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(54) Title: CARCINOMA ASSOCIATED ANTIGEN (NG1) MONOCLONAL ANTIBODIES AGAINST NG1, METHODS OF PRODUCING THESE ANTIBODIES AND USE THEREFOR

(57) Abstract

Carcinoma associated antigen (NG1) and monoclonal antibodies and methods for detecting and ameliorating malignant disease. The monoclonal antibodies are specifically reactive with epitopes present on NG1.

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CARCINOMA ASSOCIATED ANTIGEN (NG1) MONOCLONAL ANTIBODIES AGAINST NG1, METHODS OF PRODUCING THESE ANTIBODIES AND USE THEREFOR

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

This invention relates to a novel carcinoma associated antigen (NG1) which is associated with various malignancies and monoclonal antibodies specific for epitopes on NG1.

2. DESCRIPTION OF THE BACKGROUND ART

Murine monoclonal antibodies have been shown to mediate effective cytotoxicity to target cells *in vitro*; however, when utilized *in vivo* with humans, they have not achieved remarkable results. This is partially due to (i) the foreign nature of the injected murine proteins which leads to the development of a human anti-mouse antibody (HAMA) response, and (ii) the human effector functions which may not be fully activated by a murine antibody. In contrast, the dramatic effects which have been observed in systemically treating septic patients with purified human monoclonal or polyclonal antibodies (Ziegler, *et al., The New England J.Med*, 324:429-436, 1991; Kurtzberg, *et al., Am.J.Pediatr.Hematol.Oncol.*, 9:299-301, 1987) and by intralesional therapy of melanoma suggests that the clinical use of human monoclonal antibodies (Irie, *et al., Proc.Natl.Acad.Sci. USA*, 83:8694-8698, 1986) will be successful. Thus, the potential use of human Mabs for cancer therapy is attractive.

Recent progress in the field of HuMAb technology has made it possible to generate numerous hybridomas of various specificities (Glassy, [in press] *In Therapeutic Applications of Monoclonal Antibodies in Cancer*, Marcel Dekker, Inc., NY). Combined with knowledge gained in the understanding of the human immune response to cancer antigens (Lloyd, *et al., Cancer Res.*, 49:3445-3451, 1989), several HuMAbs against tumor associated

antigens (TAAs) have been produced and characterized. The reported tumor associated antigens recognized by HuMAbs include cell surface (Yoshikawa, *et al., Jpn. J. Cancer Res. (Gann)*, 80:546-553, 1989; Yamaguchi, *et al., Proc.Natl.Acad.Sci.USA*, 84:2416-2420; Haspel, *et al., Cancer Res.*, 45:3951-3961, 1985; Cote, *et al., Proc.Natl.Acad.Sci.USA*, 83:2959-2963, 1986; Glassy, *Cancer Res.*, 47:5181-5188, 1987; and Borup-Christensen, *et al., Cancer Detect. Prevent.Suppl.*, 1:207-215), cytoplasmic (Haspel, *et al., Cancer Res.*, 45:3951-3961, 1985; Cote, *et al., Proc.Natl.Acad.Sci.USA*, 83:2959-2963, 1986; Glassy, *Cancer Res.*, 47:5181-5188; Borup-Christensen, *et al., Cancer Detect Prevent Suppl.*, 1:207-215, 1987; Kan-Mitchell, *et al., Cancer Res.*, 49:4536-4541, 1989; and Yoshikawa, *et al., Jpn.J. Cancer Res.*, 77:1122-1133, 1986), and nuclear antigens (McKnight, *et al., Hum.Antibod. Hybridomas*, 1:125-129, 1990).

At present, methods of limited effectiveness exist for treatment of various malignancies. Those drugs which are administered generally have severe side effects associated with their use. Accordingly, there exists a significant need to identify and purify an antigen associated with malignant diseases and to produce monoclonal antibodies which bind to epitopes on this antigen. Further, these antibodies are suitable agents for the diagnosis and treatment of malignancies expressing the NG1 antigen.

SUMMARY OF THE INVENTION

One way to ameliorate malignancies would be to suppress cells which preferentially express an antigen associated with carcinoma. This suppression could be accomplished, for example, by active immunization using an antigen, or a derivative thereof, preferentially present in malignant cells or by passive immunization by providing antibody to the antigen.

In order to provide a means to ameliorate malignant disease the invention provides substantially purified antigen which is preferentially expressed by malignant cells and monoclonal antibodies which bind to epitopes on the antigen. These monoclonal antibodies, if desired, can be labeled for therapeutic or diagnostic use.

An object of the present invention is to provide a method of detecting the carcinoma associated antigen (NG1) preferentially expressed in various malignant cells and tissues using a detectably labeled monoclonal antibody which binds to NG1 and determining whether the detectably labeled monoclonal antibody has bound to NG1.

Another object of the present invention is to provide methods for the *in vitro* and *in vivo* diagnosis of malignancy using detectably labeled monoclonal antibodies which react with an epitope present on NG1.

