Monoclonal Antibody 1A7 and Related Polypeptides

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United States Patent
Chatterjee et al.

MONOCLONAL ANTIBODY 1A7 AND RELATED POLYPEPTIDES

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Assignee: The Board of Trustees of the University of Kentucky, Lexington, Ky.

Notice: This patent is subject to a terminal disclaimer.

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Filed: Jan. 16, 1996

Related U.S. Application Data

Continuation-in-part of application No. 08/372,676, Jan. 17, 1995, Pat. No. 5,612,030.

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Primary Examiner—Sheela Huff
Assistant Examiner—Julie E. Reeves
Attorney, Agent, or Firm—Morrison & Foerster LLP

ABSTRACT

The present invention relates to monoclonal antibody 1A7. This is an anti-idiotype produced by immunizing with an antibody specific for ganglioside GD2, and identifying a hybridoma secreting antibody with immunogenic potential in a multi-step screening process. Also disclosed are polynucleotide and polypeptide derivatives based on 1A7, including single chain variable region molecules and fusion proteins, and various pharmaceutical compositions. When administered to an individual, the 1A7 antibody overcomes immune tolerance and induces an immune response against GD2, which comprises a combination of anti-GD2 antibody and GD2-specific T cells. The invention further provides methods for treating a disease associated with altered GD2 expression, particularly melanoma, neuroblastoma, glioma, soft tissue sarcoma, and small cell carcinoma. Patients who are in remission as a result of traditional modes of cancer therapy may be treated with a composition of this invention in hopes of reducing the risk of recurrence.

32 Claims, 22 Drawing Sheets
OTHER PUBLICATIONS


Figure 1

M K L P V R L L V L M F W I P A
ATG AAG TTG CCT GTT AGG CTG TTG GTG ATG TTC TGG ATT CCT GCT
S S D
TCC ACC GAT (-1 to -19, leader)

D V L M T Q T P L S L P V S L G
GAT GTT TTG ATG ACC CAA ACT CCA CTC TCC CTG CCT GTC AGT CTT GGA
D Q A S I S C
GAT CAA GCC TCC ATC TCT TGC (1-23, Frame work 1)

R S S Q I V H S N G N T Y L E
AGA TCT AGT CAG AGC ATT GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA
(24-39, CDR 1)

W Y L Q K P G Q S P N L L I Y
TGG TAC CTA CAG AAA CCA GCC CAG TCT CCA AAC CTC CTG ATC TAC
(40-54, Frame work 2)

F V S N R F S
TTT GTT TCC AAC CGA TTT TCT (55-61, CDR 2)

G V P D R F S G S S G S G T D F T
GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA
L K I S R V E A E D L G V Y Y C
CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TAC TGC
(62-93, Frame work 3)

F Q G S H V P W T
TTT CAA GGT TCA CAT GTT CGG TGG ACG
(94-102, CDR 3)

F G G G T K L E I K
TTC GGT GGA GCC ACC AAG CTG GAA ATC AAA
(103-112, Frame work 4)

R A D A A P T V S I F P P
CGG GCT GAT GCT GCA CCA ACT GTA TCC ATC TTC CCA CCA

S S K L G
TCC AGT AAG CTT GGG (Constant region)
Figure 2

```
MAVLGLFLVFPSCT
ATGGCTGTGGGGCTGCTCTTGCCTGAGATTCACCAGTGTVLS
GTCCTGTCC(-1 to -19, Leader)

QVQVKESGPFLLVPSPQ
CAGGTGGAGTGGAAACCCTCTCTGTCGTCGCCCTCTCACT
SLSTCTVSFSLT
AGCTCCCATATCAGTGTACGTTCTTGCTTAGGTACC
(1-30, Frame work 1)

TYGVS
ACCATTGGTAAGC(31-35, CDR 1)

WIRQPGBKLEWLG
TGGATTCCGACCTCCAGGAAGGTTCTGGAGTGGCTGGGA
(36-49, Frame work 2)

AIWDGTTNTYHSATLIS
GCAATTGGGTACGGGGACCACAATATTACATCTACTGCTCTACTCC
(50-65, CDR 2)

RLSISKDNSKSQVFLK
AGACCTGAACCTAACAGTGATACCTAAAGACCAAGTCTTTATAA
LNSLTQTDATACTYCAK
CTGAACTGCTAAGTTATCGACCACCAGTACGTTCTGACCAAA
(66-97, Frame work 3)

LGNYDALDY
CTGGTACATTGGCTGACATAC
(98-106, CDR 3)

WQGTSVTVSS
TGGGTCAAGAACCCTGACCTACGCCTCTCACC
(107-117, Frame work 4)

AKTTPPVYPLVPGSL
GCCAAAAGCACCCGGGGTTCTCCTTGGTGCTAGCCTGAGTTGG
(Constant region)
```
Figure 3(A)

1A7: 1 DVLMTQTPLSLPVSLGQASICTSSGIVHSNGNTYLEWYLGKPGSSPNLLFYFSNRF 60
  1 1 ........................................... K. K. 60
  2 1 ........................................... K. K. 60
  3 1 ........................................... K. K. 60
  4 1 ........................................... K. K. 60
  5 1 ........................................... K. K. 60
  6 1 ........................................... K. K. 60
  7 1 ........................................... K. K. 60
  8 1 ........................................... S. S. 64
  9 1 ........................................... K. K. 60
 10 1 ........................................... K. K. 60
 11 1 ........................................... K. K. 60
 12 20 .......................................... K. K. 79
 13 1 ........................................... K. K. L 60
 14 1 ........................................... K. K. 60
 15 5 ........................................... S. S. 64

1A7: 61 SGVPDRFSGSGTDFTLKISRVEAEDLVYYCGSHVPFTFSGTTLIEK 112
  1 61 .......................................... 112
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  3 61 .......................................... 112
  4 61 .......................................... 111
  5 61 .......................................... 112
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  7 61 .......................................... 112
  8 61 .......................................... 111
  9 65 .......................................... T. 116
 10 61 .......................................... R. Y. 112
 11 61 .......................................... R. 112
 12 61 .......................................... R. 112
 13 61 .......................................... Y. S. 131
 14 61 .......................................... W. Y. 112
 15 65 .......................................... Q. T. 116
**Figure 3(B)**

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<td>L...</td>
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</table>
Figure 4
Figure 5

![Bar graph showing % inhibition at different concentrations of Ab3 added.](image)
Figure 6

% inhibition

μg Ab3/Ab1 added
Figure 7(B)

Fluorescence Intensity [log]

relative number of cells

100
50

$10^1$ $10^2$ $10^3$ $10^4$
Figure 7(C)

Fluorescence Intensity [log]

Relative number of cells

100
50

$10^1$  $10^2$  $10^3$  $10^4$
Figure 8

![Bar Chart]

- **Y-axis:** OD 405
- **X-axis:** Numbers 1 to 7

The chart shows a significant peak at number 1, with decreasing values for the subsequent numbers.
Figure 12(C)

>emb|X87231|MMKAPLI M. musculus mRNA for antibody light chain

89 GATGTAAAAATGACCCAAAATCTCCACTCTCCCTGCTGAGTTGAGATCAAGCCTCC 148
149 ATCTCTGTCATATGCAGATCGTGTGATACATATGAAATGGAAACACCTATTAAAG 208
209 TACCTGCAGAAGCCAGGCGGTCTCCAAAAGCAGCTCTGATCTCATAACAGTTTCAACGGATTT 268
269 TCTGAGGTCCGACACAGGTTCAGTGCAGGGACAGATTTCACACTCAAGATC 328
329 ACGAGATGGGAGGCTGAGGACATCTGGGAGTTATTACTGCTTTCAAGGGTCATGTCCG 388
389 TGGACGTTGGTGAGGGAGACGAACACGGTGGAAATCAAA 424

>gb|U29428|MMU29428 Mus musculus anti-PC rearranged Ig kappa chain V-J region

13 GATGTAAAAATGACCCAAAATCTCCACTCTCCCTGCTGAGTTGAGATCAAGCCTCC 72
73 ATCTCTGTCATATGCAGATCGTGTGATACATATGAAATGGAAACACCTATTAAAG 132
133 TACCTGCAGAAGCCAGGCGGTCTCCAAAAGCAGCTCTGATCTCATAACAGTTTCAACGGATTT 192
193 TCTGAGGTCCGACACAGGTTCAGTGCAGGGACAGATTTCACACTCAAGATC 252
253 ACGAGATGGGAGGCTGAGGACATCTGGGAGTTATTACTGCTTTCAAGGGTCATGTCCG 312
313 TGGACGTTGGTGAGGGAGACGAACACGGTGGAAATCAAA 348
1 MONOCLONAL ANTIBODY 1A7 AND RELATED POLYPEPTIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Ser. No. 08/372,676, filed Jan. 17, 1995, now issued as U.S. Pat. No. 5,612,030, which is hereby incorporated herein in its entirety.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This invention was made in part during work supported by a grant from the United States Public Health Service (CA72018). The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

In spite of numerous advances in medical research, cancer remains the second leading cause of death in the United States. Traditional modes of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Failure occurs either because the initial tumor is unresponsive, or because of recurrence due to regrowth at the original site or metastasis. Cancer remains a central focus for medical research and development.

Under the hypothesis that neoplastic cells are normally regulated by immune surveillance, an attractive approach is to re-focus the immune system in affected individuals back towards the tumor. Many types of cancers should be susceptible to the immune system, because they express unusual antigens that reflect the oncogenic transformation of the cell. Antibodies or T cells directed against a target antigen specifically expressed on tumor cells may be able to recruit immune effector functions, and thereby eliminate the tumor or mitigate the pathological consequences.

There are several potential pitfalls in this approach. The first is that the target tumor antigen may be shed from the cell, and thereby block the approach of tumor-specific immune components. The second is that the expression of the target antigen may be heterologous. In this case, a specific immune response would be ineffective in a substantial subclass of affected individuals, or the risk of escape variants would be high. The third difficulty is that tumor-specific antigens are generally antigenically related to autoantigens, or comprise autoantigens in an atypical mode of expression. This means that tumor-associated antigens are often poorly immunogenic, perhaps due to an active and ongoing immunosuppression against them. Furthermore, cancer patients tend to be immunosuppressed, and only respond to certain T-dependent antigens.

A number of features suggest that gangliosides may be preferable to other types of target antigens for antibody-mediated killing of certain tumor types. Gangliosides like GD2 have simple, well-defined structures, and the level of expression is not affected by antibody binding. In vitro studies have shown that monoclonal antibodies against gangliosides like GD2 and GD3 potentiate lymphocyte response which could potentially be directed towards tumor cells. In addition, certain gangliosides have been implicated in the adhesion of tumor cells to solid substrates, and antibodies inhibit this attachment. An immune response against them, even if not successful in eliminating the tumor cells, could reduce the extent of pathology.

2 In particular, glycosphingolipid GD2 is expressed at high density by tumors of human neuroectodermal origin; including malignant melanoma, neuroblastoma, glioma, soft tissue sarcoma and small cell carcinoma of the lung. The GD2 antigen is absent in most normal tissues, except for low levels in brain and peripheral nerve.

Melanoma is one of the human diseases for which there is an acute need of new therapeutic modalities. It is a particularly aggressive form of skin cancer, and occurs in increased frequency in individuals with regular unguarded sun exposure. In the early phases, melanoma is characterized by proliferation at the dermo-epidermal junction, which soon invades adjacent tissue and metastasizes widely. Worldwide, 70,000 patients are diagnosed and 25,000 deaths are reported each year. The American Cancer Society projects that by the year 2000, 1 out of every 75 Americans will be diagnosed with melanoma in their lifetime.

Fortunately, melanoma is one of the cancers for which gangliosides hold significant promise as a target antigen (Livingston (1995) Immunol. Rev. 145:147–160). Increased expression of GD2 has been observed in a majority of malignant melanoma cells. Several murine monoclonal anti-GD2 antibodies were reported to suppress the growth of tumors of neuroectodermal origin in athymic (nu/nu) mice or cause remission in patients with metastatic melanoma. A human-mouse chimeric anti-GD2 antibody remissions in patients with metastatic neuroblastoma. The mechanism is thought to involve antibody dependent cellular cytotoxicity (ADCC) or complement-mediated cytotoxicity (CMC).

Clinical responses have been obtained by treating with monoclonal antibodies against GM2, GD2 and GD3. Active immunization with a ganglioside vaccine comprising GM2 produced anti-GM2 antibodies in 50/58 patients, who survived longer on average than antibody negative patients.

Neuroblastoma is a highly malignant tumor occurring during infancy and early childhood. Except for Wilms’ tumor, it is the most common retroperitoneal tumor in children. This tumor metastasizes early, with widespread involvement of lymph nodes, liver, bone, lung, and marrow. While the primary tumor is resolvable by resection, the recurrence rate is high.

Small cell lung cancer is the most malignant and fastest growing form of lung cancer. It accounts for 20–25% of new cases of lung cancer, and 60,000 cases will be diagnosed in the U.S. in 1996. The primary tumor is generally responsive to chemotherapy, but is shortly followed by wide-spread metastasis. The median survival time at diagnosis is ~1 year, with a 5 year survival rate of only 5–10%.

If there was a simple and reliable therapeutic strategy for providing immune reactivity against GD2, then the clinical prospects for these types of cancers might improve.

Unfortunately, there are several reasons why GD2 is less than ideal as a component of an active vaccine. For one thing, GD2 is of limited supply, and is difficult to purify. Of course, because GD2 is a ganglioside, it cannot be generated by simple recombinant techniques. Secondly, gangliosides in general, and GD2 in particular, are poorly immunogenic. In order to render them more immunogenic in humans, it has been necessary to conjugate them to protein carriers like KLH (U.S. Pat. No. 5,308,614; WO 94/16731).

Similarly, the passive administration of anti-GD2 antibodies is less than ideal as an approach to long-term care. The amount of antibody that must be provided passively is substantial. It may lead to the formation of anti-immunoglobulin or anti-idiotypic, which in turn may lead to diminished responsiveness with each succeeding dose. And
3 perhaps most importantly, it fails to provide the host with components of the immune response which may be critical in tumor eradication; particularly tumor-directed cytotoxic T cells.

How else, then, could an active immune response against GD2 be obtained? The network hypothesis of Lindemann and Jerne suggests a way of overcoming both the natural immune tolerance against GD2, and the shortage of supply of GD2. It relies on the fact that antibodies comprise variable region epitopes that themselves may be immunogenic, leading to the generation of second-level antibodies called anti-idiotypes. According to the hypothesis, immunization with a given tumor-associated antigen will generate production of antibodies against this tumor-associated antigen (Ab1), which is purified and used in a second round of immunization to generate the anti-idotype (Ab2). Some of these Ab2 molecules (called Ab2β) fit into the paratopes of Ab1, and can be used as surrogate tumor-associated antigens. Thus, immunization with Ab2β can lead to the generation of anti-anti-idotype antibodies (Ab3) that recognize the corresponding original tumor-associated antigen identified by Ab1. The idea is that the Ab2 presents a feature that is structurally related to the tumor antigen in a different context that makes it more immunogenic.

Accordingly, efforts have been made elsewhere using anti-idiotypic to elicit a response against various tumor-associated antigens. One group of investigators raised an anti-idotype related to the melanoma associated gangloside GM$_3$, (Kanda et al., Yamamoto et al., Hastings et al.). Salch et al. and Cheung et al. have raised anti-idotypes against GD2. Other anti-idotopes have entered early clinical trials: for example, Mittelman et al. are using an anti-idotype related to a high molecular weight melanoma associated antigen (MAA), Chattopadhyay et al. have developed anti-idotopes against a melanoma-associated proteoglycan. Chapman et al are treating melanoma patients with an anti-idotype related to gangloside GD3. Most of these trials are still at about the phase I level, with results still pending.

It is important to emphasize that every potential anti-idotype for use in tumor therapy must be evaluated on a case-by-case basis. First, only a fraction of antibodies raised against an Ab1 are limited in their reactivity against the paratope of the Ab1 (i.e., non-reactive against features shared with other potential antibodies in the host individual). Second, anti-idotopes are not necessarily immunogenic. Third, only a fraction of immunogenic anti-idotopes elicit a response against the original tumor antigen and not against other antigens with less tissue specificity. These properties are related to the structure of the anti-idotype, including the amino acid sequence of its variable regions. Different anti-idotopes raised against the same Ab1 will be different in this respect, and must be evaluated separately. Furthermore, the anti-tumor response elicited will depend on the immunological status of the individual being immunized.

DISCLOSURE OF THE INVENTION

This disclosure outlines a particular monoclonal anti-idotype antibody, designated 1A7. This antibody has been established as being capable of eliciting an anti-GD2 response. It has all the desirable properties that provide for escaping immune tolerance to GD2, and is appropriate for treating GD2-associated disease.

