] Post-operatively the patient underwent conventional external beam radiotherapy of 6000 cGy in 30 fractions. Concurrent temozolomide at 75 mg/m2 was administered during radiotherapy (Stupp et al., 2005). An MR image taken at the completion of radiotherapy was unchanged and demonstrated no evidence of progression. The patient then underwent a leukapheresis to obtain sufficient cells for immunological monitoring purposes. The patient received three intradermal (i.d.) injections of PEPvIII-3 (LEEKKGNYVVTDHC), conjugated to keyhole limpet hemocyanin (KLH) at a 1:1 ratio (w/w) (PEPvIII-KLH) (500 μg/immunization) with granulocyte-macrophage colony-stimulating factor (GM-CSF) (142 μg/immunization) every two weeks over an interval of 6 weeks (the induction phase). Thereafter, he underwent a second leukapheresis for immunological monitoring purposes. At this point, the patient began maintenance cycles of temozolomide of 150 mg/m2 on day 1-5. Beginning on day 19 of each cycle, complete blood counts were monitored every other day until there was evidence of recovery of the white blood cell count nadir. At nadir recovery, the patient received the vaccine i.d., usually on day 23 (range = 19-25) of his 28-day cycle.

EXAMPLE 2

[45] Delayed type hypersensitivity (DTH) testing to common recall antigens and the components of the vaccine were evaluated prior to the start of the vaccines, after the 3rd vaccine and monthly during his maintenance cycle on day 26. Prior to the start of the vaccine and after the completion of radiation and concurrent temozolomide the patient was only reactive to Candida and had no DTH reaction to the components of the vaccine, PEPvIII or KLH. However, after the 3rd vaccination, the patient became responsive to the KLH component of the vaccine. After the 10th vaccination, and while receiving concurrent temozolomide, he became reactive to the PEPvIII component of the vaccine. For comparison, of the patients that received the vaccine without cycled temozolomide (n=22), less than 15% ever became reactive to the PEPvIII component. After the most recent follow-up and administration of the 14th vaccination, the patient was markedly indurated (16 x 15 mm) at the PEPvIII DTH site. This would indicate that the temozolomide did not negatively influence the development of DTH responses in this particular patient.

EXAMPLE 3

[46] To determine if PEPvIII-specific humoral responses were induced, serum was obtained from the patient monthly and was stored at -20°C before analysis in a PEPvIII-Dynabead® assay. PEPvIII or the extracellular domain of EGFRvIII (EGFRvIII-ECD) were covalently linked to magnetic microspheres that were used to capture specific antibodies from patient's serum (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. All serum samples are initially diluted 1:10 with phosphate-buffered saline (PBS) + 0.5% bovine serum albumin (BSA) and assayed in triplicate. To determine specificity, an additional sample set was pre-incubated for 15 minutes with 500ng of the PEPvIII peptide to block any anti-PEPvIII that would be captured by the PEPvIII conjugated Dynabeads. Standards of human-mouse chimeric anti-PEPvIII antibody (81-0.11ng/ml) are run

with each assay along with positive (patient sample ACT4) and negative (normal donor serum) controls. The flow cytometer was standardized with PE-FACS microbeads and un-reacted PEPvIII Dynabeads. Prior to the administration of the vaccine, there were no detectable humoral responses to the EGFRvIII. After the vaccination, there was a significant increase in IgG responses to EGFRvIII to a mean fluorescent intensity (MFI) of 13 and the humoral responses have been maintained despite administration the temozolomide.

EXAMPLE 4

[47] To determine if CD8+ cytotoxic responses were induced to PEPvIII, the patient's peripheral blood mononuclear cells (PBMCs) from each leukapheresis and monthly PBMCs were stimulated with either tetanus toxoid (QYIKANSKFIGITE) (SEQ ID NO: 5) (10μg/ml) (positive control), PEP-1 (HDTVYCVKGNKELE) (SEQ ID NO: 4) (10μg/mL) (negative control), PEPvIII (10μg/mL) (vaccine component), or KLH (10μg/ml) (vaccine component). A negative control included un-stimulated cells. The corresponding isotype controls were used for each condition, including γ-interferon (IFN) secretion. All wells were incubated for 6 hr at 37°C with GolgiplugTM (Pharmingen, San Diego, CA), a protein transport inhibitor that blocks the intracellular transport process. After incubation, the cells were washed and blocked for non-specific binding using purified anti-CD16 antibody (Pharmingen) and rabbit serum (Pharmingen). The cells were stained for surface markers (CD3, CD4, CD8) by incubating with the appropriate fluorescein-isothiocyanate and allophycocyanin labeled fluorescence-labeled primary antibody or isotype control (Pharmingen). Cells were then fixed with Cytofix/Cytoperm (BD Biosciences, San Jose, CA) and then incubated with phycoerythrin-labeled antibody against γ-IFN or the isotype control. After staining, cells were washed and a minimum of 1 x 10^5 live, gated events were assessed by flow cytometry on a FACSCalibur flow cytometer using Cellquest software (BD