Another object of the invention is to provide methods for ameliorating malignant disease in an animal using unlabeled or therapeutically labeled monoclonal antibodies which react with NG1.

Alternatively, the invention provides methods for ameliorating malignant disease in an animal by inducing an immune response to the malignancy by immunizing the animal with NG1.

The present invention thus relates to a method of detecting NG1 which comprises contacting a source suspected of containing NG1 with a diagnostically effective amount of detectably labeled monoclonal antibody, or fragment thereof, having the specificity of a monoclonal antibody of the invention and determining whether the antibody binds to the source.

The invention further relates to a method of suppressing malignant disease in an animal which comprises administering to the animal a therapeutically effective amount of a (1) monoclonal antibody, or fragment thereof, wherein the antibody has the specificity of a monoclonal antibody of the invention, or (2) NG1.

A major advantage in the therapeutic and diagnostic use of NG1 and monoclonal antibodies which bind to epitopes of NG1 is that the NG1 antigen occurs at high frequency in malignant cells. Consequently, there is a much greater probability of binding occurring to a malignant cell than to a normal cell. As a result of this fact, it is possible to use concentrations of the monoclonal antibody of the invention which are clinically effective, but pose minimal or no risk to normal host cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a substantially purified antigen (NG1) which is preferentially expressed by malignant cells and to monoclonal antibodies with epitopic specificity for NG1. These monoclonal antibodies are highly useful for both the *in vitro* and *in vivo* immunological detection of antigens associated with these malignancies and for immunotherapy of cells bearing NG1.

In a preferred embodiment of the invention a monoclonal antibody is disclosed which binds to an epitope on NG1. This specificity enables the monoclonal antibody, and like monoclonal antibodies with like specificity, to be used to suppress growth of malignant cells having NG1. As a consequence, these monoclonal antibodies are useful in ameliorating malignant diseases such as colorectal carcinoma, gastric cancer, pancreatic cancer, and adenocarcinoma.

Methods of Producing and Characterizing Monoclonal Antibodies to NG1

The general method used for production of hybridomas secreting monoclonal antibodies is well known (Kohler and Milstein, *Nature*, 256:495, 1975). Briefly, lymphocytes isolated from regional draining lymph nodes of five separate cancer patients with either melanoma, teratocarcinoma, cervix, glioma or lung cancer, were obtained from surgical specimens, pooled, and then fused with SHFP-1. Hybridomas were screened for production of antibody which bound to cancer cell lines.

In one aspect, the present invention is directed to monoclonal antibodies, and hybridomas which produce them, which are reactive with NG1. The isolation of hybridomas secreting monoclonal antibodies with the reactivity of the monoclonal antibodies of the invention can be accomplished using routine screening techniques to determine the elementary reaction pattern

of the monoclonal antibody of interest. Thus, if a monoclonal antibody being tested binds with NG1, then the antibody being tested and the antibody produced by the hybridomas of the invention are equivalent.

Alternatively, since the invention teaches the substantial purification of the novel NG1 antigen, it is now possible to use this antigen for purposes of immunization to produce more hybridomas which secrete monoclonal antibodies specific for the NG1 antigen. This approach would have the added advantage of decreasing the repertoire of monoclonal antibodies generated by limiting the number of antigenic determinants presented at immunization. The monoclonal antibodies so produced could be screened for specificity for NG1 using standard techniques, for example, by binding NG1 to microtiter plate and measuring binding of the monoclonal antibody by an ELISA assay. The term "substantially pure form" when applied to NG1 means that NG1 is essentially free of other proteins with which NG1 is normally associated in nature.

It is also possible to evaluate, without undue experimentation, a monoclonal antibody to determine whether it has the same specificity as a monoclonal antibody of the invention by determining whether the monoclonal antibody being tested prevents a monoclonal antibody of the invention from binding 1) to the NG1 antigen, or 2) a malignant cell expressing the NG1 antigen with which the monoclonal antibody of the invention is normally reactive. If the monoclonal antibody being tested competes with the monoclonal antibody of the invention, as shown by a decrease in binding by the monoclonal antibody of the invention, then it is likely that the two monoclonal antibodies bind to the same, or a closely related, epitope.

Still another way to determine whether a monoclonal antibody has the specificity of a monoclonal antibody of the invention is to pre-incubate the monoclonal antibody of the invention with the NG1 antigen with which it is normally reactive, and determine if the monoclonal antibody being tested is inhibited in its ability to bind the antigen. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the monoclonal antibody of the invention.