Also described are a number of other compounds derived from 1A7. These include in particular polynucleotides and polypeptides based on the 1A7 heavy and light chain variable regions, which distinguish 1A7 from other immunoglobulin molecules. A wide range of such products are contemplated. Preferred derivative compounds include a humanized version of intact 1A7, polypeptide fragments from the complementarity determining regions of the 1A7 heavy chain, and a vaccinia virus vector comprising an encoding region for a 1A7 variable region. Especially preferred compounds include a 1A7 antibody fusion molecule comprising a polypeptide region with GM-CSF or IL-2 attached to the heavy chain constant region, a single-chain V$_{H}^{-}\rightarrow$V$_{L}$ or V$_{H}^{-}\rightarrow$V$_{L}$ variable region, and polynucleotides encoding such polypeptides.

The compounds and compositions of this invention may be used inter alia for eliciting a humoral or cellular immune response in an individual. They may be used for detecting or treating a GD-2 associated disease; including therapy of advanced disease, and prophylactic care, particularly for decreasing the risk of recurrence in an adjuvant setting.

Accordingly, this invention embodies monoclonal antibody 1A7, and a hybridoma cell line that produces it. Also included are antibodies having identifying characteristics of 1A7.

Another embodiment of this invention is a polynucleotide comprising a sequence encoding a polypeptide with immunological activity of monoclonal antibody 1A7, wherein the polypeptide comprises at least 5 consecutive amino acids from a variable region of monoclonal antibody 1A7. The variable region may be from either the 1A7 light chain or heavy chain. The 5 consecutive amino acids preferably play a role in 1A7 immunologic reactivity, and may be from a complementarity determining region. Another embodiment is an isolated polynucleotide of at least 20 consecutive nucleotides capable of forming a stable duplex with the 1A7 light or heavy chain encoding sequence, but not with encoded sequences for other previously described immunoglobulin molecules. Any of these polynucleotides may be in the form of cloning vectors, expression vectors, or transfected into host cells.

Also embodied is a polypeptide having immunological activity of monoclonal antibody 1A7, wherein the polypeptide comprises at least 5 consecutive amino acids from a variable region of monoclonal antibody 1A7. The variable region may be from a light chain or heavy chain. The 5 consecutive amino acids preferably play a role in 1A7 immunologic reactivity, and may be from a complementarity determining region. Another embodiment is an isolated polynucleotide of at least 20 consecutive nucleotides capable of forming a stable duplex with the 1A7 light or heavy chain encoding sequence, but not with encoded sequences for other previously described immunoglobulin molecules. Any of these polynucleotides may be in the form of cloning vectors, expression vectors, or transfected into host cells.

Another embodiment is a pharmaceutical composition or vaccine, comprising monoclonal antibody 1A7, a polynucleotide of this invention, or a polypeptide of this invention, and a pharmacologically acceptable excipient.

Another embodiment of this invention is a method of eliciting an immune response in an individual, comprising administering to the individual an effective amount of monoclonal antibody 1A7, or a polynucleotide or polypeptide of this invention. The immune response may comprise either humoral or cellular immunity, preferably both.

Yet another embodiment is a method of treating a GD2-associated disease in an individual, comprising administering a monoclonal antibody 1A7, or a polynucleotide or polypeptide of this invention. The disease may be melanoma, neuroblastoma, glioma, soft tissue sarcoma, and small cell carcinoma. The individual may have a clinically
detectable tumor, or the tumor may have been previously treated and rendered undetectable. The method may be for palliating the disease, or for reducing the risk of recurrence.

A further embodiment of this invention is a kit for detection or quantitation of an anti-GD2 antibody or a 1A7 polynucleotide in a sample, comprising monoclonal antibody 1A7 or a polynucleotide or polypeptide of this invention in suitable packaging. Also embodied are methods for detecting such antibodies or polynucleotides by employing a reagent or kit embodied in this invention.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1 is a depiction of the cDNA sequence (SEQ ID NO:1) and the amino acid sequence (SEQ ID NO:2) of the light chain variable region of 1A7 and adjoining residues.

FIG. 2 is a depiction of the cDNA sequence (SEQ ID NO:3) and the amino acid sequence (SEQ ID NO:4) of the heavy chain variable region of 1A7 and adjoining residues.

FIGS. 3(A), 3(B), and 3(C) are listings in which the amino acid sequences of the 1A7 variable region are compared with 15 light and heavy chain immunoglobulin sequences from the prior art. Panel A (amino acids 1 through 112 of SEQ ID NO:2) shows the light chain comparison; Panel B (SEQ ID NO:5 through SEQ ID NO:14) shows the heavy chain comparison. Panel C shows variable region consensus sequences for the light and heavy chains (SEQ ID NO:15 and SEQ ID NO:16) and compares them with those of 1A7. The variable region of 1A7 shows splicing differences about the VDJ junction of the heavy chain, and an additional 16 point differences.

FIG. 4 is a graph depicting the binding of monkey Ab3 to Ab2 (1A7) by sandwich radioimmunossay. Open squares denote Ab3 from monkey PRO#805; open circles denote Ab3 from monkey PRO#778.

FIG. 5 is a bar graph depicting inhibition of Ab1-Ab2 binding by monkey Ab3 or vice-versa. For each pair of bars, the left hand bar denotes Ab2-Ab3 with labeled Ab1; the right hand bar denotes Ab1-Ab3 with labeled Ab2.

FIG. 6 is a bar graph depicting inhibition of binding of 125I-labeled 14G2a antibody to GD2 positive melanoma cell line M21/P6 in the presence of different concentrations of Ab1 and monkey Ab3. Parallel inhibition curves were obtained using either purified Ab1 or Ab3 from monkey sera. For each triad of bars, the left hand bar denotes control binding with 14G2a; the middle (hatched) bar denotes binding of Ab3 from monkey PRO#805; the right hand bar denotes binding of Ab3 from monkey PRO#778.

FIGS. 7(A), 7(B), and 7(C) is a series of graphs from a FACS analysis of the binding of monkey Ab3 to tumor cells. Panel A shows the staining observed of GD2-expressing M21/P6 cells labeled with preimmune and immune Ab3. Panel B shows the staining observed on another cell line not expressing GD2. Panel C shows control staining of M21/P6 cells using the GD2-specific antibody 14G2a, or no antibody.

FIG. 8 is a bar graph depicting binding of Ab1 and monkey Ab3 to different gangliosides by ELISA. For each pair of bars, the left hand (solid) bar denotes the binding of Ab3 from monkey PRO#805; the right hand (hatched) bar denotes control binding by anti-GD2 14G2a. This experiment shows the antibody induced upon immunization with the anti-idiotypic 1A7 is antigen specific.

FIG. 9 is a bar graph depicting inhibition of binding of 125I-labeled 14G2a antibody to purified GD2 by 14G2a and monkey Ab3. For each triad of bars, the left hand (solid) bar denotes monkey PRO#778; the middle (open) bar denotes 14G2a; the right hand (hatched) bar denotes monkey PRO#685.

FIG. 10 is a half-tone reproduction of a blot analysis showing binding of 14G2a and monkey Ab3 to different gangliosides. Lane 1, monkey PRO#685; lane 2, monkey PRO#778; lane 3, 14G2a; lane 4, monkey anti-HID10; lane 5, PBS-BSA.

FIG. 11 is a map of a model plasmid construct for creating a vaccinia vector comprising a region encoding a 1A7 heavy or light chain variable region.

**FIG. 12(A), FIG. 12(B) and FIG. 12(C) depict the 10 polynucleotide sequences that were most closely matched to the 1A7 light chain variable region encoding sequence in a database search. These sequences have the designations SEQ ID NO:17-26.**

**FIG. 13(A), FIG. 13(B), FIG. 13(C), and FIG. 13(D) depict the 10 polynucleotide sequences that were most closely matched to the 1A7 heavy chain variable region encoding sequence in a database search. These sequences have the designations SEQ ID NO:27-44."**

**MODES FOR CARRYING OUT THE INVENTION**

We have discovered an anti-idiotypic antibody that is capable of recruiting a tumor-specific response against GD2. The antibody is designated 1A7. The immune response elicited by 1A7 typically comprises both humoral and cellular components, and is therefore expected to be useful in palliating the clinical conditions related to GD2-associated tumors. The invention comprises the 1A7 antibody molecule, along with polynucleotide and polypeptide derivatives thereof, and methods for using these compounds in diagnosis and treatment.

Cancer patients are typically tolerated to various tumor associated antigens (TAA), including GD2. 1A7 successfully circumvents immune tolerance, and elicits an immune response against GD2. According to the network theory, Ab1 represents anti-tumor monoclonal antibody; Ab2 represents anti-idiotypic monoclonal antibody; and Ab3 represents anti-idiotype monoclonal antibody. 1A7 is an Ab2 that stimulus Ab3 with the same specificity as Ab1, and can therefore recruit effector functions against tumors bearing the tumor antigen. An Ab3 response reactive against the original TAA is referred to herein as Ab1'.

While not wishing to be bound by theory, one explanation is that the 1A7 combining site may present a region that at least partly resembles an epitope in GD2 in the context of one or more other epitopes which render it more immunogenic. The epitope of GD2 which may resemble that of 1A7 is identified by the Ab1 (14G2a) used to generate 1A7. As a result, 1A7 escapes the normal immune tolerance against GD2, and is able to elicit an anti-GD2 response.

FIGS. 1 and 2 show the nucleotide encoding region of the 1A7 light and heavy chain variable regions, respectively, along with the corresponding amino acid translation. These sequences were compared with those of other known immunoglobulin molecules (Example 2). We found that both the polynucleotide and polypeptide variable region sequences for 1A7 are unique.

Amongst the 50 database sequences matched most closely to that of the 1A7 light chain variable region, none was identical. 1A7 differed from the five closest sequences by 2 substitution differences at residues 50 and 55, which are contained in the second CDR. The two differences at these
positions were non-conservative substitutions, and persisted in comparisons with other light chain sequences. Amongst the 50 database sequences matched most closely to that of the 1A7 heavy chain variable region, none was identical. FIG. 3, Panels A and B). The following summarizes the main points deduced from the comparison. The closest match was with a heavy chain fragment. There were 2 deletions and 12 substitution differences. The closest match with a full length heavy chain variable region had the following features: There were 3 deletions and [17] 16 substitution differences. 1A7 differed in length from all sequences but one, due to insertions or deletions of 1 to 8 residues about the VDJ junction. For the sequence of equal length, there were [26] 25 substitution differences. All other comparisons showed a total of at least [22] 20 insertions, deletions and substitution differences. Differences appeared throughout the variable region. FIG. 3 Panel C provides a comparison of the 1A7 amino acid light and heavy chain sequences with consensus sequences derived from the database sequences. Other than splicing differences about the heavy chain VDJ junction, there appear to be about 16 differences between 1A7 and the consensus sequences that have likely arisen from somatic mutation during antibody maturation. Particularly of interest in developing 1A7 derivatives with 1A7 immunologic activity are regions of the polynucleotide or polypeptide sequence comprising part of the VDJ junction. Also of interest are regions spanning at least one, preferably 2, and more preferably 3 or more of the point differences between the 1A7 amino acid sequences and the consensus sequences. The full sequences of the 1A7 light and heavy chain constant regions have not been determined, but are expected to be identical or nearly identical to those of other mouse immunoglobulin molecules.

For the mouse kappa light chain constant region, four genetic allotypes encoding two protein allotypes have been published by Solin et al. (1993) Immunogenetics 37:401–407, which is hereby incorporated herein by reference. FIG. 1 of Solin et al. depicts mouse and rat immunoglobulin kappa chain gene sequences, comparing the sequences within the kappa chain constant region for different strains and highlighting allotypic differences. Included are kappa chain constant region sequences for BALB/c, PL, SJL, and M spreus. Other naturally occurring allotypes are possible.

The mouse γ, heavy chain constant region DNA sequence from newborn mice has been published by Honjo et al. (1979) Cell 18:559–568, which is hereby incorporated herein by reference. FIG. 5 of Honjo et al. shows the germline DNA sequence, along with the encoded protein sequence. Shown in the line above is another protein sequence obtained from the mouse myeloma MOPC 21. Other naturally occurring allotypes are possible.

The 1A7 antibody and derivatives thereof are useful, for example, for eliciting an anti-GD2 immune response, for treating a GD2-associated disease, and as reagents for detecting the presence of anti GD2.

Certain compounds, compositions and methods described in this application relate generally to 1A7 and derivatives thereof which are routinely generated by classical techniques of immunochemistry. This includes 1A7 which has been coupled to another compound by chemical conjugation, or by mixing with an excipient or an adjuvant. It includes such immunoglobulin fragments as Fab, Fab′2, Fab′, and isolated heavy and light chains. First generation therapies are those based on such compounds and compositions.

Other compounds, compositions and method described in this invention relate to polynucleotide and polypeptide derivatives of 1A7 that fall outside the preceding classification. The compositions are typically generated by genetic engineering, although they may alternatively be obtained by other methods and combinations of methods. This classification includes (but is not limited to) isolated polynucleotide fragments, recombinant polynucleotides, engineered peptide fragments and fusion peptides, and polynucleotides or polypeptides prepared by chemical synthesis based on the sequence data. Preferred compounds include polypeptide fragments of the 1A7 CDRs, antibody fusion proteins comprising cytokine effector components, single-chain variable region proteins and the polynucleotides encoding them, therapeutic plasmids comprising 1A7 polynucleotides, and vectors such as vaccinia vectors.

Unless explicitly stated otherwise or required by their nature, methods of making or using certain compounds embodied in this invention may be applied to other compounds of this invention, as appropriate.

Pharmaceutical compositions and treatment modalities of this invention may be brought to bear whenever it is desirable to elicit a response against GD2, especially in humans. Human patients with GD2-associated tumors, including melanoma, neuroblastoma, glioma, soft tissue sarcoma, and small cell carcinoma (including small cell lung cancer) are especially appropriate subjects. The subjects may have an advanced form of disease, in which case the objective may include mitigation or reversal of disease progression, and amelioration of side effects. The subjects may have had a history of the condition, for which they have already been treated, in which case the objective will typically include a decrease or delay in the risk of recurrence of clinical pathology. Additionally, the antibodies and derivatives of this invention may also be used as probes, primers, and polypeptides for use as diagnostic reagents. These applications are described in more detail in the sections that follow.

Definitions

“1A7” is a particular anti-idiotypic antibody raised against the anti-GD2 monoclonal antibody with the designation 14G2a. The generation and characterization of 1A7 is described in Example 1. “Immunologic activity” of 1A7 refers to any of the following activities: high ability to specifically bind monoclonal antibody 14G2a; ability to inhibit the binding of 1A7 to 14G2a or 14G2a to GD2 in a specific manner; and an ability to elicit an immune response against GD2. A specific immune response may comprise antibody, B cells, T cells, and any combination thereof, and effector functions resulting therefrom. Included are the antibody-mediated functions ADCC and complement-mediated cytolsis (CDC). The T cell response includes T helper cell function, cytokotic T cell function, inflammation/inducer T cell function, and T cell mediated suppression. A compound able to elicit a specific immune response according to any of these criteria is referred to as “immunogenic”. 1A7 “activity” or “function” refers to any of the immunological activities of 1A7, or to any other biological activity ascribed to 1A7 in this disclosure, including the role of 1A7 in the amelioration or palliation of GD2-associated disease.

The “variable region” of 1A7 refers to the variable region of the 1A7 light chain or the variable region of the 1A7 heavy chain, either alone or in combination.

GM-CSF, IL-2, and other biologically active molecules referred to herein are meant to include fragments and derivatives based on the respective parent molecule that have the same biologic or physiologic function.
A "polynucleotide" is a polymeric form of nucleotides of any length, which contain deoxyribonucleotides, ribonucleotides, and analogs in any combination analogs. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes double-, single-stranded, and triple-helical molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double stranded form.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, rRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracil, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be phosphorylated, or any other manipulation or modification, such as conjugation with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching to proteins, metal ions, labeling components, other polynucleotides, or a solid support.

The term "recombinant" polynucleotide as used herein intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in an arrangement not found in nature.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids or amino acid analogs, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Unless stated or implied otherwise, the term 1A7 polypeptide includes any polypeptide monomer or polymer with 1A7 activity, including the intact 1A7 antibody, and smaller and larger functionally equivalent polypeptides, as described herein.

A "fusion polypeptide" is a polypeptide comprising regions in a different position in the sequence than occurs in nature. The regions may normally exist in separate proteins and are brought together in the fusion polypeptide; or they may normally exist in the same protein but are placed in a new arrangement in the fusion polypeptide.

A "functionally equivalent fragment" of a 1A7 polypeptide or polynucleotide varies from the native sequence by any combination of additions deletions, or substitutions while preserving at least one functional property of the fragment relevant to the context in which it is being used. A functionally equivalent fragment of a 1A7 polynucleotide either encodes a polypeptide that is functionally equivalent to 1A7 when used in an expression system, or has similar hybridization specificity as a 1A7 polynucleotide when used in a hybridization assay. A functionally equivalent fragment of a 1A7 polypeptide typically has one or more of the following properties: ability to bind monoclonal antibody 14G2a; ability to inhibit the binding of 1A7 to 14G2a or 14G2a to GD2 in a specific manner; and an ability to elicit an immune response with a similar antigen specificity as that elicited by 1A7.