Immunocytometry systems, San Jose, CA). Prior to receiving the vaccine the patient had minimal response in the un-stimulated controls and with the PEP-1 negative control. After receiving the vaccine, and during administration of the temozolomide, there was an increase in PEPvIII-specific γ-IFN producing $CD8^+$ T cells.

EXAMPLE 5

[48] To characterize the response of the various T cell populations during a cycle of temozolomide (5/21 schedule) and concurrently administered vaccine (day 19 on this example), we obtained peripheral blood on days 0, 3, 5, 12, 19, 23, 25 and 26. By flow analysis cytometry, we investigated the percentage of the CD8+ T cell and CD4+CD25+FoxP3+ regulatory T cells subsets during an immunochemotherapy cycle. All fluorescence-conjugated monoclonal antibodies (mAb) (PerCP-Cy5.5-CD3, FITC-CD8, APC-CD4 and PE-CD25) were purchased from BD Biosciences except the FITC-labeled mAb of FoxP3 was made by eBioscience. The surface and intracellular staining of peripheral blood cells were performed according to the standard procedures provided by the manufacturer. Results were analyzed by FACSCalibur flow cytometer using Cellquest Pro software (BD Biosciences). In contrast to the decline of the CD8+ T cell subset, the Treg population started to increase after the administration of temozolomide for 3 days and reached its peak (0.9% of total $CD4^+$ T cells) on day 12. The Tregs then began to drop until day 23 while the $CD8^+$ T cell numbers started to recover. At the end of the course, both of $CD8^+$ T cell and Treg populations recovered to pre-treatment levels. The vaccination resulted in a boost of CD8+ cytotoxic T cells during a period of relative diminished Tregs.

EXAMPLE 6

[49] Over the last 15 months, the patient underwent complete physical examination and brain MR imaging at two-month intervals. His exam has remained stable and MR imaging has failed to demonstrate any evidence of recurrence. He works full time without impairment and has a Karnofsky performance status (KPS) of 100% and mini-mental status exam score of 30/30. His neurological exam is completely normal.

[50] This report suggests that concurrent administration of chemotherapy with immunotherapy may be possible if the timing of the treatments are carefully monitored. In the case reported, there are several findings that indicate that the co-administration of the temozolomide has not affected the efficacy of the PEPvIII-KLH vaccine. First, the patient has not yet progressed at 15 months of follow-up. This was the median TTP for patients (n=22) that received only vaccination therapy. Thus, the clinical efficacy does not appear to have been effected compared to patients that did not receive the concurrently administered temozolomide. The patient developed DTH responses to the PEPvIII component of the vaccine, even while receiving temozolomide, whereas only 15% of the patients receiving the vaccine alone developed these types of responses. Furthermore, the area of PEPvIII DTH reactivity has continued to increase with subsequent vaccinations. Third, IgG specific responses to PEPvIII were induced after the 3rd vaccination and have been maintained while receiving the concurrent temozolomide. Fourth, the induced PEP-3 specific $CD3^+CD8^+\gamma$-IFN producing T cells do not appear to be diminished during cycles of concurrently administered temozolomide but appear enhanced during the concurrently administered temozolomide. Finally, we have followed the $CD8^+$ T cell and Treg populations during a single treatment cycle and found that there appears to be a window of T effector ($CD8^+$ T cell) responsiveness with a relative diminution of Tregs. Thus, the concurrent administration of temozolomide and

vaccine does not appear to diminish the induced immune responses, in the manner in which we have described.