While the *in vivo* use of a monoclonal antibody from a foreign donor species in a different host recipient species is usually uncomplicated, a potential problem which may arise is the appearance of an adverse immunological response by the host to antigenic determinants present on the donor antibody. In some instances, this adverse response can be so severe as to curtail the *in vivo* use of the donor antibody in the host. Further, the adverse host response may serve to hinder the malignancy suppressing efficacy of the donor antibody. One way in which it is possible to circumvent the likelihood of an adverse immune response occurring in the host is by using chimeric antibodies (Sun, *et al., Hybridoma*, 5 (Supplement 1):S17, 1986; Oi, *et al., Bio Techniques*, 4(3): 214, 1986). Chimeric antibodies are antibodies in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species. Typically, a chimeric antibody will comprise the variable domains of the heavy (V_H) and light (V_L) chains derived from the donor species producing the antibody of desired antigenic specificity, and the constant domains of the heavy (C_H) and light (C_L) chains derived from the host recipient species. It is believed that by reducing the exposure of the host immune system to the antigenic determinants of the donor antibody domains, especially those in the C_H region, the possibility of an adverse immunological response occurring in the recipient species will be reduced. Thus, for example, it is possible to produce a chimeric antibody for *in vivo* clinical use in humans which comprises mouse V_H and V_L domains coded for by DNA isolated from a hybridoma of the invention, such as ATCC HB 11230, and C_H and C_L domains coded for with DNA isolated from a human leukocyte.

Although the present invention encompasses all monoclonal antibodies which recognize the novel carcinoma antigen NG1, especially preferred are monoclonal antibodies of human origin. Such human monoclonal antibodies are exemplified by HuMAb(NG1) which is an IgG antibody produced by a hybridoma having accession number ATCC HB 11230.

Under certain circumstances, monoclonal antibodies of one isotype might be more preferable than those of another in terms of their diagnostic or therapeutic efficacy. For example, from studies on antibody-mediated

cytolysis, it is known that unmodified mouse monoclonal antibodies of isotype gamma-2a and gamma-3 are generally more effective in lysing target cells than are antibodies of the gamma-1 isotype. This differential efficacy is thought to be due to the ability of the gamma-2a and gamma-3 isotypes to more actively participate in the cytolytic destruction of target cells. Particular isotypes of a monoclonal antibody can be prepared either directly, by selecting from the initial fusion, or prepared secondarily, from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection technique to isolate class-switch variants (Steplewski, *et al., Proceedings of the National Academy of Science,* U.S.A., 82:8653, 1985; Spira, *et al., Journal of Immunological Methods,* 74:307, 1984). Thus, the monoclonal antibodies of the invention would include class-switch variants having specificity for an epitope on NG1.

The isolation of other hybridomas secreting monoclonal antibodies with the specificity of the monoclonal antibodies of the invention can also be accomplished by one of ordinary skill in the art by producing anti-idiotypic antibodies (Herlyn, *et al., Science*, 232:100, 1986). An anti-idiotypic antibody is an antibody which recognizes unique determinants present on the monoclonal antibody produced by the hybridoma of interest. These determinants are located in the hypervariable region of the antibody. It is this region which binds to a given epitope and, thus, it is responsible for the specificity of the antibody. The anti-idiotypic antibody can be prepared by immunizing an animal with the monoclonal antibody of interest. The animal immunized will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants. By using the anti-idiotypic antibodies of the second animal, which are specific for the monoclonal antibodies produced by a single hybridoma which was used to immunize the second animal, it is now possible to identify other clones with the same idiotype as the antibody of the hybridoma used for immunization.

Idiotypic identity between monoclonal antibodies of two hybridomas demonstrates that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using

antibodies to the epitopic determinants on a monoclonal antibody it is possible to identify other hybridomas expressing monoclonal antibodies of the same epitopic specificity.

It is also possible to use the anti-idiotype technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody. Thus, in this instance, the anti-idiotypic monoclonal antibody could be used for immunization since the anti-idiotype monoclonal antibody binding domain effectively acts as an antigen.

When the monoclonal antibodies of the invention are used in the form of fragments, such as, for example, Fab and $F(ab')_2$, and especially when these fragments are therapeutically labeled, any isotype can be used since amelioration of the malignancy in these situations is not dependent upon complement-mediated cytolytic destruction of those cells bearing the NG1 antigen.

The monoclonal antibodies of the invention can be used in any animal in which it is desirable to administer *in vitro* or *in vivo* immunodiagnosis or immunotherapy. The term "animal" as used herein is meant to include both humans as well as non-humans.

The term "antibody" as used in this invention is meant to include intact molecules as well as fragments thereof, such as for example, Fab and $F(ab')_2$, which are capable of binding the epitopic determinant.

DIAGNOSTIC USES

The monoclonal antibodies of the invention are suited for use, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the monoclonal antibodies in these immunoassays can be detectably labeled in various ways. Examples of

types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of the antigens using the monoclonal antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

The monoclonal antibodies of the invention can be bound to many different carriers and used to detect the presence of NG1. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, and bioluminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the monoclonal antibody, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the monoclonal antibody of the invention can be done using standard techniques common to those of ordinary skill in the art.

For purposes of the invention, NG1 may be detected by the monoclonal antibodies of the invention when present in biological fluids and tissues. Any sample containing a detectable amount of NG1 can be used. A sample can be a liquid such as urine, saliva, cerebrospinal fluid, blood, serum and

the like, or a solid or semi-solid such as tissues, feces, and the like, or, alternatively, a solid tissue such as those commonly used in histological diagnosis.

Another technique which may also result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin, which reacts with avidin, or dinitrophenyl, pyridoxal, and fluorescein, which can react with specific anti-hapten antibodies.