A "cell line" or "cell culture" denotes higher eukaryotic cells grown or maintained in vitro. It is understood that the descendant of a cell may not be completely identical (either morphologically, genotypically, or phenotypically) to the parent cell. A "vector" refers to a recombinant DNA or RNA plasmid or virus that comprises a heterologous polynucleotide to be delivered into a target cell, either in vitro or in vivo. The heterologous polynucleotide may comprise a sequence of interest for purposes of therapy, and may optionally be in the form of an expression cassette. As used herein, a vector need not be capable of replication in the ultimate target cell or subject. The term includes cloning vectors for the replication of a polynucleotide, and expression vectors for translation of a polynucleotide encoding sequence. Also included are viral vectors, which comprise a polynucleotide encapsidated or enveloped in a viral particle.

A "host cell" denotes a eukaryotic cell that has been genetically altered. A cell is capable of being genetically altered by administration of an exogenous polynucleotide, such as a recombinant plasmid or vector. When referring to genetically altered cells, the term refers both to the originally altered cell, and to the progeny thereof.

"Heterologous" means derived from a genotypically distinct entity from the rest of the entity to which it is being compared. For example, a polynucleotide may be placed by genetic engineering techniques into a plasmid or vector derived from a different source, and is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence with which it is not naturally found linked is a heterologous promoter.

A "signal sequence" or a "leader sequence" is a short amino acid sequence that directs a newly synthesized secretory or membrane protein through a cellular membrane, usually the endoplasmic reticulum. Signal sequences are typically in the N-terminal portion of a polypeptide and are typically removed between biosynthesis and secretion of the polypeptide from the cell.

An "isolated" polynucleotide or polypeptide is one that is substantially free of the materials with which it is associated in nature. By substantially free is meant at least 50%, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90% free of the materials with which it is associated in nature.

A "stable duplex" of polynucleotides, or a "stable complex" formed between any two or more components in a biochemical reaction, refers to a duplex or complex that is sufficiently long-lasting to persist between the formation of the duplex or complex and subsequent detection, including any optional washing steps or other manipulation that may take place in the interim.

A "biological sample" encompasses a variety of sample types, including blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures, or cells derived therefrom and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain compounds, such as proteins or polynucleotides. The term encompasses various kinds of clinical samples obtained from any species, and also includes cells in culture, cell supernatants, and cell lysates.
A “vaccine” is a pharmaceutical composition for human or animal use, which is administered with the intention of conferring the recipient with a degree of specific immunological reactivity against a particular target, or group of targets. The immunological reactivity may be antibodies or cells particularly B cells, plasma cells, T helper cells, and cytotoxic T lymphocytes, and their precursors) that are immunologically reactive against the target, or any combination thereof. For purposes of this invention, the target is primarily tumor associated antigen GD2, but also includes any tumor associated antigen bound by 14G2a. The immunological reactivity may be desired for experimental purposes, for treatment of a particular condition, for the elimination of a particular substance, or for prophylaxis. An active vaccine is a vaccine intended to elicit an immune response in the recipient that persists in the absence of the vaccine components.

“Adjuvant” as used herein has two meanings, depending on the context in which it is used. In the context of a pharmaceutical preparation, an adjuvant is a chemical or biological agent given in combination with an antibody, polynucleotide or polypeptide to enhance its immunogenicity. In the context of cancer diagnosis or management, it refers to a class of subjects with no clinically detectable tumor mass, but who are suspected of being at risk of recurrence because of a history of cancer.

When referring to a type of cancer that normally manifests as a solid tumor, a “clinchably detectable” tumor is one that is detectable on the basis of tumor mass; i.e., by such procedures as CAT scan, X-ray, or palpation. Positive biochemical, histological or immunological findings on their own are insufficient to meet this definition.

An effective amount is an amount sufficient to effect a beneficial or desired clinical result. An effective amount can be administered in one or more doses. For purposes of this invention, an effective amount of a 1A7 polynucleotide or polypeptide is an amount that induces an immune response, particularly an anti-GD2 response. In terms of treatment, an effective amount is amount that is sufficient to palliate, ameliorate, stabilize, reverse or slow the progression of the GD2-associated disease, or otherwise reduce the pathological consequences of the disease.

An “individual” is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, and pets.

General techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, “Molecular Cloning: A Laboratory Manual”, second edition (Sambrook et al., 1989); “Oligonucleotide Synthesis” (M. J. Gait, ed., 1984); “Animal Cell Culture” (R. I. Freshney, ed., 1987); “Methods in Enzymology” (Academic Press, Inc.); “Handbook of Experimental Immunology” (D. M. Weil & C. C. Blackwell, eds.); “Gene Transfer Vectors for Mammalian Cells” (J. M. Miller & M. P. Calos, eds., 1987); “Current Protocols in Molecular Biology” (F. M. Ausubel et al., eds., 1987); “PCR: The Polymerase Chain Reaction” (Mullis et al., eds., 1994); “Current Protocols in Immunology” (J. E. Coligan et al., eds., 1991).

All patents, patent applications, articles and publications mentioned herein, both supra and infra, are hereby incorporated herein by reference.

These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

How monoclonal antibody IA7 was generated and selected

1A7 was obtained by immunizing naive mice with 14G2a anti-GD2 antibody to obtain an anti-idiotypic response. 14G2a binds to a unique epitope of GD2. Syngeneic BALB/c mice were immunized four times with 14G2a (Ab1) and their spleen cells were fused with the non-secreting myeloma P3/NS-1 cells.

To obtain an anti-idiotypic with all the features we desired, an extensive screening process was employed, comprising the following four important steps: (1) Positive selection for antibody binding to 14G2a; (2) Negative selection against antibody recognizing isotypic or allotypic determinants; (3) Positive selection for an ability to inhibit the binding of 14G2a to GD2; and (4) Positive selection for an ability to induce a humoral immune response against the original tumor-associated antigen (GD2) in both mice and rabbits.

Several anti-idiotypic (Ab2) hybridomas were obtained that were specific for the idiotypic components of the 14G2a immunogen, and did not react with its isotypic or allotypic determinants. To determine whether the anti-1 4G2a were directed against the paratope of 14G2a, the binding of radiolabeled 14G2a to the GD2-positive cell line M21/P6 was studied in the presence of varying amounts of Ab2 hybridoma culture supernatants. With as little as 5 μl of culture supernatant, three of the Ab2 tested inhibited the binding by at least 70%. 1A7 showed the highest level of inhibition. Accordingly, 1A7 was grown and purified from ascites fluid for further studies, while the others were kept in reserve.

The purified Ab2 was prepared as a vaccine and injected into naive mice and rabbits. After 4 injections, serum samples were titered for the presence of Ab3 that bound not only to the immunizing Ab2, but also to GD2. The Ab2 passing all of these screening stages was designated 1A7.

Further details of the method used to obtain 1A7 are provided in Example 1.

Ab3 produced in animals immunized with 1A7 has been further characterized. The immune sera from both mice and rabbits competed with 14G2a for binding to the GD2-associated cell line M21/P6 and inhibited the binding of radiodinated 14G2a to 1A7. This indicated that anti-anti-
Id (Ab3) in mice and rabbits may share idiotopes with 14G2a and they probably bind to the same epitope as Ab1. Administration of 1A7 to non-human primates (cynomolgus monkeys) also generated a specific immune response, comprising activity against GD2 (Example 3).

We have also deduced the nucleic acid sequence encoding the light and heavy chain variable regions of 1A7, along with the translated protein sequences (Example 2).

The invention also encompasses 1A7 conjugated to a label capable of producing a detectable signal. These conjugated antibodies are useful, for example, in detection systems such as quantitation of anti-GD2 or tumor imaging. Such labels are known in the art and include, but are not limited to, radioisotopes, enzymes, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds. The labels may be covalently linked to 1A7, or conjugated to the 1A7 through a secondary reagent, such as a second antibody, protein A, or a biotin-avidin complex. Preparation of 1A7 antibody

The 1A7 antibody of this invention can be prepared in several ways. It is most conveniently obtained from the hybridoma deposited with the ATCC under Accession No. HB-11786, or the expression in vitro/expressed in vitro. For example, this can be cultured in a suitable medium, and spent medium can be used as an antibody source. Optionally, matrix-coated channels or beads and cell cultures may be utilized to enhance growth of antibody-producing cells. For the production of large amounts of antibody, it is generally more convenient to obtain an ascites fluid. The method of raising ascites generally comprises injecting hybridoma cells into an immunologically naive histocompatible or immunotolerant mammal, especially a mouse. The antibody is optionally primed for ascites production by prior administration of a suitable composition; for example, Freund's

Alternatively, 1A7 can be chemically synthesized using sequence data and other information provided in this disclosure, in conjunction with standard methods of protein synthesis. A suitable method is the solid-phase Merrifield technique. Automated peptide synthesizers are commercially available, such as those manufactured by Applied Biosystems, Inc. (Foster City, Calif.).

1A7 may also be obtained by employing routine recombinant methods such as described in Sambrook et al. (1989). For example, this can be cultured herein, a polynucleotide encoding either the 1A7 heavy or light chain can be cloned into a suitable expression vector (which contains control sequences for transcription, such as a promoter). The expression vector is in turn introduced into a host cell. The host cell is grown under suitable conditions such that the polynucleotide is transcribed and translated into a protein. Heavy and light chains of 1A7 may be produced separately, and then combined by disulfide bond rearrangement. Alternatively, vectors with separate polynucleotides encoding each chain of 1A7, or a vector with a single polynucleotide encoding both chains as separate transcripts, may be transcribed into a single host cell which may then produce and assemble the entire molecule. Preferably, the host cell is a higher eukaryotic cell that can provide the normal carbohydrate complement of the molecule. The 1A7 thus produced in the host cell can be purified using standard techniques in the art. A polynucleotide encoding 1A7 for use in the production of 1A7 by any of these methods can in turn be obtained from the hybridoma producing 1A7, or be produced synthetically or recombinantly from the DNA sequence provided herein.

Methods of antibody isolation are well known in the art, for example, Harlow and Lane (1988) Antibodies: A
The invention provides various polynucleotides encoding the anti-idiotyp antibody 1A7 or fragments of 1A7, based on the polynucleotide sequences provided herein. Various embodiments are described in this section, comprising a number of different combinations of the 1A7 heavy or light chain variable region sequences. In general, a 1A7 polynucleotide of this invention encodes at least one feature that is unique to the 1A7 molecule (in comparison with other immunoglobulins). Preferably, this feature is related in some way to an immunological reactivity of 1A7.

The invention encompasses a polynucleotide encoding a portion of the 1A7 light chain variable region, comprising at least about 70 consecutive nucleotides, preferably at least about 80 consecutive nucleotides, even more preferably at least about 100 consecutive nucleotides, even more preferably at least about 150 nucleotides of SEQ ID NO:1. The invention also encompasses a polynucleotide encoding a portion of the 1A7 light chain variable region, comprising at least about 25 consecutive nucleotides, preferably at least about 30 consecutive nucleotides, even more preferably at least about 35 consecutive nucleotides of the CDR1 encoding sequence thereof. The invention also encompasses a polynucleotide encoding a portion of the 1A7 light chain variable region, comprising at least about 20 consecutive nucleotides, preferably at least about 25 consecutive nucleotides, even more preferably at least about 35 consecutive nucleotides of the CDR2 or CDR3 encoding sequence thereof.

The invention also encompasses a polynucleotide encoding a portion of the 1A7 heavy chain variable region, comprising at least about 70 consecutive nucleotides, preferably at least about 80 consecutive nucleotides, even more preferably at least about 100 consecutive nucleotides, even more preferably at least about 150 nucleotides of SEQ ID NO:3. The invention also encompasses a polynucleotide encoding a portion of the 1A7 light chain variable region, comprising at least about 20 consecutive nucleotides of the CDR1 encoding sequence thereof. The invention also encompasses a polynucleotide encoding a portion of the 1A7 light chain variable region, comprising at least about 20 consecutive nucleotides, preferably at least about 25 consecutive nucleotides, even more preferably at least about 35 consecutive nucleotides of the CDR2 or CDR3 encoding sequence thereof.

The invention includes isolated 1A7 polynucleotides encoding a polypeptide having immunological activity of 1A7, wherein the polypeptide encodes at least 5 amino acids of a variable light chain of 1A7 as depicted in SEQ. ID. NO:2. In another embodiment, the invention includes isolated 1A7 polynucleotides encoding a polypeptide having immunological activity of 1A7, wherein the polynucleotide encodes at least 5 amino acids of a variable heavy chain of 1A7 as depicted in SEQ. ID. NO:4. The polynucleotide sequence may be similar to those depicted in SEQ ID NO:1 (FIG. 1) or SEQ ID NO:3 (FIG. 2) with changes designed to optimize codon usage, stability, or to facilitate cloning, or for some other purpose. It is within the skill of one in the art, given the amino acid sequence in SEQ ID NO:2 or SEQ ID NO:4, to design such polynucleotides. Preferred are polynucleotides encoding at least five amino acids of a 1A7 complementarity determining region (CDR).

The invention also encompasses polynucleotides encoding for functionally equivalent variants and derivatives of 1A7 and functionally equivalent fragments thereof which may enhance, decrease or not significantly affect properties of the polypeptides encoded thereby. These functionally equivalent variants, derivatives, and fragments display the
ability to induce an immune response, preferably an anti-GD2 immune response. For instance, changes in a DNA sequence that do not change the encoded amino acid sequence, as well as those that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are those which will not significantly affect properties of the encoded polypeptide.

The IA7 polynucleotides of the invention may comprise additional sequences, such as additional encoding sequences within the same transcription unit, controlling elements such as promoters, ribosome binding sites, and polyadenylation sites, additional transcription units under control of the same or a different promoter, sequences that permit cloning, expression, and transformation of a host cell, and any such construct as may be desirable to provide embodiments of this invention.

The invention includes a polynucleotide of at least about 15 consecutive nucleotides, preferably at least about 20 nucleotides, more preferably at least about 25 consecutive nucleotides, more preferably at least about 35 consecutive nucleotides, even more preferably at least about 50 nucleotides, still more preferably at least about 75 nucleotides, still more preferably at least about 100 nucleotides, still more preferably at least about 200 nucleotides, and even more preferably at least about 300 nucleotides that forms a stable hybrid with a polynucleotide encoding the light chain or heavy chain variable region of 1A7, but not with another immunoglobulin encoding regions known at the time of filing of this application. Any set of conditions may be used for this test, as long as at least one set of conditions exist wherein the test polynucleotide demonstrates the required specificity. Preferably, the IA7 encoding sequences to which the test polynucleotide binds are those shown in SEQ. ID NO:1 and 3. Since the known immunoglobulin sequences fall into a hierarchy of similarity with that of IA7, the test may be performed by comparing the hybridization of the test polynucleotide with the IA7 sequence with the hybridization with the most closely related sequences. Preferred is a panel of about 10 of the most closely related sequences to SEQ. ID NO:1 or 3, as appropriate. Sequences to which the test polynucleotide should not form a duplex are the light chain variable region encoding sequences listed in SEQ. ID NO:7 through SEQ. ID NO:26 and the heavy chain variable region encoding sequences listed in SEQ. ID NO:27 through SEQ. ID NO:44.

Hybridization reactions can be performed under conditions of different “stringency”. Conditions that increase stringency of a hybridization reaction are published, see, for example, Sam勃rook and Maniatis. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25°C, 37°C, 50°C and 68°C; buffer concentrations of 10x SSC, 6x SSC, 1x SSC, 0.1x SSC (where SSC is 0.15M NaCl and 15 mM citrate buffer) and their equivalent using other buffer systems: formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 2 hours; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 0x SSC, 1x SSC, 0.1x SSC, or deionized water. "Tm" is the temperature in degrees Centigrade at which 50% of a polynucleotide duplex made of complementary strands hydrogen bonded in antiparallel direction by Watson-Crick base pairing dissociates into single strands under conditions of the experiment. Tm may be predicted according to a standard formula, such as:

\[ Tm = 81.5 + 16.6 \log[Na] + 0.41(\%GC) - 0.61(\%GC)^2 - 6.0L \]

where [Na+] is the cation concentration (usually sodium ion) in mol/L; (%GC) is the number of G and C residues as a percentage of total residues in the duplex; (\%GC) is the percent formamide in solution (wt/vol); and L is the number of nucleotides in each strand of the duplex.

Useful IA7 polynucleotides encoding fragments of IA7 may be identified by generating polynucleotide fragments (based on SEQ ID NO:1 or SEQ ID NO:3, for example) and testing the polypeptides encoded thereby for a function of interest. Alternatively, the polypeptide fragment encoded by a particular polynucleotide may be prepared and tested for a function of interest. Alternatively, given a IA7 polypeptide with desirable properties, a polynucleotide could be designed that encodes it.

Included in all these embodiments are polynucleotides with encoding regions for IA7 polymers, fusion proteins, humanized immunoglobulins, single-chain variable regions, and other particular polynucleotides of interest. These polypeptides are described in a later section.

The invention also provides polynucleotides covalently linked with a detectable label. Such polynucleotides are useful, for example, as probes for detection of related nucleotide sequences.