[51] The use of lymphodepletion to augment immunological responses has been described in both murine model systems (Berenson et al., 1975; Cheever et al., 1980; North, 1982) and in human cancer patients (Dudley et al., 2002; Dudley et al., 2005). Multiple mechanisms have been proposed to be responsible for these enhanced anti-tumor responses. Lymphodepletion may remove competition at the surface of antigen presenting cells (Kedl et al., 2000), enhance the availability of cytokines such as IL-7 and IL-15, which augment T cell activity (Gattinoni et al., 2005) and deplete the immune inhibitory Tregs (Anthony et al., 2005). Chemotherapy could also potentially augment immunological responsiveness by enhancing immune priming and presentation (Nowak et al., 2002), enhancing antigen expression (Aquino et al., 1998), and enhancing targets for immune eradication (Ciusani et al., 2002). When a vaccination is administered during the nadir of temozolomide, we hypothesized that there may be an enhanced effector response. Those effector responses may be secondary to a lag in the recovery of Tregs thus allowing a greater clonotypic expansion than would have otherwise been seen without the temozolomide. This was certainly observed during a monitored chemoimmunotherapy cycle on this particular patient. The lag of recovery of Tregs relative to effector T cells is not surprising given the normal physiological roles of immune cell responses. In order to mount an immune response, T effectors would need to become activated, proliferate and mediate their response. If this remained unchecked by homeostatic mechanisms such as Tregs, then the T cell proliferation would escalate unabated. Therefore, the delay of Treg response would allow for efficacious immune responses but eventual down-modulation/regulation of this response.

[52] In conclusion, this case report suggests that co-administration of chemotherapy and immunotherapy may not be deleterious.

References

The disclosure of each reference cited is expressly incorporated herein.

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Liu, M., B. Dai, et al. (2006). "FoxM1B is overexpressed in human gliobastomas and critically regulates the tumorigenicity of glioma cells." Can Res **66**(7): 3593-3602.

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We Claim:

1. The use of a treatment effective amount of an EGFRvIII peptide in the manufacture of a medicament for treating a tumor in a subject, wherein said medicament is to be administered with a treatment effective amount of a chemotherapeutic agent which induces lymphopenia, said chemotherapeutic agent is temozolomide or a pharmaceutically acceptable salt thereof.

2. The use of claim 1 wherein the EGFRvIII peptide is conjugated to keyhole limpet hemocyanin (KLH).

3. The use of claim 1 wherein said medicament is to be administered concurrently with an effective amount of GM-CSF as an adjuvant.

4. The use of claim 1 wherein the EGFRvIII peptide has the sequence H-LEEKKGNYWTDHS-OH (SEQ ID NO: 1).

5. The use of claim 1 wherein the EGFRvIII peptide has the sequence LEU-GLU- GLU-LYS-LYS-GLY-ASN-TYR-VAL-VAL-THR-ASP-HIS (SEQ ID NO: 2)

6. The use of claim 1 wherein the EGFRvIII peptide has the sequence LEU-GLU- GLU-LYS-LYS-GLY-ASN-TYR-VAL-VAL-THR-ASP-HIS-CYS (SEQ ID NO: 3) and wherein KLH is conjugated to the CYS residue.

7. The use of claim 6 wherein the EGFRvIII peptide is conjugated to the KLH with a heterobifunctional cross-linker.

8. The use of claim 7 wherein the heterobifunctional cross-linker is sulfosuccinimidyl 6-[3 '(2-pyridyldithio)-propionamido] hexanoate.

9. The use of claim 1 wherein the tumor is a malignant glioma.

10. The use of claim 1 wherein the tumor is an astrocytoma.

11. The use of claim 1 wherein the chemotherapeutic agent is a purine analogue.

12. The use of claim 1 wherein the chemotherapeutic agent is fludarabine and/or 2-chlorodeoxyadenosine.

13. The use of claim 1 wherein the tumor is a lung tumor.

14. The use of claim 1 wherein the tumor is a breast tumor.

15. The use of claim 1 wherein the tumor is a head and neck cancer.

16. The use of a treatment effective amount of an EGFRvIII peptide, the EGFRvIII peptide conjugated to KLH, in the manufacture of a medicament for treating tumor in a subject, wherein said medicament is to be administered concurrently with an effective amount of GM-CSF as an adjuvant and wherein said medicament is to be administered with an effective amount of temozolomide or a pharmaceutically acceptable salt thereof.