As used in this invention, the term "epitope" is meant to include any determinant capable of specific interaction with the monoclonal antibodies of the invention. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

In using the monoclonal antibodies of the invention for the *in vivo* detection of antigen, the detectably labeled monoclonal antibody is given in a dose which is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled monoclonal antibody is administered in sufficient quantity to enable detection of the site having the NG1 antigen for which the monoclonal antibodies are specific.

The concentration of detectably labeled monoclonal antibody which is administered should be sufficient such that the binding to those cells having NG1 is detectable compared to the background. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

As a rule, the dosage of detectably labeled monoclonal antibody for *in vivo* diagnosis will vary depending on such factors as age, sex, and extent of disease of the individual. The dosage of monoclonal antibody can vary from about 0.01 mg/m^2 to about 500 mg/m^2, preferably 0.1 mg/m^2 to about 200

mg/m^2, most preferably about 0.1 mg/m^2 to about 10 mg/m^2. Such dosages may vary, for example, depending on whether multiple injections are given, tumor burden, and other factors known to those of skill in the art.

For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay which is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 keV range, which may be readily detected by conventional gamma cameras.

For *in vivo* diagnosis radioisotopes may be bound to immunoglobulin either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar molecules. Typical examples of metallic ions which can be bound to the monoclonal antibodies of the invention are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, ^{90}Y, and ^{201}Tl.

The monoclonal antibodies of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

The monoclonal antibodies of the invention can be used to monitor the course of amelioration of malignancy in an animal. Thus, by measuring the increase or decrease in the number of cells expressing NG1 or changes in

the concentration of NG1 present in various body fluids, it would be possible to determine whether a particular therapeutic regimen aimed at ameliorating the malignancy is effective.

THERAPEUTIC USES

The term "ameliorate" denotes a lessening of the detrimental affect of the malignancy in the animal receiving therapy. The term "therapeutically effective" means that the amount of monoclonal antibody or NG1 used is of sufficient quantity to ameliorate the malignancy.

The term "immunogenically effective amount," as used in the invention, is meant to denote that amount of NG1 antigen which is necessary to induce an ameliorative immune response to the malignancy, for example, by stimulating the production of antibodies which will bind to NG1 epitopes.

NG1 can be administered parenterally by injection, rapid infusion, nasopharyngeal absorption, dermal absorption, and orally. Preparations for parenteral administration include sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers for occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable forms for suspending the liposomes include emulsions, suspensions, solutions, syrups, and elixirs containing inert diluents commonly used in the art, such as purified water. Besides the inert diluents, such compositions can also include adjuvants, wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

It is also possible for the antigenic preparations containing NG1 to include an adjuvant. Adjuvants are substances that can be used to non-specifically augment a specific immune response. Normally, the adjuvant and the antigen are mixed prior to presentation to the immune system, or presented

separately, but into the same site of the animal being immunized. Adjuvants can be loosely divided into several groups based on their composition. These groups include oil adjuvants (for example, Freund's Complete and Incomplete), mineral salts (for example, $AlK(SO_4)_2$, $AlNa(SO_4)_2$, $AlNH_4(SO_4)$, silica, alum, $Al(OH)_3$, $Ca_3(PO_4)_2$, kaolin, and carbon), polynucleotides (for example, poly IC and poly AU acids), and certain natural substances (for example, wax D from *Mycobacterium tuberculosis*, as well as substances found in *Corynebacterium parvum, Bordetella pertussis,* and members of the genus *Brucella*).

The physical form of the NG1 antigen which is used to immunize an animal can be either aggregated or non-aggregated. Aggregated NG1 can be produced from non-aggregated NG1 by such common techniques as, for example, treatment with glutaraldehyde or other cross-linking agents. The aggregated NG1 thus derived could then be used for purposes of producing a malignancy ameliorating composition effective in inducing an active immune reaction.

However, regardless of whether an animal is immunized with aggregated or non-aggregated, both of these forms of NG1 should cause the production of antibodies to NG1. Thus, it is possible to use these anti-NG1 antibodies diagnostically as, for example, in a kit to detect the presence of NG1 in a specimen.

The NG1 antigen preparations of the invention can be used to induce the production of antibodies which will bind to epitopic determinants of NG1. A particularly useful method in enhancing the production of antibodies to NG1 is to first immunize with the NG1 antigenic preparation of the invention followed by a later immunization.

Many different techniques exist for the timing of the immunizations when a multiple immunization regimen is utilized. It is possible to use the antigenic preparation of the invention more than once to increase the levels and

diversity of expression of the immunoglobulin repertoire expressed by the immunized animal. Typically, if multiple immunizations are given, they will be spaced one to two months apart.

Generally, the dosage of NG1 administered to an animal will vary depending on such factors as age, condition, sex and extent of disease, if any, and other variables which can be adjusted by one of ordinary skill in the art.