Preparation of IA7 polynucleotides:
The polynucleotides of this invention can be obtained using chemical synthesis, recombinant cloning methods, PCR, or any combination thereof.

Methods of chemical polynucleotide synthesis are well known in the art and need not be described in detail herein. One of skill in the art can use the sequence data provided herein to obtain a desired polynucleotide by employing a DNA synthesizer or ordering from a commercial service.

Alternatively, IA7 polynucleotide sequences can be obtained from a IA7 antibody producing cell line, IA7 cloning vector, or IA7 expression vector. RNA or DNA encoding the desired sequence may be isolated, amplified, and processed by standard recombinant techniques. Such techniques include digestion with restriction nucleases, and amplification by polymerase chain reaction (PCR), or a suitable combination thereof. PCR technology is described in U.S. Pat. Nos. 4,683,195, 4,800,150, 4,754,065 and 4,683,202, as well as PCR: The Polymerase Chain Reaction, Mullis et al. eds., Birkauwen Press, Boston (1994). A polynucleotide comprising a desired sequence can be inserted into a suitable vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification. Polynucleotides may be inserted into host cells by any means known in the art. Cells are transformed by introducing an exogenous polynucleotide by direct uptake, endocytosis, transfection, f-mating or electroporation. Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell genome. Amplified DNA can be isolated from the host cell by standard methods see, e.g., Sambrook et al. (1989). RNA may also be obtained from transformed host cell, it may be obtained by using a DNA-dependent RNA polymerase.

It used as a vaccine, plasmids containing IA7 polynucleotides are preferably prepared by the method of Horn et al. (1995) Human Gene Therapy 6:565-573), which produces a pharmaceutical grade plasmid DNA suitable for administration.

Cloning and expression vectors comprising a IA7 polynucleotide

The present invention further includes a variety of vectors comprising a IA7 polynucleotide. These vectors can be used for expression of recombinant polypeptides as well as a source of IA7 polynucleotides. Cloning vectors can be used to obtain replicate copies of the IA7 polynucleotides they
contain, or as a means of storing the polynucleotides in a depository for future recovery. Expression vectors (and host cells containing these expression vectors) can be used to obtain polypeptides produced from the polynucleotides they contain. They may also be used where it is desirable to express 1A7 polypeptides in an individual and thus have intact cells capable of synthesizing the polypeptide, such as in gene therapy. Suitable cloning and expression vectors include any known in the art, e.g., those for use in bacterial, mammalian, yeast and insect expression systems. Specific vectors and suitable host cells are known in the art and need not be described in detail herein. For example, see Gacesa and Ranji, *Vectors*, John Wiley & Sons (1994).

Cloning and expression vectors typically contain a selectable marker (for example, a gene encoding a protein necessary for the survival or growth of a host cell transformed with the vector), although such a marker gene can be carried on another polynucleotide sequence co-introduced into the host cell. Only those host cells into which a selectable gene has been introduced will grow under selective conditions. Typical selection genes either: (a) confer resistance to antibiotics or other toxins substances, e.g., ampicillin, neomycin, tetracycline; (b) complex nutrients deficiencies; or (c) supply critical nutrients not available from complex media. The choice of the proper marker gene will depend on the host cell, and appropriate genes for different hosts are known in the art. Cloning and expression vectors also typically contain a replication system recognized by the host.

Suitable cloning vectors may be constructed according to standard techniques, or may be selected from a large number of cloning vectors available in the art. While the cloning vector may vary in detail herein, it is expected that the host cell intended to be used, useful cloning vectors will generally have the ability to self-replicate, may possess a single target for a particular restriction endonuclease, or may carry genes for a marker that can be used in selecting clones containing the vector. Suitable examples include plasmids and bacterial viruses, e.g., pUC18, mp18, mp19, pBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors such as pSA3 and pAT28. These and many other cloning vectors are available from commercial vendors such as BioRad, Stratagene, and Invitrogen.

Expression vectors generally are replicable polynucleotide constructs that contain a polynucleotide encoding a 1A7 polypeptide of interest. The polynucleotide encoding the 1A7 polypeptide is operatively linked to suitable transcriptional controlling elements, such as promoters, enhancers and terminators. For expression (i.e., translation), one or more translational controlling elements are also usually required, such as ribosome binding sites, translation initiation sites, and stop codons. These controlling elements (transcriptional and translational) may be derived from the 1A7 gene, or they may be heterologous (i.e., derived from other genes or other organisms). A polynucleotide sequence encoding a signal peptide can also be included to allow a 1A7 polypeptide to cross or lodge in cell membranes or be secreted from the cell. A number of expression vectors suitable for expression in eukaryotic cells including yeast, avian, and mammalian cells are known in the art. One example of an expression vector is pCDNA3 (Invitrogen, San Diego, Calif., in which transcription is driven by the cytomegalovirus (CMV) early promoter/enhancer. This vector also contains recognition sites for multiple restriction enzymes for insertion of the 1A7 polynucleotide of interest. Another example of an expression vector (system) is the baculovirus/insect system.

The vectors containing the polynucleotides of interest can be introduced into the host cell by any of a number of appropriate means, including electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (where the vector is an infectious agent, such as vaccinia virus, which is discussed below). The choice of means of introducing vectors or 1A7 polynucleotides will often depend features of the on the host cell.

Once introduced into a suitable host cell, for example, *E. coli* or COS-7, expression of a 1A7 polypeptide can be determined using any of the assays described herein. For example, presence of 1A7 polypeptide can be detected by RIA or ELISA of the culture supernatant (if the 1A7 polypeptide is secreted) or cell lysates.

A particularly useful expression vector for 1A7 polynucleotides is a vaccinia virus comprised of a 1A7 polynucleotide sequence, which can also be used in vaccine preparations (Moss (1991) *Science* 252:1662-1667). To introduce polynucleotide sequences encoding 1A7 polypeptide, including 1A7 polypeptide fragments, into vaccinia, the gene of interest is first inserted into a plasmid containing a vaccinia virus promoter with flanking sequences homologous to vaccinia DNA inessential for replication. Plasmid-containing cells are then infected with vaccinia, which leads to a low level of homologous recombination between plasmid and virus, with resultant transfer of the vaccinia promoter and 1A7 polypeptide-encoding polynucleotide sequence into the vaccinia virus genome. Typically, the 1A7 polynucleotide is inserted into the viral tk (thymidine kinase) gene. Insertion into the tk site attenuates the virus more than 1,000 fold compared to wild type (Flexner et al. (1980) *Vaccine* 8 (Cold Spring Harbor Laboratory), 179-184). Recombinant virus is identified by the tk phenotype. Preferably, expression of the 1A7 polynucleotide is under the control of the vaccinia early/late promoter (7.5K), whereby the resultant 1A7 polypeptides can be expressed in infected cells throughout the life cycle of the virus. However, other promoters known in the art can be used, such as ph6, or synthetic promoters. Expression of the 1A7 polypeptide occurs in cells infected with the recombinant vaccinia or individuals which are immunized with the live recombinant vaccinia virus. Any one of several strains of vaccinia can be used, including but not limited to WR, ALVAC, and NYVAC.

A vaccinia vector of this invention can contain one or more polynucleotides encoding a 1A7 polypeptide. It can also contain polynucleotide sequences encoding other polypeptides that enhance, facilitate, or modulate the desired result, such as lymphokines, including, but not limited to, IL-2, IL-4 and GM-CSF. A preferred lymphokine is GM-CSF. Preferred GM-CSF constructs are those which have been deleted for the AU-rich elements from the 3' untranslated regions and sequences in the 5' untranslated region that are capable of forming a hairpin loop. Also embodied in this invention is vaccinia vectors encoding for recombinant 1A7 variants containing 1A7 polypeptides, such as scFvs, chimeras, and polymers, as described below. Host cells transformed with 1A7 polynucleotides

Other embodiments of this invention are host cells transformed with 1A7 polynucleotides and vectors comprising 1A7 polynucleotide sequences, as described above. Both prokaryotic and eukaryotic host cells may be used. Prokaryotic hosts include bacterial cells, for example *E. coli* and mycobacteria. Among eukaryotic hosts are yeast, insect, avian, plant and mammalian cells. Host systems are known
in the art and need not be described in detail herein. One example of a mammalian host cell is NS0, obtainable from the European Collection of Cell Cultures (England). Transfection of NS0 cells with a plasmid, for example, which is driven by a CMV promoter, followed by amplification of this plasmid in using glutamine synthetase provides a useful system for protein production. (Cockett et al. (1990) Bio/Technology 8:662–667).

The host cells of this invention can be used, inter alia, as repositories of 1A7 polynucleotides, or as vehicles for production of 1A7 polynucleotides and polypeptides. They may also be used as vehicles for in vivo expression of 1A7 polypeptides.

Uses for 1A7 polynucleotides

The polynucleotides of this invention have several uses. 1A7 polynucleotides are useful, for example, in expression systems for the production of 1A7 or 1A7 fragments. They are also useful as hybridization probes to assay for the presence of 1A7 polynucleotide or related sequences in a sample using methods well known to those in the art. Further, 1A7 polynucleotides are also useful as primers to effect amplification of desired polynucleotides. The polynucleotides may be used as therapeutically active agents, as pharmaceutical compositions including vaccines and for gene therapy. 1A7 polynucleotides can also be used as hybridization probes for detection of 1A7 encoding sequences. Suitable samples include cells transfected ex vivo for use in gene therapy. In one illustration, DNA or RNA is extracted from a sample, and optionally run on a gel and/or digested with restriction nucleases. The processed sample polynucleotide is typically transferred to a medium suitable for washing. The sample polynucleotide is then contacted with the 1A7 polynucleotide probe under conditions that permit a stable duplex to form if the sample contains a matching 1A7 sequence. Any stable duplexes formed are detected by any suitable means. For example, the 1A7 polynucleotide probe may be supplied in labeled form, and label remaining with the sample after washing will directly reflect the amount of stable duplex formed. In a second illustration, hybridization is performed in situ. A suitable tissue slice is overlaid with a labeled 1A7 probe to indicate the location of 1A7 encoding sequences comprised therein.

A short 1A7 polynucleotide may also be used as a primer for PCR reaction, particularly to amplify a longer sequence comprising a region hybridizing with the primer. This may be conducted preparatively, in order to produce polynucleotide for further genetic manipulation. It may also be conducted analytically, to determine whether a 1A7 encoding polynucleotide is present, for example, in a sample of diagnostic interest.

The 1A7 polynucleotides of this invention can be used in expression systems to produce 1A7 polypeptides, intact 1A7, or recombinant forms of 1A7, such as are described below.

Another use of 1A7 polynucleotides is in vaccines and gene therapy. The general principle is to administer the polynucleotide so that it either promotes or attenuates the expression of the polypeptide encoded therein. Thus, the present invention includes methods of inducing an immune response and methods of treatment comprising administration of an effective amount 1A7 polynucleotides to an individual. In these methods, a 1A7 polynucleotide encoding a 1A7 polypeptide is administered to an individual, either directly or via cells transfected with the 1A7 polynucleotide. Preferably, the 1A7 polynucleotide is in the form of a circular plasmid, preferably in a supercoiled configuration. Preferably, the 1A7 polynucleotide is replicated inside a cell. Thus, the 1A7 polynucleotide is operatively linked to a suitable promoter, such as a heterologous promoter that is intrinsically active in cells of the target tissue type. Preferably, once in cell nuclei, plasmids persist as circular non-replicating episomal molecules. In vitro mutation may be carried out with plasmid constructs to provide, for example, molecules that are more immunogenic or that comprise a T cell epitope with a desirable HLA motif.

To determine whether plasmids containing 1A7 polynucleotides are capable of expression in eukaryotic cells, eukaryotic cells such as COS-7, CHO, or HeLa cells can be transfected with the plasmids. Expression of 1A7 polypeptide is then determined by immunosay, for example, by Western blot. Smaller 1A7 polypeptides can be detected, for example, by constructing the plasmid so that the resultant 1A7 polypeptide is fused with a tag, such as a target epitope or enzyme label. Further characterization of the expressed 1A7 polypeptide can be achieved by purifying the peptide and then conducting one of the functional assays described herein.

In one mode of gene therapy, the polynucleotides of this invention are used for genetically altering cells ex vivo. In this strategy, cells are grown or harvested from a cell line are transfected or transduced with vectors encoding a 7 polypeptide, and then administered to a recipient. Suitable cells for transfection include peripheral blood mononuclear cells.

In another mode of gene therapy, the polynucleotides of this invention are used for genetically altering cells in vivo. The purpose may include (but is not limited to) eliciting an antibody response to 1A7 or GD2, eliciting a T cell response to 1A7 or GD2, and treating various types of cancer. These uses are elaborated in more fully in a later section.

Polypeptide Derivatives of 1A7

The present invention encompasses polypeptide fragments of 1A7 containing at least a portion of a variable region of 1A7. Preferred fragments are those with immunological activity of 1A7.

Also preferred are fragments which comprise amino acid sequences substantially different from other immunoglobulins, and fragments comprising a CDR. In one embodiment, the invention includes a polypeptide fragment of the 1A7 heavy chain variable region, comprising at least 25 consecutive amino acids, more preferably 30 consecutive amino acids of SEQ ID NO:2, or 5 consecutive amino acids of the CDR1 thereof, or at least 7 consecutive amino acids, preferably at least 9 consecutive amino acids of the CDR2 or CDR3 thereof. The invention also includes a polypeptide fragment of the 1A7 light chain variable region, comprising at least 25 consecutive amino acids, more preferably 30 consecutive amino acids of SEQ ID NO:4, or 7 consecutive amino acids of the CDR2 thereof, or at least 8 consecutive amino acids, preferably 10 consecutive amino acids of the CDR1 or CDR3 thereof.

The size of the 1A7 polypeptide fragments may be only the minimum size required to provide a desired function. It may optionally comprise additional sequence, either native to 1A7, or from a heterologous source, as desired. 1A7 fragments may contain only 5 consecutive amino acids from a 1A7 variable region sequence. Polypeptides comprising 7 amino acids, more preferably about 10 amino acids, more preferably about 15 amino acids, more preferably about 25 amino acids, more preferably about 50 amino acids, more preferably about 75 amino acids from the 1A7 light or heavy chain variable region are also included. Even more preferred are polypeptides comprising the entire 1A7 light or heavy chain variable region.
The invention includes modified 1A7 polypeptides which are functionally equivalent to 1A7, or have altered but measurable 1A7 immunologic activity. Fragments with improved 1A7 immunologic activity are preferred. Examples of modified polypeptides include polypeptides with conservative substitutions of amino acid residues, and one or more deletions or additions of amino acids which do not significantly deleteriously change the functional activity. One example of this is 1A7 polypeptides comprising one or more amino acid substitution in comparison with the prototype 1A7 sequence. Substitutions can range from changing or modifying one or more amino acids to complete redesign of a region, such as the variable region. Amino acid substitutions, if present, are preferably conservative substitutions that do not deleteriously affect folding or functional properties of the peptide. Groups of functionally related amino acids within which conservative substitutions may be made are glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine/methionine; lysine/arginine; and phenylalanine/tyrosine/tryptophan. Polypeptides of this invention may be in glycosylated or unglycosylated form, may be modified post-translationally (e.g., acetylation, and phosphorylation) or may be modified synthetically (e.g., the attachment of a labeling group).

The invention also encompasses fusion proteins comprising one or more 1A7 polyepitope. A 1A7 fusion polyepitope can be prepared, for example, by chemical synthesis, or by creating and translating a polynucleotide in which the peptide regions are encoded in the desired relationship. Alternatively, fusion proteins may be provided in expression systems constructed by co-transfection with plasmids comprising encoding regions for different functional regions of the protein. Useful heterologous sequences for inclusion in a fusion polyepitope include sequences that provide for secretion from a host cell, enhance immunological reactivity, or facilitate the coupling of the polyepitope to an immunosassay support or a vaccine carrier. One example is a bacterial “super antigens,” such as staphylococcal enterotoxin A (SEA) (Dohlslien et al. (1994) Proc. Natl. Acad. Sci. USA 91:8945–8949). In a preferred example, a 1A7 polyepitope is fused with a bioreponse modifier, particularly a cytokine, which may enhance immunogenicity. Examples of bioreponse modifiers include cytokines and lymophokines such as GM-CSF, interleukin-2 (IL-2), interleukin 4 (IL-4), and γ-interferon. GM-CSF and IL-2 are especially preferred. The preferred arrangement is for the cytokine effector unit to be fused to the C-terminal of the immunoglobulin heavy chain.