17. The use of claim 16 wherein the tumor is a malignant glioma.

18. The use of claim 16 wherein the tumor is an astrocytoma.

19. The use of claim 16 wherein the tumor is a lung tumor.

20. The use of claim 16 wherein the tumor is a breast tumor.

21. The use of claim 16 wherein the tumor is a head and neck cancer.

22. The use of a treatment effective amount of an EGFRvIII peptide in the manufacture of a medicament for treating a tumor in a subject, wherein said medicament is to be administered with a treatment effective amount of temozolomide or a pharmaceutically acceptable salt thereof.

23. The use of claim 22 wherein the EGFRvIII peptide is conjugated to keyhole limpet hemocyanin (KLH).

24. The use of claim 22 wherein said medicament is to be administered concurrently with an effective amount of GM-CSF as an adjuvant.

25. The use of claim 22 wherein the EGFRvIII peptide has the sequence H-LEEKKGNYWTDHS-OH (SEQ ID NO: 1).

26. The use of claim 22 wherein the EGFRvIII peptide has the sequence LEU-GLU-GLU-LYS-LYS-GLY-ASN-TYR-VAL-VAL-THR-ASP-HIS (SEQ ID NO: 2)

27. The use of claim 22 wherein the EGFRvIII peptide has the sequence LEU-GLU-GLU-LYS-LYS-GLY-ASN-TYR-VAL-VAL-THR-ASP-HIS-CYS (SEQ ID NO: 3) and wherein KLH is conjugated to the CYS residue.

28. The use of claim 27 wherein the EGFRvIII peptide is conjugated to the KLH with a heterobifunctional cross-linker.

29. The use of claim 28 wherein the heterobifunctional cross-linker is sulfosuccinimidyl 6-[3 '(2-pyridyldithio)-propionamido] hexanoate.

30. The use of claim 22 wherein the tumor is a malignant glioma.

31. The use of claim 22 wherein the tumor is an astrocytoma.

32. The use of claim 22 wherein the tumor is a lung tumor.

33. The use of claim 22 wherein the tumor is a breast tumor.

34. The use of claim 22 wherein the tumor is a head and neck cancer.

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Ser Ile Gln Trp Arg Asp Ile Val Ser Ser Asp Phe Leu Ser Asn Met
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Ser Met Asp Phe Gln Asn His Leu Gly Ser Cys Gln Lys Cys Asp Pro
            180                 185                 190
Ser Cys Pro Asn Gly Ser Cys Trp Gly Ala Gly Glu Glu Asn Cys Gln
        195                 200                 205
Lys Leu Thr Lys Ile Ile Cys Ala Gln Gln Cys Ser Gly Arg Cys Arg
    210                 215                 220
Gly Lys Ser Pro Ser Asp Cys Cys His Asn Gln Cys Ala Ala Gly Cys
225                 230                 235                 240
Thr Gly Pro Arg Glu Ser Asp Cys Leu Val Cys Arg Lys Phe Arg Asp
                245                 250                 255
Glu Ala Thr Cys Lys Asp Thr Cys Pro Pro Leu Met Leu Tyr Asn Pro
            260                 265                 270
Thr Thr Tyr Gln Met Asp Val Asn Pro Glu Gly Lys Tyr Ser Phe Gly
        275                 280                 285
Ala Thr Cys Val Lys Lys Cys Pro Arg Asn Tyr Val Val Thr Asp His
    290                 295                 300
Gly Ser Cys Val Arg Ala Cys Gly Ala Asp Ser Tyr Glu Met Glu Glu
305                 310                 315                 320
Asp Gly Val Arg Lys Cys Lys Lys Cys Glu Gly Pro Cys Arg Lys Val
                325                 330                 335
Cys Asn Gly Ile Gly Ile Gly Glu Phe Lys Asp Ser Leu Ser Ile Asn
            340                 345                 350
Ala Thr Asn Ile Lys His Phe Lys Asn Cys Thr Ser Ile Ser Gly Asp
        355                 360                 365
Leu His Ile Leu Pro Val Ala Phe Arg Gly Asp Ser Phe Thr His Thr
    370                 375                 380
Pro Pro Leu Asp Pro Gln Glu Leu Asp Ile Leu Lys Thr Val Lys Glu
385                 390                 395                 400
Ile Thr Gly Leu Ser
                405
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