The antigenic NG1 preparations of the invention can be administered as either single or multiple dosages and can vary from about 50 mg to about 500 mg for the NG1 antigen per dose, more preferably about 50 mg to about 300 mg NG1 antigen per dose, most preferably about 100 mg to about 200 mg NG1 antigen per dose.

The monoclonal antibodies of the invention can also be used, alone or in combination with effector cells or molecules (Douillard, *et al., Hybridoma,* 5 (Supp. 1: S139, 1986), for immunotherapy in an animal having a tumor which expresses NG1 antigen with epitopes reactive with the monoclonal antibodies of the invention.

When used for immunotherapy, the monoclonal antibodies of the invention may be unlabeled or labeled with a therapeutic agent. These agents can be coupled either directly or indirectly to the monoclonal antibodies of the invention. One example of indirect coupling is by use of a spacer moiety. These spacer moieties, in turn, can be either insoluble or soluble (Diener, *et al., Science,* 231:148, 1986) and can be selected to enable drug release from the monoclonal antibody molecule at the target site. Examples of therapeutic agents which can be coupled to the monoclonal antibodies of the invention for immunotherapy are drugs, radioisotopes, lectins, and toxins.

The drugs with which can be conjugated to the monoclonal antibodies of the invention include non-proteinaceous as well as proteinaceous drugs. The terms "non-proteinaceous drugs" encompasses compounds which are classically referred to as drugs, for example, mitomycin C, daunorubicin, and vinblastine.

The proteinaceous drugs with which the monoclonal antibodies of the invention can be labeled include immunomodulators and other biological response modifiers. The term "biological response modifiers" is meant to encompass substances which are involved in modifying the immune response in such manner as to enhance the destruction of the antigen bearing tumor for which the monoclonal antibodies of the invention are specific. Examples of immune response modifiers include such compounds as lymphokines. Lymphokines include tumor necrosis factor, interleukins 1, 2, and 3, lymphotoxin, macrophage activating factor, migration inhibition factor, colony stimulating factor, and interferon. Interferons with which the monoclonal antibodies of the invention can be labeled include alpha-interferon, beta-interferon, and gamma-interferon and their subtypes.

In using radioisotopically conjugated monoclonal antibodies of the invention for immunotherapy certain isotypes may be more preferable than others depending on such factors as leukocyte distribution as well as isotype stability and emission. If desired, the tumor cell distribution can be evaluated by the *in vivo* diagnostic techniques described above. Depending on the malignancy some emitters may be preferable to others. In general, alpha and beta particle-emitting radioisotopes are preferred in immunotherapy. For example, if an animal has solid tumor foci, as in a carcinoma, a high energy beta emitter capable of penetrating several millimeters of tissue, such as ^{90}Y, may be preferable. On the other hand, if the malignancy consists of simple target cells, as in the case of leukemia, a short range, high energy alpha emitter, such as ^{212}Bi, may be preferable. Examples of radioisotopes which can be bound to the monoclonal antibodies of the invention for therapeutic purposes are ^{125}I, ^{131}I, ^{90}Y, ^{67}Cu, ^{212}Bi, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, and ^{188}Re.

Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. However, ricin is a toxic lectin which has been used immunotherapeutically. This is preferably accomplished by binding the alpha-peptide chain of ricin, which is responsible for toxicity, to the antibody molecule to enable site specific delivery of the toxic effect.

Toxins are poisonous substances produced by plants, animals, or microorganisms that, in sufficient dose, are often lethal. Diphtheria toxin is a substance produced by *Corynebacterium diphtheria* which can be used therapeutically. This toxin consists of an alpha and beta subunit which under proper conditions can be separated. The toxic A component can be bound to an antibody and used for site specific delivery to a NG1 bearing cell for which the monoclonal antibodies of the invention are specific. Other therapeutic agents which can be coupled to the monoclonal antibodies of the invention are known, or can be easily ascertained, by those of ordinary skill in the art.

The labeled or unlabeled monoclonal antibodies of the invention can also be used in combination with therapeutic agents such as those described above. Especially preferred are therapeutic combinations comprising the monoclonal antibody of the invention and immunomodulators and other biological response modifiers.

Thus, for example, the monoclonal antibodies of the invention can be used in combination with alpha-interferon. This treatment modality enhances monoclonal antibody targeting of carcinomas by increasing the expression of monoclonal antibody reactive antigen by the carcinoma cells (Greiner, *et al.*, *Science,* 235:895, 1987). Alternatively, the monoclonal antibody of the invention could be used, for example, in combination with gamma-interferon to thereby activate and increase the expression of Fc receptors by effector cells which, in turn, results in an enhanced binding of the monoclonal antibody to the effector cell and killing of target tumor cells. Those of skill

in the art will be able to select from the various biological response modifiers to create a desired effector function which enhances the efficacy of the monoclonal antibody of the invention.

When the monoclonal antibody of the invention is used in combination with various therapeutic agents, such as those described herein, the administration of the monoclonal antibody and the therapeutic agent usually occurs substantially contemporaneously. The term "substantially contemporaneously" means that the monoclonal antibody and the therapeutic agent are administered reasonably close together with respect to time. Usually, it is preferred to administer the therapeutic agent before the monoclonal antibody. For example, the therapeutic agent can be administered 1 to 6 days before the monoclonal antibody. The administration of the therapeutic agent can be daily, or at any other interval, depending upon such factors for example, as the nature of the tumor, the condition of the patient and half-life of the agent.