1A7 polyepitope derivatives comprising both a 1A7 light chain and a 1A7 heavy chain may be formed as separate light and heavy chains and then assembled, or assembled in situ by an expression system for both chains. Such expression systems may be created by transfecing with a plasmid comprising separate transcribable regions for the light and heavy chain, or by co-transfecting the same cell with plasmids for each chain. In a third method, a suitable plasmid with a heavy chain encoding region is transfected into a heavy chain loss mutant. For example, heavy chain loss mutants may be obtained by treating 2x10⁵ 1A7 cells with fluorescein-labeled rabbit anti-mouse IgG (H chain specific, DAKO Corporation, Carpinteria, Calif.) according to the supplier’s instruction. The stained and unstained cell populations are analyzed in a fluorescence-activated cell sorter. The unstained cells are collected in a sterilized tube and placed in 96-well plates with 1 cell/well by limiting dilution. The culture supernatants are then assayed by ELISA using goat anti-mouse IgG (heavy chain specific) and goat anti-mouse kappa. The clones with kappa-positive and IgG-negative phenotype are subcloned at least 3 times to obtain stable 1A7 (–) mutants. MRNA from putative heavy chain loss mutant 1A7 (–) clones can be isolated and the sequence of the light chain variable region cDNA determined. Reverse PCR of the MRNA for the 1A7 VFab is performed with 2 sets of 5’ and 3’ primers, used for cloning of 1A7 (–) cDNA (Example 2). A heavy chain loss mutant should yield no detectable DNA band. Transfection of these cells may then proceed using a suitable heavy chain plasmid construct. The invention also encompasses a hybrid antibody, in which one pair of heavy and light chains is obtained from a first antibody, which the other pair of heavy and light chains is obtained from a different second antibody. For purposes of this invention, one pair of light and heavy chains is from 1A7. In one example, each light-heavy chain pair binds different epitopes of GD2. Such hybrids may also be formed using chimeric heavy or light chains.

Another embodiment is a humanized version of 1A7. “Humanized” antibodies are antibodies in which at least part of the sequence has been altered from its initial form to render it more like human immunoglobulin, and the heavy chain and light chain constant regions are replaced with human sequence. This is a fusion polyepitope comprising a 1A7 variable region and a heterogeneous immunoglobulin constant region. In another version, the CDR regions comprise 1A7 amino acid sequences, while the variable framework regions have also been converted human sequences. See, for example, EP0329400. In a third version, variable regions are humanized by designing consensus sequences of variable and mouse variable regions, and converting residues outside the CDRs that are different between the consensus sequences.

Another 1A7 derivative contemplated by this invention is an antibody in which the 1A7 heavy or light chain has been modified to provide additional properties. For instance, a change in amino acid sequence can result in greater immunogenicity of the resultant 1A7 polyepitope. The changes range from changing of one or more amino acids to the complete redesign of a region such as a constant region domain. Changes contemplated affect complement fixation, interaction with membrane receptors, and other effector functions. A recombinant 1A7 antibody may also be designed to aid the specific delivery of a substance (such as a lymphokine) to an effector cell. Also contemplated are proteins in which various immunoglobulin domains have been placed in an order other than occurs in nature.

The invention also encompasses single chain variable region fragments (“scFv”) of 1A7. Single chain variable region fragments are made by linking light and/or heavy chain variable regions by using a short linking peptide (Bird et al. (1986) Science 242: 423–426). Any peptide having sufficient flexibility and length can be used as a linker in a scFv. Usually the linker is selected to have little to no immunogenicity. An example of a linking peptide is (GGGGS), (SEQ ID NO:45), which bridges approximately 3.5 nm between the carboxy terminus of one variable region and the amino terminus of another variable region. Other linker sequences may also be used, and may provide additional fuctions, such as for a means of attaching a drug or to a solid support.

All or any portion of the heavy or light chain can be used in any combination. Typically, the entire variable regions are included in the scFv. For instance, the light chain variable region can be linked to the heavy chain variable region. Alternatively, a portion of the light chain variable region can
be linked to the heavy chain variable region, or thereof. Also contemplated are scFvs in which the heavy chain variable region is from 1A7, and the light chain variable region is from another immunoglobulin. It is also possible to construct a biphasic, scFv in which one component is a 1A7 polypeptide and another component is a different polypeptide, such as a T cell epitope.

The scFvs can be assembled in any order, for example, Vν → (linker) → Vβ or Vβ → (linker) → Vβ. There may be a difference in the level of expression of these two configurations in particular expression systems, in which case one of these forms may be preferred. Tandem scFvs can also be made, such as (X) → (linker) → (X) → (linker) → (X), in which X are 1A7 polypeptides, or combinations of 1A7 polypeptides with other polypeptides. In another embodiment, single chain 1A7 antibody polypeptides have no linker polypeptide, or just a short, inflexible linker. Possible configurations are Vλ → Vβ and Vβ → Vλ. The linkage is too short to permit interaction between Vλ and Vβ within the chain, and the chains form homodimers with a Vλ/NH₂ and an N/β antigen binding site at each end. Such molecules are referred to in the art as “diabodies”.

Single chain variable regions may be produced either recombinitely or synthetically. For synthetic production of scFv, an automated synthesizer can be used. For recombinant production of scFv, a suitable plasmid containing polynucleotide that encodes the scFv can be introduced into a suitable host cell, either eukaryotic, such as yeast, plant, insect or mammalian cells, or prokaryotic, such as E. coli, and the expressed protein may be isolated using standard protein purification techniques.

A particularly useful system for the production of 1A7 scFv is a plasmid vector pET-22b (+) (Novagen, Madison, WIs.) in E. coli. pET-22b (+) contains a nickel ion binding domain consisting of 6 sequential histidine residues, which allows the expressed protein to be purified on a suitable affinity resin. Another example of a vector that can be used is pcDNA3 (Invitrogen, San Diego, Calif.), described above.

Conditions of expression should be such that the scFv polypeptide can assume optimal tertiary structure. Depending on the plasmid used (especially the activity of the promoter) and the host cell, it may be necessary to modulate the rate of production. For instance, use of a weaker promoter, or expression at lower temperatures, may be necessary to optimize production of properly folded scFv in prokaryotic systems; or it may be preferably to express scFv in eukaryotic cells.

Preferred single chain variable regions comprise at least 10 consecutive amino acids of SEQ. ID NO:2 and at least 10 consecutive amino acids of SEQ. ID NO:4, especially wherein the amino acids of SEQ. ID NO:2 and the amino acids of SEQ. ID NO:4 are joined by a linker polypeptide of 5 to 20 amino acids, or comprising the light chain variable region and the heavy chain variable region of monoclonal antibody 1A7.

The invention also encompasses polymeric forms of 1A7 polypeptides, containing a plurality of 1A7 polypeptides. One embodiment is a linear polymer of 1A7 polypeptides, optionally conjugated to carrier. These linear polymers can comprise multiple copies of a single 1A7 polypeptide, or combinations of different 1A7 polypeptides, and can have tandem 1A7 polypeptides, or 1A7 polypeptides separated by other amino acid sequences. Another embodiment is 1A7 multiple antigen peptides (MAPs). MAPs have a small immunologically inert core having radially branching lysine dendrites, onto which a number of 1A7 polypeptides are covalently attached. See Posnett et al. (1988) J. Biol. Chem. 263:1719-1725; Tam (1989) Meth. Enz. 168:7-15. The result is a large macromolecule having a high molar ratio of 1A7 polypeptides to core. MAPs are efficient immunogens and useful antigens for immunoassays. The core for creating an 1A7 MAP can be made by standard peptide synthesis techniques, or obtained commercially (Quality Controlled Biochemicals, Inc., Hopkinton, Mass.). A typical core matrix is made up of three levels of lysine and eight amino acids.

In another embodiment of the invention, the immunogenicity of the 1A7 polypeptides is enhanced by preparing them in expression systems in which they are assembled with particle-stabilizing proteins such as, for example, those associated with hepatitis B virus. See, e.g., U.S. Pat. No. 4,722,840. Constructs wherein the 1A7 polypeptide is linked directly to particle-forming protein coding sequences produce hybrids which are immunogenic for an anti-GD2 response. The vectors also comprise immunogenic HBV epitopes; for example, the pre-S peptide and stimulate a response against HBV. Such expression systems may be provided in eukaryotic cells, including yeast or mammalian cells.

In another embodiment, 1A7 polypeptides are conjugated to a carrier molecule. This is desirable for a 1A7 peptide that comprises a suitable epitope for eliciting anti-GD2, but is too small to be immunogenic. Any conjugation method known in the art may be used. Any carrier can be used which is not harmful to the host. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins; polysaccharides (such as latex functionalized Sepharose, agarose, cellulose, cellulose beads and the like); polymeric amino acids (such as polyglutamic acid, polylysine, and the like); amino acid copolymers, and inactive virus particles or attenuated bacteria, such as Salmonella. Especially useful carrier proteins are serum albumins, keyhole limpet hemocyanin (KLH), certain immunoglobulin molecules, thyroglobulin, ovalbumin, and tetanus toxoid. KLH is especially preferred.

1A7 polypeptides of the invention can be identified in a number of ways. For example, the variable regions of the light and heavy chains can be screened by preparing a series of short polypeptides that together span the entire variable region amino acid sequence. Using a series of polypeptides of 20 or 50 amino acids in length, each 1A7 variable region may be surveyed for useful functional properties. It is also possible to carry out a computer analysis of a protein sequence to identify potentially interesting polypeptides, such as those that bear the shape of D2, or those involved in idiotype-anti-idiotype contact.

Those skilled in the art will readily appreciate that the various adaptations of 1A7 described in this section may be combined in various fashions to yield other 1A7 polypeptides with desirable properties. For instance, 1A7 polypeptides with modified residues may be comprised in a MAP. In another example, a 1A7 scFv is fused to a cytokine, such as IL-2. All such combinations are contemplated in this invention.

Preparation of 1A7 polypeptides

The polypeptides of this invention can be made by any suitable procedure, including proteolysis of the 1A7 antibody, by recombinant methods or by chemical synthesis. 1A7 polypeptides, especially shorter polypeptides up to about 50 amino acids, are conveniently made by chemical synthesis, based on the sequence data and other information provided herein.

Certain 1A7 polypeptides which are fragments of the whole molecule may alternatively be prepared from enzymatic cleavage of intact 1A7. Examples of proteolytic
enzymes include, but are not limited to, trypsin, chymotrypsin, pepsin, papain, V8 protease, subtilisin, plasmin, and thrombin. Intact 1A7 can be incubated with one or more proteinases simultaneously or sequentially. Alternatively, or in addition, intact 1A7 can be treated with disulfide reducing agents. Peptides may then be separated from each other by techniques known in the art, including but not limited to gel filtration chromatography, gel electrophoresis, and reverse-phase HPLC.

A 1A7 polypeptide can also be made by obtaining a nucleotide encoding it according to the information provided elsewhere in this application, and introducing it into a suitable expression system. Typically, polynucleotides encoding a 1A7 polypeptide are ligated into an expression vector under control of a suitable promoter and used to genetically alter the intended host cell. Both eukaryotic and prokaryotic host systems can be used. The polypeptide is then isolated from lysed cells or from the culture medium and purified to the extent needed for its intended use. Examples of prokaryotic host cells appropriate for use with this invention include E. coli. Examples of eukaryotic host cells include avian, insect, plant, and animal cells such as COS7, HeLa, and CHO cells.

In certain situations, such as when a 1A7 polypeptide is expressed in a suitable storage medium such as a plant seed, the 1A7 polypeptide can be used without purification (Fiedler et al. (1995) Biotechnology 13:1090–1093). For most applications, it is generally preferable that the polypeptide is at least partially purified from other cellular constituents. Preferably, the polypeptide is at least about 50% pure, as a weight percent of total protein. More preferably, the protein is at least about 50–75% pure. For clinical use, the polypeptide is preferably at least about 80% pure, according to the criteria given in another section.

Characterization of 1A7 polypeptides

The 1A7 polypeptides of this invention can be characterized in several ways. For instance, a 1A7 polypeptide may be tested for its ability to bind specifically to 14G2a, for its ability to specifically inhibit the binding between 14G2a and intact 1A7, or for its ability to specifically inhibit the binding between 14G2a and GD2. Alternatively, a 1A7 polypeptide can be tested for its ability to elicit an immune response, preferably an anti-GD2 response. 1A7 polypeptides can also be tested for their ability to palliate or ameliorate GD2-associated diseases, such as GD2-associated tumors. It is understood that only one of these properties need be present in order for a polypeptide to come within this invention, although preferably more than one of these properties is present.

The ability of a 1A7 polypeptide to bind 14G2a may be tested by immunosassay. Any form of direct binding assay is suitable. In one such assay, the 14G2a or alternatively the 1A7 polypeptide is labeled. Suitable labels include radioisotopes such as 125I, enzymes such as peroxidase, fluorescent labels, such as fluorescein, and chemiluminescent labels. Typically, the other binding partner is insolubilized (for example, by coating onto a microtiter plate) to facilitate washing. After combining the labeled component with the insolubilized component, the solid phase is washed and the amount of bound label is determined. Another such assay is a sandwich assay, in which the putative 1A7 polypeptide is captured by a first anti-immunoglobulin on a solid phase and developed with a second anti-immunoglobulin. Either the insolubilized or labeled anti-immunoglobulin or both is 14G2a.

To certain of these examples, the extent of binding of 1A7 is directly related to the amount of label bound to the solid phase.

To conduct the inhibition assays, the putative 1A7 polypeptide is titrated for its ability to decrease the binding of 1A7 to 14G2a, or 14G2a to GD2. Either of the binding pairs in the reaction to be inhibited is labeled, while the other is typically insolubilized in order to facilitate washing. GD2, if it is used, may be provided as the purified ganglioside, or as a GD2-expressing cell line, like M21P6. The 1A7 polypeptide is typically mixed with the labeled component, and then the mixture is combined with the solid phase. Polypeptides with the characteristics of 1A7 will proportionately decrease the amount of label attached to the solid phase, compared with control polypeptides. This test may be more sensitive than measuring direct binding, because lower affinity interaction between 1A7 and Ab1 may be too weak to form a stable bond, but adequate to interfere with the binding of another ligand-receptor pair when present at sufficient concentration.

A 1A7 polypeptide or a molecule comprising such a peptide that has an Fc-like effector component may be tested for their ability to mediate immune effector reactions, particularly complement-mediated cytosis (CMC) and antibody-dependent cellular cytotoxicity (ADCC). Suitable assays are described elsewhere in this disclosure.

Another way of characterizing 1A7 polypeptides, particularly those intended for use in therapy, is to test their ability to generate an immune response. Suitable techniques for characterizing the immune response are given in a later section.

Uses of polypeptides

Polypeptide fragments, fusion proteins, and other derivatives of 1A7 have many uses. The uses generally parallel those of the intact 1A7 antibody as outlined earlier in this disclosure. Certain derivatives may have desirable effects for particular uses. For example, humanized or single-chain variable region molecules may be desirable for human administration, because they are less likely to stimulate an anti-mouse isotype response. Fusion proteins comprising a cytokine may be more immunogenic.

Preferred uses of these compounds include eliciting an antibody response to 1A7 or more preferably GD2, eliciting a T cell response to 1A7 or more preferably GD2, and treating various types of GD2-associated cancer. These uses are elaborated more fully in a later section.

Pharmaceutical compositions and vaccines comprising 1A7 antibody and polynucleotide and polypeptide derivatives.

The present invention encompasses pharmaceutical compositions and vaccines containing 1A7 antibody, or polynucleotide or polypeptide derivatives of 1A7 either alone or in combination. Such pharmaceutical compositions and vaccines are useful for eliciting an immune response and treating GD2-associated diseases, either alone or in conjunction with other forms of therapy, such as chemotherapy or radiotherapy.

Pharmaceutical compositions include vaccines for direct administration to an individual. Vaccines may comprise 1A7 antibodies, polynucleotides, or polypeptide derivatives, or a combination thereof.

Vaccines containing naked 1A7 polynucleotides can be used for genetic immunization (see generally Tang et al. (1992) Nature 356: 152–154). Once in the cell nuclei, plasmids comprising 1A7 encoding regions may persist as circular non-replicating episomes leading to dose-dependent and long-lived expression. (Spooner et al. (1995) Gene Therapy 2: 173–180). Immunization using polynucleotides has been shown to generate cellular as well as humoral responses (Spooner et al.; Wang et al. (1995) Human Gene Therapy 6:407–418). Genetic immunization has many of the
advantages of live or attenuated microorganisms as vehicles for eliciting an immune response, without the risk of infection.

Preferably, I Avenue polynucleotides are in the form of plasmid vectors containing appropriate control sequences for transcription and translation, such as promoters, enhancers, and signal sequences. One or more I Avenue polynucleotides can be used within a single cloning vector, or multiple vectors can be used. A preferred I Avenue encoding region for use in a polynucleotide vaccine encodes a 1 Avenue scFv. The polynucleotide vaccine may also comprise encoding regions for other substances which will enhance the immune response. A preferred example is GM-CSF.

Another preferred embodiment of an I Avenue polynucleotide suitable for use as a vaccine is a viral vector. Examples include adenovirus, adeno-associated retroviruses (AAV), and SV40. Especially preferred is a vaccinia vector, whereby the polypeptide encoded by the I Avenue polynucleotide is expressed along with the immunogenic viral particle. Recombinant vaccinia comprising polynucleotides encoding I Avenue polypeptides such as scFv may be used for direct vaccination at about 10⁷ to 10⁸ plaque forming units per dose. Other vectors, such as baculoviral or murine retroviral vectors, can also be used. Alternatively, vaccinia can be administered via vaccinia-infected cells. In this method, suitable cells, such as tumor cells, are infected with vaccinia in culture. The infected cells are then reintroduced to the individual. Methods for infecting cells with vaccinia and reintroducing these infected cells have been described (see, e.g., Moss (1991)).