Using the monoclonal antibodies of the invention, it is possible to design therapies combining all of the characteristics described herein. For example, in a given situation it may be desirable to administer a therapeutic agent, or agents, prior to the administration of the monoclonal antibodies of the invention in combination with effector cells and the same, or different, therapeutic agent or agents. For example, it may be desirable to treat patients with malignant disease by first administering gamma-interferon and interleukin-2 daily for 3 to 5 days, and on day 5 administer the monoclonal antibody of the invention in combination with effector cells as well as gamma-interferon, and interleukin-2.

It is also possible to utilize liposomes with the monoclonal antibodies of the invention in their membrane to specifically deliver the liposome to the area of the tumor expressing NG1. These liposomes can be produced such that they contain, in addition to the monoclonal antibody, such immunotherapeutic agents as those described above which would then be released at the tumor site (Wolff, *et al.*, *Biochemical et Biophysical Acta,* 802:259, 1984).

The dosage ranges for the administration of the monoclonal antibodies of the invention are those large enough to produce the desired effect in which the symptoms of the malignant disease are ameliorated. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any complication. Dosage can vary from about 0.1 mg/kg to about 2000 mg/kg, preferably about 0.1 mg/kg to about 500 mg/kg, in one or more dose administrations daily, for one or several days. Generally, when the monoclonal antibodies of the invention are administered conjugated with therapeutic agents lower dosages, comparable to those used for *in vivo* immunodiagnostic imaging, can be used.

The monoclonal antibodies of the invention can be administered parenterally by injection or by gradual perfusion over time. The monoclonal antibodies of the invention can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally, alone or in combination with effector cells.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising the NG1 antigen, or the monoclonal antibodies of the invention, the medicament being used for therapy of malignant disorders.

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

EXAMPLE 1

PREPARATION OF HYBRIDOMA CELL LINES PRODUCING MONOCLONAL ANTIBODY TO NG1

Halves of five lymph nodes of five separate patients with cancer were harvested and pooled from fresh surgical specimens. The remaining halves were submitted for histopathologic study. The lymphocytes were cryopreserved at -70°C until use. The viabilities before and after cryopreservation ranged from approximately 90%-99%.

Lymphocytes from the cancer patients were cultured in complete medium overnight prior to their fusion with human cell line SHFP-1. At the end of the culture, the viability of the lymphocytes was determined by 0.05% trypan blue exclusion prior to fusion with SHFP-1. The mean viability was approximately 70%.

A human B lymphoblastoid cell line derived from parental WIL-2 cells (Glassy, *J.Tissue Culture Methods*, 12:85, 1989), called SHFP-1, was the human fusion partner cell line. Cancer cell lines U87.MG, SK-OV-3, HT144, SK-N-SH, Kato-III, and HT-29 were obtained from American Type Culture Collection, Rockville, MD. These cell lines were cultured in RPMI 1640 medium supplemented with 10% to 20% fetal calf serum.

In the fusion procedure, one part of lymphocytes from the pooled lymph nodes and two parts of SHFP-1 cells were fused with 50% polyethylene glycol 1500 (Behringer Mannheim, Germany). The hybrids were selected by medium containing hypoxanthine, aminopterin, and thymidine (Behringer Mannheim). Reactivity to cancer cells and isotype of immunoglobulin by enzyme-linked immunosorbent assay (ELISA) were determined for the supernatant of each visible hybridoma. The reactive clones were subcloned by limiting dilution (Glassy, *et al., Cancer Invest.*, 5:449, 1987).

Immunoglobulin production and tumor reactivity were evaluated by enzyme-linked immunosorbent assay (ELISA). The ELISA plates were prepared by coating the plates with target cancer cells at 5×10^4 cells per well. After blocking with phosphate-buffered saline-0.5% bovine serum albumin solution, 50 μL of the testing supernatant was added to each well for 1 to 2 hours at room temperature. After washing, horseradish peroxidase-conjugated goat anti-human immunoglobulin (anti-IgM and anti-IgG were from Jackson, West Grove, PA, and anti-IgA was from Zymed, San Francisco, CA) was added for an incubation of 45 minutes. The substrate, orthophenylenediamine, was added for color reaction. The plates were read at 492 nm with a microplate reader (MR 700, Dynatech Laboratories, Inc., Chantilly, VA). The background optical density (OD_b) was obtained by substituting phosphate-buffered saline-bovine serum albumin solution for the testing supernatant and was found to be 0.050 or lower. At the 96-well stage, supernatants with ODs greater than 0.250 were regarded as positive. Subsequently, because few IgM and IgG-secreting clones bound nonspecifically to the ELISA plates, new criteria were established to evaluate the ELISA results:

Criterion A: $\Delta OD < 1.000$ and $OD_s - OD_f > 0.200$, and
Criterion B: $\Delta OD \geq 1.000$ and $OD_s - OD_f > 0.150$,

where $\Delta OD = OD_s - OD_b$, OD_s (sample OD) represents the OD obtained from specific binding of supernatant to the antigen-coated ELISA plates, and OD_f (false OD) represents the OD obtained from nonspecific binding of supernatant to the ELISA plates without antigen coating.

EXAMPLE 2

IMMUNOLOGICAL REACTIVITY OF HuMAb(NG1)

Various human carcinoma cell lines were tested for their reactivity with HuMAb(NG1). Cell lines tested were HT29, Panc-1, KATO III, Calu-1, U87.MG, SK-OV-3, SK-N-SH, M21, A375, and HTB63 (ATCC, Rockville, MD) (Table 1).

All the cells were maintained in RPMI 1640 medium (Whittaker, Wallsville, MD) supplemented with 10-20% FCS (Hyclone Lab., Logan, UT) and 2 mM L-glutamine (Whittaker). Twenty ml of cell suspension at 5 x 10^4/ml were added to each petri dish containing a sterile glass slide. After various periods of culture, the slides were harvested and used for either immunoperoxidase (IP) or immunofluorescence (IF) staining.

In preparing the slides for staining, the slides with seeded cells were washed in PBS and fixed with cold acetone. For indirect IP staining, fixed cells were incubated with the HuMAb (NG1) supernatant (~5 μg/ml) and subsequently developed with horseradish peroxidase-conjugated goat anti-human IgG, followed by diaminobenzidine (DAB; Sigma) containing 0.01% H_2O_2. After counterstaining with Mayer's hematoxylin, the specimens were cleared with ammonium peroxide and mounted with aqua-mount. In IF stainings, a 1:200 dilution of FITC conjugated goat antibody to human IgG (Boehringer Mannheim) was used as second antibody and reactivities were evaluated by Nikon fluorescence microscopy. Controls consisted of secondary antibody and cells.

TABLE 1

CELL PANEL FOR NO. 1.245

CELL LINES	DESCRIPTION	NG-1.245Ab
U87-MG	Neuroblastoma	+++
SK-N-SH	Neuroblastoma	++
NMB-7	Neuroblastoma	++
UCLA-P3	Lung Adenocarcinoma	-
Sk-Lu-1	Lung Adenocarcinoma	-
NCI-H128	Lung Small Cell Carcinoma	+
SK-Mes-1	Lung Squamous Carcinoma	+
A427	Lung Carcinoma	++
T293	Small Cell Carcinoma	++
SK-Br-3	Breast Adenocarcinoma	++
SK-OV-3	Ovarian Adenocarcinoma	++
HT-29	Adenocarcinoma Colon	-
M-21	Melanoma	-
M-14	Melanoma	++
HT-144	Melanoma	-
SK-Mel-24	Melanoma	++
SK-Mel-28	Melanoma	-

EXAMPLE 3

CHARACTERIZATION OF NG1

NG1 was evaluated using lysates of various cell lines. In preparing the lysates, U87.MG, SK-OV-3, and KATO III cells were washed 3 times in Tris-buffered saline (TBS) and resuspended with TBS at 5 x 10^7 cells/ml. The cell lysates were prepared by repeated freezing and thawing (4X) and centrifugation at 3000 xg for 10 min. The supernatants were collected and stored at -70°C until use. The cell lysates were then tested by SDS-PAGE and Western Blot.

Electrophoresis was performed using the Novex X-cell mini-gel system (Novex, San Diego, CA). Precast 8-16% Tris-glycine gels (Novex) were used to run all samples. Following electrophoresis, proteins were transferred onto nitrocellulose membranes using the Bio-Rad transfer system (Richmond, CA). Protein standards and prestained standards (Sigma) were used simultaneously. After blocking with TBA (TBS - 1% albumin) the membranes were washed, dried, and incubated in hybridoma-supernatants at 25 μg/ml. Alkaline phosphatase conjugated goat anti-human IgG (American, Qualex, La Marada, CA) at a dilution of 1:3000 was used as the secondary antibody. The bands were developed using the Proto Blot System from Promega.

These studies showed that NG1 is a two chain structure with molecular weights estimated to be 50-55 kDa by gel electrophoresis and Western blot under reducing conditions and a single chain with a molecular weight of 100-110 kDA under non-reducing conditions. The antigen was identified in cell lysates of 1 to 2 day old cultures of the following cells: U87.MG and SK-OV-3. In contrast to the above carcinoma cells, the melanoma cell lines HTB63, HTB66, HTB71, HTB72, HTB73, UCLA M21, and UCLA M14 had no detectable antigen NG1 by both gel electrophoresis with Western blot and cytoimmunoperoxidase staining.