Other I Avenue polynucleotide vaccines may be designed using viral vectors known in the art. Another such delivery vehicle is cationic liposomes, to which DNA may be readily attached by virtue of its charge.

Vaccines can also be prepared from the I Avenue antibody, polypeptide derivatives thereof, or an combination thereof. A protein can optionally be treated chemically to enhance its immunogenicity, especially if 100 amino acids or less. This may include cross-linking, for example, with glutaraldehyde; or linking to a protein carrier, such as keyhole limpet hemocyanin (KLH) or tetanus toxoid.

The preparation of pharmaceutical compositions that contain I Avenue antibody or polypeptide derivatives as an active ingredient is conducted in accordance with generally accepted procedures for the preparation of pharmaceutical preparations. See, for example, Remington's Pharmaceutical Sciences 18th Edition (1990), E. W. Martin ed., Mack Publishing Co., PA. Depending on the intended use and mode of administration, it may be desirable to process the active ingredient further in the preparation of pharmaceutical compositions. Appropriate processing may include sterilizing, mixing with appropriate non-toxic and non-interfering components, dividing into dose units, and encapsulating in a delivery device.

Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving or dispersing a vector embodied herein in a liquid excipient, such as water, saline, aqueous dextrose, glycerol, or ethanol. The composition may optionally also contain other medicinal agents, pharmaceutical agents, adjuvants, carriers, and auxiliary substances such as wetting or emulsifying agents, and pH buffering agents.

Protein vaccines of this invention typically comprise an adjuvant, which may be the same as or in addition to the excipient or carrier. Examples of adjuvants include but are not limited to aluminum hydroxide, alum, QS-21 (U.S. Pat. No. 5,057,540), DHEA (U.S. Pat. Nos. 5,407,684 and 5,077,284) including its precursors and modified forms (e.g., DHEA-S, the sulfonated form of DHEA), beta-2 microglobulin (WO 91/16924), muramyl dipeptides, muramyl tripeptides (U.S. Pat. No. 5,171,588), monophosphoryl lipid A (U.S. Pat. No. 4,436,728; WO 92/16231) and its derivatives, such as various forms and generations of Detox™ and BCG (U.S. Pat. No. 4,726,947). Other suitable adjuvants are aluminum salts, squalene mixtures (SAF-1), muramyl peptide, saponin derivatives, mycobacterium wall preparations, mycetic acid derivatives, nonionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) Nature 344:873-875. For veterinary use and for production of antibodies in animals, complete and incomplete Freund's adjuvant can be used. The choice of an adjuvant will depend, in part, on the stability of the vaccine in the presence of the adjuvant, the route of administration, and the regulatory acceptability of the adjuvant, particularly when intended for human use. For instance, alum is approved by the United States Food and Drug Administration (FDA) for use in human vaccines.

A preferred vaccine composition comprising I Avenue antibody or polypeptide derivative is prepared by mixing with aluminum hydroxide and incubated to about 48⁰C. for about 30 min. Even more preferred are vaccine compositions comprising I Avenue and QS-21 or Ribi™PC. QS-21 and Ribi™PC are equally preferred; and the selection between them is made on the basis of availability.

Pharmaceutical compositions of the present invention are administered by a mode appropriate for the form of composition. Typical routes include subcutaneous, intramuscular, intraperitoneal, intradermal, oral, intranasal, intradermal, and intrapulmonary (i.e., by aerosol). Protein vaccines of this invention for human use are typically administered by a parenteral route, most typically intracutaneous, subcutaneous, intramuscular.

Pharmaceutical compositions for oral, intranasal, or topical administration can be supplied in solid, semi-solid or liquid forms, including tablets, capsules, powders, liquids, and suspensions. Compositions for injection can be supplied as liquid solutions or suspensions, or as solid forms suitable for dissolution or suspension in liquid prior to injection. For administration via the respiratory tract, a preferred composition is one that provides either a solid or liquid aerosol when used with an appropriate aerosolizer device. Although not required, pharmaceutical compositions are preferably supplied in unit dosage form suitable for administration of a precise amount. Also contemplated by this invention are slow release or sustained release forms, whereby a relatively consistent level of the active compound are provided over an extended period.

It is recognized that a number of alternative vaccine compositions, not limited to those described herein, may be efficacious in inducing an immune response. All such compositions are embodied within the present invention, providing they include a I Avenue polynucleotide or polypeptide as an active ingredient. The pharmaceutical compositions of this invention can be used in conjunction with other modes of therapy, whether established or experimental, whenever this is clinically desirable.

Testing I Avenue compounds and compositions for the ability to elicit a specific immune response is important. Compounds embodied in this invention may be assessed for their ability to elicit a specific immune response. Accordingly, test compounds are prepared as a suitable
pharmaceutical composition and administered to test subjects. Initial studies are preferably done in small animals such as mice or rabbits, optionally next in non-human primates and then ultimately in humans. Immunogenicity is preferably tested in individuals without a previous anti-IgA response.

A test composition in an appropriate test dose is administered on an appropriate immunization schedule. It may be appropriate to compare different doses and schedules within the predicted range.

Samples (usually blood samples) are collected regularly during treatment, and assayed for a specific immune response. The response may include antibody, helper-inducer T cells, or cytotoxic T cells, or a combination thereof. As a screening test, the samples may be measured for an anti-IgA response. Since the objective is typically to identify compositions useful in cancer therapy, the samples are preferably measured for an anti-GD2 response, as manifest in direct or inhibition type experiments.

This section outlines some non-limiting examples of assays that are suitable to monitor the immune response. Presence of anti-IgA (Ab3) and anti-GD2 (Ab1) activity in a humoral response is preferably determined after first pre-incubation of serum with autologous or adsorbing on a suitable affinity resin to remove antibody activity against isotopic and allotypic determinants. The adsorbed serum is then tested for Ab3 or Ab1 activity, for example, using ELISA or RIA. For instance, different dilutions of pre-reacted sera are reacted with IgA (or IgA polypeptide) coated on microtiter plates. An unrelated Ab2 serves as a control. After washing, the Ab3-IgA complex is labeled using, for example, 32P]-labeled IgA in a homogeneous sandwich assay. Results from this assay are compared to those obtained before administration of the IgA polypeptide (Example 1). Alternatively, binding to GD2 positive cells, such as M21-P6 cells, can be tested using immune flow cytometry. In a third example, the specificity of Ab3 is determined by Western blot. GD2 is separated by SDS-PAGE and blotted to a nitrocellulose filter. The filter is then incubated with sera containing Ab3, and the reaction developed by a suitably labeled anti-immunoglobulin. If the Ab3 binds to GD2, a band at the appropriate molecular weight should appear.

If desired, the specificity of the Ab3 can be further characterized. For example, competition assays can be performed to determine whether Ab3 share Ab1 idiotopes. Accordingly, competition experiments are conducted in which Ab3 is tested for inhibition of binding between IgA and 14G2a. Inhibition indicates that Ab3 and 14G2a contain at least some similar binding determinants. Competition of Ab3 with the binding of 14G2a to GD2 may also be measured.

Another way of characterizing a composition of this invention is testing its ability to elicit an antibody that is cytotoxic. For determination of complement mediated cytotoxicity (CMC), M21/P6 target cells expressing GD2 are labeled with 51Cr. Labeling may be accomplished by incubating about 10⁶ cells with approximately 200 μCi Na₂⁵¹CrO₄ for 60 minutes at 37°C, followed by washing. The assay is conducted by adding and incubating serum suspected of containing antibody. Guinea pig serum pre-adsorbed with M21/P6 cells (or other source of complement) is then added. After a suitable incubation period at 37°C, extent of 51Cr release is then measured and compared with that of unadsorbed control cells. Release of 51Cr correlates with CMC activity (Herlyn et al. 1981 Int. J. Cancer 27:769).

Another way of characterizing a composition of this invention is by testing its ability to elicit an anti-GD2 antibody that participates in an ADCC response (Cheros et al. 1986 Cancer Research 46:5112–5118). In this assay, cultured human M21/P6 cells (which express GD2 in their surface) are labeled with 51Cr and are used as target cells. Normal human peripheral blood mononuclear cells (PBMC) are used as effector cells. The Ab3 containing serum from immunized subjects is subjected to mediate the ADCC reaction. Preferably, the ADCC assay is conducted in the presence of heat-inactivated serum with an effector to target cell ratio of 100:1 for 4 hours, although other suitable conditions may be used. The amount of 51Cr released is then measured.

The IA7 antibodies, polynucleotides and polypeptides of this invention can also be characterized by their ability to elicit a cellular immune response. As used herein, a “cellular immune response” is a response that involves T cells, and can be observed in vitro or in vivo. Subjects are immunized with the IA7 composition, and cells are recovered for assaying the response. Where the subject is a small animal, cells are typically obtained from the spleen. For larger animals including humans, cells are typically obtained from peripheral blood. A suitable cell population is recovered from the sample by standard separation techniques, typically involving centrifugation over an appropriate medium such as Ficoll Hypaque.

One way of detecting a cellular immune response is by assaying for T cell proliferative activity. In this test, cellular immune response is measured by proliferation of peripheral blood mononuclear cells (PBMCs) incubated with IA7 polypeptide. PBMCs are isolated after a requisite number of administrations of IA7 polypeptide, and are incubated with varying concentrations of IA7 polypeptide. A non-specific mitogen such as PHA serves as a positive control; incubation with an unrelated non-idiotypic antibody serves as a negative control. Preferably, the stimulator cells are autologous with the responder cells, particularly in terms of histocompatibility Class II antigens. After incubation of the PBMCs for an appropriate period (typically 5 days), [³H]thymidine incorporation is measured. If desired, determination of which subset of T cells are proliferating can be performed using flow cytometry. Optionally, splenic T cells can be pre-depleted of either CD4+ or CD8+ cells before the proliferation assay by incubation with monoclonal antibody RL172 (anti-CD4) or monoclonal antibody 168 (anti-CD8) and complement.

Another way of detecting a cellular immune response is to test for T cell cytotoxicity (CTL) activity. In this test, an enriched T cell population are used as effectors in a standard ⁵¹Cr release assay (Kantor et al. 1992 J. Natl. Cancer Inst. 84:1084–1091). An example of a ⁵¹Cr release assay is the following. GD2-positive tumor cells (typically 1–2x10⁶ cells) are radiolabeled as target cells with about 200 μCi of Na₂⁵¹CrO₄ (Amersham Corp., Arlington Heights, Ill.) for 60 minutes at 37°C, followed by thorough washing to remove unincorporated isotopes. T cells and targets (1x10⁶/well), both resuspended in culture medium, are then be combined at various effector-to-target ratios in 96-well, U-bottom plates (Costar Corp.). The plates are centrifuged at 100g for 5 minutes to initiate cell contact, and are incubated for 4 or 6 hours at 37°C with 5% CO₂. After incubation, supernatants are collected using a Supernatant Collection System (Skatron, Inc., Sterling, Va.) and radioactivity will be quantitated in a gamma counter (Beckman Instruments). Spontaneous release of ⁵¹Cr is determined by incubation of targets in the absence of effectors, while maximum or total release of ⁵¹Cr will be determined by incubation of targets in 0.1% Triton™ X-100. Percentage of specific release of ⁵¹Cr is calculated in relation to spontaneous and maximal release.
Another way of characterizing a 1A7 polypeptide is testing its ability to ameliorate, delay the progression or reduce the extent of GD2-associated disease, as outlined in the following section. Use of pharmaceutical compositions for eliciting an immune response and treating disease

Compositions embodied in this invention such as those outlined in the previous section may be used for administration to individuals. They may be administered for experimental purposes, or to obtain a source of anti-GD2.

Compositions of this invention are particularly suitable for administration means for individuals with a GD2-associated disease. A GD2-associated disease is one in which expression of the GD2 ganglioside is altered at the affected tissue site, usually an elevation in cell-surface expression. Relevant diseases are those in which an active immune response against GD2 would confer a clinical benefit. Especially relevant are GD2-associated cancers; particularly melanoma, neuroblastoma, glioma, sarcoma, and small cell lung cancer.

The compositions of this invention may be administered to an individual with several objectives in mind. For example, the various compositions of this invention may be used to elicit an immune response. This includes an anti-1A7 specific response, and more preferably an anti-GD2 response. The desired response may be a specific antibody response; a specific T helper-inducer response, or a specific cytotoxic T cell response. An ADCC response or a cytotoxic T cell response is especially preferred in the context of cancer therapy, since these arms of the immune system are believed to be important effector factors in immune surveillance. The presence of an antibody response may provide a convenient means for routine clinical monitoring. Thus, a response that involves several components of the immune response in combination is especially preferred.

Assays for measuring and characterizing antibody response, ADCC, antibody-mediated cytolytic activity, T cell proliferative activity, and cytotoxic T cell activity are all described elsewhere in this disclosure.

Also included in this invention are methods for treating GD2-associated disease, such as a tumor expressing GD2. The method comprises administering an amount of a pharmaceutical composition effective to achieve the desired effect, be it palliation of an existing tumor mass or prevention of recurrence.

Dose

For treatment of a GD2-associated disease in vivo, the amount of a pharmaceutical composition administered is an amount effective in producing the desired effect. An effective amount may be provided in one or a series of administrations.

For intact 1A7, a mouse requires approximately 100 µg of KLH-coupled 1A7 emulsified in CFA and IFA in each of at least about three and typically at least four administrations. Monkeys require approximately 2 mg. The range of intact 1A7 that can be appropriately administered to humans is from about 10 µg to 20 µg, preferably 200 µg to 15 mg, more preferably 500 µg to 10 mg, still more preferably 1 mg to 4 mg, and even more preferably 2 mg. Smaller peptides and fusion proteins may be more potent on a per-weight basis, and the preferred dose may be lower than with the intact molecule. Appropriate doses can easily be determined by comparing various doses of intact 1A7 and derivatives thereof, in animal models, and scaling appropriately for human use.

The amount of 1A7 polynucleotide to be administered will depend upon several factors, such as the route of administration, the condition of the individual, and the desired objective. Typically, if administered directly, the amount per administration is about 10 µg to 1 mg, preferably 25 µg to 500 µg, more preferably 30 µg to 250 µg, even more preferably 50 to 100 µg.

Admissions are typically conducted on a weekly or biweekly basis until a desired, measurable parameter is detected, such as elicitation of an immune response. Administration can then be continued on a less frequent basis, such as biweekly or monthly, as appropriate.

The various compounds of this invention can be used alone, or in conjunction with other active agents that promote the desired objective, or provide a desirable adjunct therapy.

The exact dose and timing for the administration of any composition of this invention depends on the individual to be treated, the capacity of the individual’s immune system to synthesize antibodies, the route of administration, the degree of protection desired, and the immunological and clinical response to previous doses. The immunological response may be assessed by assays given in the previous section. Choosing an appropriate amount to administer in accordance with the previously stated are the responsibility of the administering physician.

Appropriate subjects for therapy, and desirable effects

Suitable subjects include those who are suspected of being at risk of a pathological effect of any GD2-associated condition are suitable for treatment with the pharmaceutical compositions of this invention. Those with a history of a GD2-associated cancer are especially suitable.

The clinical studies described in the Example section are designed to exclude subjects who have not been treated previously with mouse immunoglobulin. The concept is that a proportion of subjects who have been so treated may have circulating anti-mouse immunoglobulin (HAMA). This selection criteria has been implemented to facilitate initial testing. However, we do not believe that the presence of HAMA is an impediment to therapy, since the purpose of the 1A7 is to elicit a response, not remain in the circulation.

Patients are more typically chosen for therapy irrespective of their history of previous treatment with mouse immunoglobulin. Of course, for certain engineered compounds like humanized 1A7 and scFv, most mouse antigenic determinants have been deleted, and the presence of HAMA in a potential recipient is even less of a consideration.

Suitable human subjects for therapy comprise two groups, which may be distinguished by clinical criteria.

Patients with “advanced disease” or “high tumor burden” are those who bear a clinically measurable tumor. A clinically measurable tumor is one that can be detected on the basis of tumor mass (e.g., by palpation, CAT scan, or X-ray; positive biochemical or histopathological markers on their own are insufficient to identify this population). A pharmaceutical composition embodied in this invention is administered to these patients to elicit an anti-GD2 response, with the objective of palliating their condition. Ideally, reduction in tumor mass occurs as a result, but any clinical improvement constitutes a benefit. Clinical improvement includes decreased risk or rate of progression or reduction in pathological consequences of the tumor.

A second group of suitable subjects is known in the art as the “adjuvant group”. These are individuals who have had a history of a GD2-associated cancer, but have been responsive to another mode of therapy. The prior therapy may have included (but is not restricted to) surgical resection, radiotherapy, and traditional chemotherapy. As a result, these individuals have no clinically measurable tumor.
However, they are suspected of being at risk for progression of the disease, either near the original tumor site, or by metastasis.

This group may be further subdivided into high-risk and low-risk individuals. The subdivision is made on the basis of features observed before or after the initial treatment. These features are known in the clinical arts, and are suitably defined for each different GD2-associated cancer. Features typical of high risk subgroups are those in which the tumor has invaded neighboring tissues, or who show involvement of lymph nodes.

A pharmacological composition embodied in this invention is administered to patients in the adjuvant group, or in either of these subgroups, in order to elicit an anti-GD2 response. Ideally, the composition delays recurrence of the cancer, or even better, reduces the risk of recurrence (i.e., improves the cure rate). Such parameters may be determined in comparison with other patient populations and other modes of therapy.

Of course, cross-overs between these two patient groups are possible, and the pharmaceutical compositions of this invention may be administered at any time that is appropriate. Previous GD2 therapy may be administered before or during traditional therapy of a patient with high tumor burden, and continued after the tumor becomes clinically undetectable. GD2 therapy may be continued in a patient who initially fell in the adjuvant group, but is showing signs of recurrence. The attending physician has the discretion to determine how or when the compositions of this invention are to be used.

It is recognized in the art that the immunological status of each of the aforementioned category differs one from another by several criteria. For example, patients with active disease are generally immunosuppressed, either due to tumor-related pathology or to recent radiotherapy or chemotherapy. Their immune system may be under a barrage of tumor-associated antigen from the tumor site. On the other hand, patients who are in remission may have stronger active suppression against autoantigens. Accordingly, the ability of an anti-idiotypic based vaccine to elicit an anti-tumor response, or improve the clinical condition, must be determined separately for each patient category.

Other clinical indications

Various compounds and compositions of this invention have other clinical indications, of which this section provides only a survey.

One indication is the treatment of cells ex vivo. This may be desirable for experimental purposes, or for treatment of an individual with a GD2-associated disease. In one example, the 1A7 antibody, or a polynucleotide or polypeptide derivative are administered to a culture of cells, such as peripheral blood cells obtained from a donor, or a suitable cell line. This may be done, for example, with the objective of stimulating T cell activity. About 0.5 to 2 μg/mL of 1A7 is an effective dose for this purpose. If desired, the stimulated cells may then be administered to a recipient, in an effort to convey passive immunity. In a second example, donor cells are genetically altered with an expression vector of this invention, to provide for ongoing secretion of 1A7 antibody after administration of the cells to the recipient.

The invention also encompasses compositions and methods using 1A7 antibodies and polypeptide derivatives to remove a label (particularly a radionuclide) from an individual who has received a labeled anti-GD2 antibody before as 14G2a in the course of radiocintigraphy or radiotherapy. Effective imaging using radiolabeled antibody is hampered due to excess circulating radiolabeled antibody, which often takes several days to clear. Accordingly, 1A7 antibody or a polypeptide derivative is administered to the individual at a specified time after administration of the labeled anti-GD2. The intention is for the 1A7 polypeptide to complex with anti-GD2 at sites other than the tumor, such as in the circulation and interstitial spaces, and thereby promote its clearance. As a result, the level of label in unaffected tissues is reduced, and the image of the tumor (in comparison to neighboring tissues) is enhanced. Similarly, when radionuclides are given to subjects for irradiation of a tumor site, it is desirable to reduce collateral exposure of unaffected tissue. This intention thus includes cell kill by treatment in which a radiolabeled anti-GD2 antibody is administered in a therapeutic dose, and followed by a molar excess of 1A7.

In either of these applications, an amount of 1A7 polypeptide is chosen that is in sufficient molar excess over the labeled anti-GD2 to locate and bind any anti-GD2 that is not localized at the tumor site. The timing of administration and amount of 1A7 polypeptide will depend upon the nature of the radiolabeled antibody, the type of radioisotope used and the condition of the individual. Preferably, the molar ratio of 1A7 polypeptide to the anti-GD2 antibody is at least about 5:1, more preferably between 25:1 to 200:1. Preferably, 1A7 polypeptide is administered 5 to 24 hours after the individual has received the anti-GD2 antibody.

The invention also includes methods of detecting the presence of an anti-GD2 antibody bound to a tumor cell comprising the steps of treating an individual with 1A7 for a sufficient time to allow binding to the anti-GD2 antibody, and detecting the presence of any complex formed. The intention is for the 1A7 to detect anti-GD2 that has pre-attached to the tumor cell, or alternatively, to promote the binding. This intention thus includes cell kill by treatment in which a radiolabeled anti-GD2/1A7 immune complex. In the former instance, the anti-GD2 is provided with a detectable label or a means by which a label can be attached. In the latter instance, either the anti-GD2 or the 1A7 is provided with a label. Suitable labels include radionuclues such as 32P, 35S and 32P. The anti-GD2 and 1A7 are administered (usually sequentially) into the subject and allowed to accumulate at the tumor site. The tumor is then detected or visualized using standard techniques of radiocintigraphy.

Diagnostic kits

The present invention encompasses kits containing 1A7 antibodies, polynucleotides, or polypeptides. Diagnostic procedures using the 1A7 polynucleotides or polypeptides of this invention can be performed by diagnostic laboratories, experimental laboratories, practitioners, or private individuals. Kits embodied by this invention include those that allow someone to conduct an assay for anti-GD2 or anti-1A7 activity, or for an 1A7 encoding sequence. An alteration in one of these components resulting, for example, from the presence of a GD2-associated disease or treatment directed towards it is typically compared with that in a sample from a healthy individual. The clinical sample is optionally pretreated for enrichment of the target being tested for. The user then applies a reagent contained in the kit in order to detect the altered level or alteration in the diagnostic component. Each kit necessarily comprises the reagent which renders the procedure specific: a reagent 1A7 antibody or polypeptide, used for detecting anti-1A7 or anti-GD2 in the sample; or a reagent 1A7 encoding polynucleotide, used for detecting a 1A7 encoding polynucleotide in the sample. Optionally, the reagent may be conjugated with a label to permit detection of any complex formed with the target in the sample. In another option, a second reagent is provided that is capable of combining with the first reagent after it has
found its target. For example, labeled anti-mouse IgG may be provided as a secondary reagent for use with intact 1A7. Labeled avidin may be provided as a secondary reagent when the primary reagent has been conjugated with biotin.

The kits may be employed on a variety of biological samples, including both liquid samples, cell suspensions and tissue samples. Suitable assays using 1A7 antibodies, polypeptides, and polynucleotides that can be supplied in kit form include those described elsewhere in this disclosure.

Each reagent is supplied in a solid form or liquid buffer that is suitable for inventory storage, and later for exchange or addition into the reaction medium when the test is performed. Suitable packaging is provided. The kit may optionally provide additional components that are useful in the procedure. These optional components include buffers, capture reagents, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

Deposit

The foregoing description provides, inter alia, detailed methods for preparing monoclonal antibody 1A7, along with 1A7 encoding polynucleotides, 1A7 polypeptide fragments, and other derivatives.

A practitioner of ordinary skill in the art may practice embodiments of this invention by referring to the sequence data for 1A7, which is provided herein. Alternatively, a practitioner may practice the invention by first purifying the 1A7 antibody, or a 1A7 encoding polynucleotide from a 1A7 antibody producing cell. A hybridoma cell line producing 1A7 antibody was deposited on Dec. 28, 1994 with the American Type Culture Collection (ATCC), now located at 10801 University Blvd, Manassas, Va. 20110-2209, and has been accorded Accession No. HB-11786.

The following examples are provided to illustrate but not limit the present invention.

EXAMPLES

Example 1

Generation and Characterization of 1A7 Anti-Idiotype Antibody

The monoclonal anti-idiotype antibody producing hybridoma cell line 1A7 was created and identified according to the following description. Aspects of both the immunization procedure and the screening procedure were important to obtain an antibody with the desired specificity and functionality. 1A7 was one of a number of Ab2 that were initially produced, and was identified as the candidate with the most desirable features.

1A7 was obtained by using the 14G2a mouse monoclonal antibody as immunogen for an anti-idiotype response. 14G2a binds to a unique epitope of GD2 that is not present on other members of the ganglioside family. Since the responding animal was also a mouse, the Ab2 generated were expected to be directed against idiotypeic features of 14G2a. However, only a fraction of those would be directed against the 14G2a paratope, an even smaller proportion would be immunogenic and capable of eliciting an Ab3, and a still smaller proportion would elicit Ab3 that cross-reacted with the tumor-associated antigen.

To render 14G2a sufficiently immunogenic in an autologous species, it was conjugated to the carrier KLH, and emulsified in Freund's adjuvant. It was administered repetitively into the recipient animals on an unusual schedule with only 2 weeks between doses. Five mice were immunized according to this schedule. Substantial responses arose in about 3 mice only after the fourth immunization. Responding animals were boosted with a fifth dose of 14G2a intravenously, spleen cells were isolated, and hybridomas were prepared separately from each animal. Cloning was performed according to standard techniques.

The screening procedure comprised four important steps: (1) Positive selection for antibody binding to 14G2a; (2) Negative selection against antibody recognizing isotypic or allotypic determinants; (3) Positive selection for an ability to inhibit the binding of 14G2a to GD2; and (4) Positive selection for an ability to induce a humoral immune response against the original tumor-associated antigen (GD2) in both mice and rabbits. The rest of this section provides an overview of the screening procedure, which is given in more detail in the sections that follow.

Initial screening was conducted by immunoprecipitation to identify the clones that reacted with 14G2a, but not with other target monoclonal antibodies sharing the same allotypic or isotypic determinants. A critical assay was a sandwich RIA in which 14G2a is attached to a solid phase, overlaid with culture supernatant, and developed with radiiodinated 14G2a. This assay requires the antibody in the hybridoma supernatant to be functionally bivalent, and be able to span between the capture 14G2a and the developing 14G2a.

Several clones that were allotype specific and gave a strong signal in this assay were selected for further study.

Subsequent screening was conducted by competition assays, in which the Ab2 was required to block the binding of 14G2a to GD2. This established that Ab2 recognized the paratope of 14G2a. GD2 was provided in the form of M21/P6 cells, a human melanoma cell line expressing GD2 at the cell surface. The nature of the assay requires the Ab2 to block the interaction between 14G2a and the tumor antigen in its particular manner of presentation on tumor cells. At a minimum, candidate Ab2 which had passed the earlier screening tests were required to inhibit the binding of 14G2a to the cells by at least 75%. There were about three Ab2 that substantially exceeded the minimum, with 1A7 providing about the highest level of inhibition.

The ultimate screening test was a determination of whether the candidate Ab2 were capable of eliciting an Ab3 of the desired specificity when injected into a recipient. Sufficient quantities of Ab2 were prepared from mouse ascites, and tested in mice and rabbits. Sera from the test animals were first assayed for the presence of Ab3 in a sandwich immunoprecipitation using the same labeled Ab2 used for immunization. Sera testing positively were then assayed for ability of the Ab3 to react against the tumor-associated antigen; namely GD2. A preparation of GD2 was used to coat microtiter plates, overlaid with the test serum in serial dilutions, and the Ab3 that bound was detected using labeled anti-immunoglobulin. The titer of the Ab3 binding to GD2 defined the “quality” of Ab2, as a reflection of its capacity as an inducer of anti-GD2.

Monoclonal antibody 1A7 emerged as the anti-idiotype with the highest quality, and is the original basis for various compounds, compositions, and procedures embodied in this invention. The cell line producing 1A7 was recloned by limiting dilution twice, to ensure the stability of the line.

Materials

Antibody: The hybridoma cell line producing monoclonal antibody 14G2a was obtained from the Scripps Research Institute. 14G2a has been subsumed as an IgG2aK. The specificity of 14G2a was reconfirmed by immunoperoxidase staining and flow microfluorimetry analysis using cells expressing GD2. Other monoclonal and myeloma mouse
immunoglobulins were used as controls in various experiments herein described.

Ascitese of 14G2a hybridomas and other cell lines were prepared by injecting individual pristane-primed mice i.p. with 2–10 × 10^7 viable cells. The IgG fraction was isolated from ascites by 45% saturated ammonium sulfate precipitation and subsequent chromatography on Protein A Sepharose(TM) CL-4B (EY et al. (1978) Immunochemistry 15:429). The purity of the isolated IgG was checked by immunodiffusion, immunoelectrophoresis, and high pressure liquid chromatography (HPLC) fractionation.

Coupling of antibody with KLH: 14G2a was coupled to keyhole limpet hemocyanin (KLH) according to a method described by Maloney et al. (1985; Hybridoma 4:191). Antibody stock solution (1 mg/ml) was mixed with KLH (1 mg/ml) in PBS in the presence of freshly diluted glutaraldehyde solution (final concentration 0.05%). The mixture was rotated end-over-end for 1 h at room temperature, and then dialyzed exhaustively against PBS at 4°C.

Immunization of syngeneic BALB/c mice: BALB/c females were immunized four times over a period of 2 months. The first injection was given i.p. using 100 μg of 14G2a, emulsified in complete Freund’s adjuvant. The next two injections were given in a pool of 100 μg of 14G2a coupled to KLH in incomplete Freund’s adjuvant, either s.c. or i.p. Mice were bled from time to time, and sera were checked for anti-Id activity by ELISA in a binding assay by using F(ab')2 fragments of 14G2a and normal pooled BALB/c mouse serum IgG as control. Three days before the fusion, the mice were boosted i.v. with 14G2a in PBS.

Production of Anti-Idiotypic Hybridomas

The fusion partner used to produce the hybridoma lines was the mouse non-secretory myeloma cell line P3-653, anestomically related to P3X63Ag8.653, available from the ATCC as No. CR1-1580. Established human cell lines were cultured in RPMI 1640 supplemented with 5% fetal calf serum as described elsewhere (Seon et al. (1984) J. Immunol. 132:2089).

Hybridomas were produced essentially following the method of Oi and Herzenberg ((1980) Selected Methods of Cellular Immunology, Mishell & Shiigi eds., Freeman Publs., at 315–372). Spleen cells from immunized mice were mixed with P3-653 cells at a ratio of 1:1 to 10:1, in the presence of 0.5% polyethylene glycol (PEG, mw–4000). Fused cells were then washed and cultured. Hybrid lines were selected using hypoxanthine-aminopterin-thymidine media.

Initial Selection of Anti-Idiotypic Antibody (Ab2)Secreting Hybridoma Clones

Initial screening of the hybridoma clones was performed by RIA. Purified 14G2a was radiiodinated by the chloramine T method (Hunter (1970) Proc. Soc. Exp. Biol. Med. 133:989). The assay was conducted by coating microtiter plate wells with 14G2a antibody (or control) at 500 ng/well. After incubating overnight at 4°C, the plates were blocked with 1% bovine serum albumin (BSA) in PBS. 100 μl of hybridoma culture supernates or 20 μl of serum was added by incubated in the well for 4 h at room temperature. After washing with PBS, the plates were further incubated for 4 h at room temperature or overnight at 4°C. With labeled 14G2a, washed, and counted. This RIA is a stringent test for antibody specificity, since it requires that the antibody be able to span between two 14G2a molecules.

An ELISA was conducted in a similar fashion, using subcell-specific anti-immunoglobulin as both the plate coat and detecting reagent. Generally, antibody to certain IgG subclasses is desired because it is stable, easily purified by protein A chromatography, and may have useful effector functions.

A number of monoclonal Ab2 secreting cell lines emerged from these screening assays with the desired properties. Amongst them was monoclonal antibody 1A7. Confirmation that Ab2 is Specific for 14G2a Idiotypic

Idiotype specificity of Ab2 was confirmed by direct binding to Ab1. Various purified Ab2 were labeled with 125I, and tested for binding to plates coated with a panel of monoclonal anti-TAA Ab1. 1A7 bound almost exclusively to 14G2a; there was virtually no cross-reactivity with any of the other Ab1 tested.

Specificity for the 14G2a idiotype was further established in competition experiments. ~25,000 cpm of various labeled Ab2 was mixed with different members of a panel of unlabeled competitors comprising Ab2, Ab1, and other mouse immunoglobulins. The Ab2 was then tested for binding to 14G2a coated plates. For the best Ab2, greater than 90% inhibition was obtained using either Ab2 or 14G2a as competitor. Virtually no inhibition was obtained, up to a concentration of 5 μg, using control immunoglobulins as potential competitors.

Screening for Anti-Idiotypic Antibodies Directed Against the 14G2a Paratope

to determine whether the Ab2 were directed against the paratope of 14G2a, the Ab2 were used to compete for the binding of radiolabeled 14G2a to GD2. This was performed conducted using M21/P6 cells, a human cancer cell line expressing GD2 as a membrane constituent.

To conduct the assay, M21/P6 cells were grown as confluent monolayer in 96-well tissue culture plates. Various dilutions of the test Ab2 (either culture supernatant or purified antibody) were mixed with the labeled 14G2a, and then added to the cultured cells. Percent inhibition of the assay was calculated according to the formula:

\[
\%
\text{inhibition} = \left[1 - \left(\frac{R_{f} - R_{c}}{R_{\text{Max}} - R_{c}}\right)\right] \times 100\%
\]

where \(R_f\) is the average cpm of the experimental well with inhibitors; \(R_c\) is the average background cpm; and \(R_{\text{Max}}\) is the average maximum binding without any inhibitors.