Deposit of Materials

The following cell line has been deposited with the American Type Culture Collection, 1301 Parklawn Drive, Rockville, MD, USA (ATCC):

Cell Line	ATCC Accession No.	Deposit Date
NG1	HB11230	January 7, 1993

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty which assures permanent and unrestricted availability of the progeny of the culture to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if the culture deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable specimen of the same culture. Availability of a deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the cell lines deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any cell lines that are functionally equivalent are within the scope of this invention. The deposit of material does not constitute an admission that the

written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustration that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

CLAIMS

1. A continuous hybridoma cell line capable of secreting monoclonal antibodies which specifically bind to carcinoma antigen NG1.

2. The hybridoma of claim 1, wherein the hybridoma is ATCC HB 11230.

3. A monoclonal antibody which specifically binds to NG1.

4. The monoclonal antibody of claim 3, which is human.

5. The monoclonal antibody of claim 4, having the specificity of a monoclonal antibody produced by hybridoma cell line ATCC HB 11230.

6. The monoclonal antibody of claim 4, wherein the monoclonal antibody is produced by hybridoma cell line ATCC HB 11230.

7. An anti-idiotypic antibody to the antibody of claim 3.

8. A method of detecting NG1 which comprises contacting a source suspected of containing NG1 with a diagnostically effective amount of detectable labeled antibody or fragment thereof, wherein the antibody specifically binds to NG1 and determining whether the antibody binds to the source.

9. The method of claim 8, wherein the antibody is produced by hybridoma cell line ATCC HB 11230.

10. The method of claim 8, wherein the detecting is *in vivo.*

11. The method of claim 10, wherein the detectable label is selected from the group consisting of a radioisotope and a paramagnetic label.

12. The method of claim 8, wherein the detecting is *in vitro.*

13. The method of claim 12, wherein the detectable label is selected from the group consisting of a radioisotope, a fluorescent compound, a colloidal metal, a chemiluminescent compound, a bioluminescent compound and an enzyme.

14. A method of ameliorating malignant disease in an animal which comprises administering to the animal a therapeutically effective amount of a monoclonal antibody or fragment thereof, wherein said antibody specifically binds to NG1.

15. The method of claim 14, wherein the malignant disease is a carcinoma.

16. The method of claim 14, wherein the antibody is human.

17. The method of claim 14, wherein the monoclonal antibody has the specificity of the monoclonal antibody produced by hybridoma cell line ATCC HB 11230.

18. The method of claim 14, wherein the monoclonal antibody is produced by hybridoma cell line ATCC HB 11230.

19. The method of claim 14, wherein the administration is parenteral.

20. The method of claim 18, wherein the parenteral administration is by subcutaneous, intramuscular, intraperitoneal, intracavity, transdermal, or intravenous injection.

21. The method of claim 14, wherein the administration is at a dosage of about 0.01 mg/kg to about 2000 mg/kg/dose.

22. The method of claim 14, wherein the antibody is administered in combination with effector cells.

23. The method of claim 14, wherein the monoclonal antibody is therapeutically labeled.

24. The method of claim 14, wherein the therapeutic label is selected from the group consisting of a radioisotope, a drug, an immunomodulator, a biological response modifier, a lectin, and a toxin.

25. The method as in any of claims 14, 22 and 23, wherein the antibody is administered substantially contemporaneously in combination with a therapeutic agent.

26. The method of claim 23, wherein the therapeutic agent is selected from the group consisting of a radioisotope, a drug, an immunomodulator, a biological response modifier, a lectin, and a toxin.

27. Antigen NG1 in substantially pure form and mixtures and salts thereof.

28. The NG1 of claim 27 having a molecular weight of 50-55 kD as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions.

29. The NG1 of claim 27 comprising an epitope which is specifically bound by a monoclonal antibody having the specificity of a monoclonal antibody produced by hybridoma cell line ATCC HB 11230.

30. A method of ameliorating malignant disease in an animal which comprises immunizing the animal with an immunogenically effective amount of NG1.

31. A method of ameliorating malignant disease in an animal which comprises immunizing the animal with an immunogenically effective amount of the anti-idiotypic antibody of claim 7.

32. A pharmaceutical composition which comprises a therapeutically effective amount of the monoclonal antibodies as in any of claims 3-7, together with a pharmacological carrier.

33. A pharmaceutical composition comprising an immunogenically effective amount of NG1, together with a pharmacological carrier.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00488

A. **CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :A61K 39/00, 39/395, 45/05; C07K 15/28; C12N 5/24; C12P 21/08

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. **FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8; 514/2; 435/240.27; 530/388.8, 388.85, 391.1, 391.3, 391.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

search terms: tumor antigen, carcinoma, immunotoxin, immunodiagnosis, anti-idiotype, monoclonal antibody, conjugate

C. **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,761,377 (GLASSY ET AL) 02 August 1988, see entire document.	1-33
Y	US, A, 5,134,075 (HELLSTROM ET AL) 28 July 1992, see Column 2, line 67 to Column 3, line 60; Column 7, lines 1-10; Column 7, line 44 to Column 10, line 20; Column 10, lines 41-61; Column 13, Table 1; Column 14-15, Table 2; and Column 16, lines 9-25.	1-33

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

08 MAY 1994

Date of mailing of the international search report

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A. CLASSIFICATION OF SUBJECT MATTER:
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