Three Ab2, including 1A7, inhibited the binding of labeled 14G2a to the GD2 expressing cells at amounts as low as about 25 ng. Purified control antibody demonstrated no inhibition.

Antibody-producing clones testing positively in the screening tests described so far were used to prepare mouse ascites as a source of Ab2. The Ab2 were purified by chromatography using Protein A and Protein G affinity resins by standard techniques.

Screening for Anti-Idiotypic Antibodies Capable of Eliciting a Tumor-Specific Immune Response

Since a central purpose of these experiments was to find an anti-idiotypic capable of eliciting an anti-GD2 immune response, the next screening step was to test its immunogenicity in animal models. The Ab2 would have to be not only immunogenic, but capable of raising Ab3 that cross-reacted back to the tumor antigen GD2.

Accordingly, the monoclonal antibody that gave the strongest result in the competition experiments with the GD2-expressing cells was brought forward for testing in this part of the study. The other two antibodies showing specific inhibition were held in reserve in case 1A7 failed to demonstrate the desired properties in this test.
Accordingly, 1A7 was prepared as a vaccine composition and injected into test animals. First, we immunized syngeneic BALB/c mice (6-8 weeks old) with 50 μg of 1A7 coupled to the carrier KLH (1:1 ratio) in the presence of equal volume (0.1 ml) of Freund's Complete or Incomplete adjuvant on days 0, 14, 28, and 42, subcutaneously. Blood samples were drawn from each mouse 10 days after the 4th immunization and analyzed for total Ab3 response (anti-iso/allo/idiotype) by sandwich RIA and anti-anti-idiotype response by the inhibition of Ab1 (14G2a) binding to Ab2 (1A7 on the plate) by Ab3 sera. In addition, serum was checked for inhibition of 14T1-14G2a binding to GD2 positive melanoma cells (M21/P6). Also, direct binding of sera to purified GD2, coated onto microtiter plate, was determined by ELISA assay. Representative data from 3 BALB/c mice are shown in Table 1.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Serum Dilution</th>
<th>Mouse #1</th>
<th>Mouse #2</th>
<th>Mouse #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandwich RIA (CFP)</td>
<td>1:50</td>
<td>16,700</td>
<td>24,576</td>
<td>26,214</td>
</tr>
<tr>
<td>% inhibition of Ab1-Ab2 Binding by Ab3 Ser</td>
<td>1:50</td>
<td>87</td>
<td>95</td>
<td>97</td>
</tr>
<tr>
<td>% inhibition of Ab1 Binding by M21/P6 Melanoma Cells</td>
<td>1:50</td>
<td>28</td>
<td>32</td>
<td>27</td>
</tr>
<tr>
<td>Direct binding to GD2 by ELISA (OD405 nm)</td>
<td>1:10</td>
<td>0.70</td>
<td>0.76</td>
<td>0.71</td>
</tr>
<tr>
<td>PBS-BSA Control</td>
<td></td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There was no reactivity with GD-2 negative cell lines or unrelated gangliosides such as GD3 and GM3. Results are expressed as mean value of triplicate determinations (S.D. <10%).

Next, we immunized allogeneic C57BL/6 mice (6-8 weeks old) with three different formulations of anti-id 1A7 vaccine: (i) 1A7+KLH+Freund's Adjuvant; (ii) 1A7-KLH+QS-21 (10 μg per mouse); (iii) 1A7+QS-21 (10 μg per mouse). Results shown in Table 2.

<table>
<thead>
<tr>
<th>Immunized With</th>
<th>Sandwich RIA of Ab3 Sera (1:50 dil) (CFP)</th>
<th>% inhibition of Ab3-Ab2 Binding by Ab3 Sera (1:50 dil)</th>
<th>% inhibition of Ab1 Binding to M21/P6 Melanoma Cells by Ab3 Sera (1:50 dil)</th>
<th>Direct Binding of Ab3 Sera (1:10 dil) to GD2 by ELISA (OD 450 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A7-KLH + Freund's</td>
<td>4,729</td>
<td>80</td>
<td>27</td>
<td>1.28</td>
</tr>
<tr>
<td>Mouse #1</td>
<td>6,067</td>
<td>83</td>
<td>14</td>
<td>0.62</td>
</tr>
<tr>
<td>Mouse #2</td>
<td>9,591</td>
<td>96</td>
<td>13</td>
<td>0.41</td>
</tr>
<tr>
<td>PBS-BSA Control</td>
<td>650</td>
<td>—</td>
<td>—</td>
<td>0.10</td>
</tr>
<tr>
<td>1A7-KLH + QS-21</td>
<td>5,506</td>
<td>79</td>
<td>37</td>
<td>0.64</td>
</tr>
<tr>
<td>Mouse #1</td>
<td>5,831</td>
<td>95</td>
<td>58</td>
<td>0.63</td>
</tr>
<tr>
<td>Mouse #2</td>
<td>7,315</td>
<td>94</td>
<td>43</td>
<td>0.63</td>
</tr>
<tr>
<td>PBS-BSA Control</td>
<td>549</td>
<td>—</td>
<td>—</td>
<td>0.09</td>
</tr>
<tr>
<td>1A7 + QS-21</td>
<td>587</td>
<td>70</td>
<td>73</td>
<td>0.66</td>
</tr>
<tr>
<td>Mouse #1</td>
<td>738</td>
<td>77</td>
<td>79</td>
<td>0.62</td>
</tr>
<tr>
<td>Mouse #2</td>
<td>1,000</td>
<td>74</td>
<td>80</td>
<td>0.64</td>
</tr>
<tr>
<td>PBS-BSA Control</td>
<td>153</td>
<td>—</td>
<td>—</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Results are expressed as mean value of the triplicate determinations (S.D. <10%). There was no reactivity with GD2-negative cell lines or unrelated gangliosides, such as GD3 and GM3.

Comparison between the three vaccine preparations suggests that in C57BL/6 allogeneic mice 1A7-KLH and Freund's adjuvant induced almost identical humoral immune responses as 1A7-KLH and QS-21. The production of total Ab3 response was less in 1A7+QS-21 immunized mice; however, the binding of Ab1 to melanoma cells was inhibited much more strongly and the production of anti-GD2 antibodies (Ab1) was comparable to the other two groups. Thus, there was no additional advantage of coupling of KLH to 1A7. KLH apparently induced strong anti-isotypic and anti-allotypic responses in C57BL/6 mice. Immune mice sera at 1:100 dil reacted strongly with M21/P6 melanoma cells and EL4 lymphoma cells, but not with the unrelated colon carcinoma LS174-T cells by FACS analysis.

We also compared the T cell responses of the spleen cells from the differently immunized groups of mice to various stimuli by T cell proliferation assay. Representative data from all three groups of C57BL/6 mice and BALB/c mice are presented in Table 3.
The data are expressed as mean CPM of triplicate wells (S.D.<10%). S.I.=Stimulation Index. S.I.>3.0 was arbitrarily considered as positive.

The results indicated that in C57BL/6 mice, immunization with all three regimens induced 1A7 specific, M21/P6 melanoma cell specific and EL4 cells specific proliferative responses, some reactivity against control 3H1 and no reaction against control cell line LS174-T cells or ganglioside GD2 or GD3. These data support the postulate that for T cell activation, GD2 needs to be associated with cell surface oligopeptides. There was also no significant difference in Stimulation Index obtained with any of these three regimens. 1A7+QS-21 was as good as 1A7-KLH+QS-21 or 1A7-KLH +Friends in C57BL/6 mice.

In order to assess the ability of these vaccines to induce T-cell mediated DTH response, C57BL/6 mice immunized

with 1A7-KLH +Friends’, 1A7-KLH +QS-21 and 1A7 +QS-21 were given intradermal foot pad injection of irradiated M21/P6 cells or irradiated LS174-T (control) cells. In another experiment, mice received intradermal foot pad injection of purified GD2 or purified GD3. Mice were observed for development of DTH response at the inoculation site at 24 hours and 48 hours. There were significant DTH responses directed at GD2-positive M21/P6 cells but not GD2-negative LS174-T cells in all three groups of immunized mice (data not shown). There was, however, no DTH reactivity directed at GD2 or GD3 in any of the groups of immunized mice.

Rabbits were selected next for the immunization studies with 1A7-KLH+QS-21 and 1A7+QS-21. The relative composition and tissue distribution of gangliosides in rabbits is similar to that of humans (75). Each group of three rabbits were immunized with either 500 μg of 1A7 mixed with 50 μg of QS-21, or 500 μg of 1A7-KLH+50 μg of QS-21. The injections were given intramuscularly on days 0, 14, 28 and 42. Blood samples were collected 10 days after the fourth immunization, and analyzed for Ab3 (Ab1) responses and T-cell proliferation. Table 4 shows the humoral immune response induced.

<table>
<thead>
<tr>
<th>TABLE 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell proliferation</td>
</tr>
<tr>
<td>Stimulant</td>
</tr>
<tr>
<td>CPM (S.I.)</td>
</tr>
<tr>
<td>Anti-id 1A7 (2 μg)</td>
</tr>
<tr>
<td>Anti-id 3H1, Control 2 μg</td>
</tr>
<tr>
<td>M21/P6 irradiated Melanoma Cells (1 × 10⁶)</td>
</tr>
<tr>
<td>EL4 Marine Lymphoma Irradiated Cells (1 × 10⁶)</td>
</tr>
<tr>
<td>LS174-T Control Colon Carcinoma Irradiated Cells (1 × 10⁶)</td>
</tr>
<tr>
<td>GD2 (1 μg)</td>
</tr>
<tr>
<td>GD3 (1 μg)</td>
</tr>
<tr>
<td>Median</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding studies using rabbit serum</td>
</tr>
<tr>
<td>% Serum ( \times 10^5 )</td>
</tr>
<tr>
<td>Assays</td>
</tr>
<tr>
<td>Sandwich RIA (CPM)</td>
</tr>
<tr>
<td>% Inhibition of Ab1-Ab2 Binding by Ab3</td>
</tr>
<tr>
<td>% Inhibition of Ab1 Binding to M21/P6 Melanoma Cells</td>
</tr>
<tr>
<td>Direct Binding to GD2 by ELISA (OD 450 nm)</td>
</tr>
</tbody>
</table>

Results are expressed as the mean value of triplicate determinations (S.D.<10%). There was no reactivity with GD2-negative cell lines or unrelated gangliosides (GD3, GM3, etc.). The O.D. value obtained with PBS-BSA control was 0.08.

KLH-coupled 1A7 plus QS-21 induced higher levels of anti-isotypic and anti-allotypic responses in all three rabbits. Ab3 and GD2-positive cell binding inhibition reactions were better in all three 1A7+QS-21 immunized rabbits. Two out of 3 rabbits in each group raised anti-GD2 antibodies, and
the response was better in 1A7+QS-21 immunized group as compared to 1A7-KLH +QS-21 group.

Thus, in these experiments, 1A7+QS-21 was capable of raising desired anti-tumor responses in mice and rabbits, and there was no additional advantage of KLH-coupling to 1A7. The rabbit Ab3 sera were cytotoxic to M21/P6 and EL4 cells by in vitro ADCC assay, and the isotype of the anti-GD2 antibodies in the rabbit sera was mostly of IgG type with trace amount of IgM. The Ab1 antibody in rabbit sera also reacted with melanoma cells but not with GD2-negative carcinoma cells by FACS analysis. Table 5 demonstrates representative data from immunized rabbits for PBL-transformation assay.

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>1A7-KLH + QS-21</th>
<th>1A7 + QS-21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-id 1A7 (2 µg)</td>
<td>21,329</td>
<td>9,794</td>
</tr>
<tr>
<td>Anti-id KLH</td>
<td>13,501</td>
<td>5,691</td>
</tr>
<tr>
<td>Control (2 µg)</td>
<td>4,235</td>
<td>2,442</td>
</tr>
<tr>
<td>M21/P6</td>
<td>73,596</td>
<td>28,845</td>
</tr>
<tr>
<td>Malignant Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1 x 10⁶),</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imiditated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EL4 Murine</td>
<td>44,619</td>
<td>28,040</td>
</tr>
<tr>
<td>Lymphoma Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1 x 10⁶),</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imiditated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS174-T Colon</td>
<td>5,196</td>
<td>3,131</td>
</tr>
<tr>
<td>Carcinoma Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1 x 10⁶),</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imiditated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GD2 (1 µg)</td>
<td>11,345</td>
<td>5,888</td>
</tr>
<tr>
<td>GD3 (1 µg)</td>
<td>7,329</td>
<td>4,678</td>
</tr>
<tr>
<td>Medium</td>
<td>4,816</td>
<td>1,991</td>
</tr>
</tbody>
</table>

Results are expressed as the mean value of triplicate determinations (S.D.<10%). Stimulation Index>3.0 was arbitrarily considered as positive.

Results shown in Table 5 demonstrate that immunization of rabbits with both 1A7-KLH+QS-21 and 1A7-QS-21 induced T cell proliferation in PBL against anti-id 1A7, irradiated GD2-positive M21/P6 cells and EL4 cells but not against GD2-negative LS174-T cells or against GD2 and GD3. There was insignificant stimulation against normal isotype-matched control Ab2 (S.I.<3.0). Stimulation Index against various stimuli was almost identical in both groups of immunized rabbits.

EXAMPLE 2
Obtaining the 1A7 Heavy and Light Chain Sequences

The polynucleotide sequence was obtained for the 1A7 antibody by isolating messenger RNA from the 1A7 producing cell line. For each sequence determination, total RNA was isolated from 1x10⁷ 1A7 hybridoma cells. Messenger RNA was prepared by passage through two cycles of Chromatography of oligothymidylate-cellulose columns. The yield of mRNA was about 10 µg. First strand cDNA was synthesized using SuperScript Preamplification kit ( Gibco/BRL).

To sequence the heavy chain variable region, PCRs were conducted on the cDNA using a reverse primer corresponding to amino acids 126 to 119 of the murine γ1 constant region: 5'-CCCAAGCCTCCAGGGRCCARKGGATARACGRTGG-3' (SEQ ID NO:46) and various mixtures of forward primers, corresponding to the N-terminal leader sequences of murine variable region subgroups. The forward primer that gave a positive reaction was: 5'-ACTAGTGCAATGACGGCTTCYTRGBGCTGTYCTC- YTCGTG-3' (SEQ ID NO:47) corresponding to amino acids -20 to -12.

The amplified fragment of cDNA was subcloned into pT7 plasmid and NovaBlue competent cells were transformed using a protocol provided by the supplier (Novagen). Recombinant colonies were picked up by color selection and plasmid DNA was prepared by miniprep procedure. The DNA sequence of the double stranded plasmid was determined using a Sequenase Version 2.0 kit (USB, Cleveland, Ohio). The sequence of the DNA insert in the plasmid was determined from both orientations using primers specific for the plasmid: namely T7 promoter (TAATACGACTCACTATAGGG) (SEQ ID NO:48) and U-19 (GTTTTCCCCAGTCAGGATC) (SEQ ID NO:49). At least 8 clones were picked for sequence determination.

The sequence of the 1A7 light chain variable region was determined in a similar fashion. The forward and reverse primers giving a positive result in the PCR were: 5'-ACTAGTGCAATGACGGCTTCYTRGBGCTGTYCTC- YTCGTG-3' (SEQ ID NO:50) corresponding to amino acids -19 to -10 of the leader sequence, and 122 to 116 of the mouse κ chain constant region.

In order to minimize the error rates in PCR amplification, pT7 DNA polymerase (Stratagene, San Diego) was used for amplification in all subsequent experiments. Mutant frequency with this thermostable DNA polymerase is 1/10 compared to Taq DNA polymerase.

Confirmation that the isolated cDNA correspond to the 1A7 heavy and light chains is obtained by amino acid sequencing of the N-terminal of the isolated antibody. Fifty µg of purified 1A7 antibody is diluted with sample loading buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 1% glycerol, 500 mM NaCl) and loaded onto a gel.
0.1% β-mercaptoethanol and heated to 100°C for 3 minutes. The denatured protein is loaded onto a 7.5% polyacrylamide gel (BioRad Miniprotein II Dual Slab Cell) containing SDS and subjected to electrophoresis at 200 V for 1 hour. Proteins in the gels are transferred to polyvinylidene difluoride (PVDF) membranes by the procedure described by Towbin et al. ((1979) Proc. Natl. Acad. Sci. USA. 76: 4350–4354) at 150 mA overnight. The transfer buffer contains 25 mM Tris, 192 mM glycine, 20% (v/v) methanol. The membranes are stained by quick dipping in 0.1% Coomassie Brilliant blue in 50% methanol-50% acetic acid, followed by washing in a solution containing 40% methanol plus 10% acetic acid. After drying the membrane at room temperature, the stained heavy and light chain bands are excised with a clean razor blade. The proteins on the membrane slices are subjected to N-terminal microsequencing by automated Edman degradation using an Applied Biosystem Model 477A protein sequencer employing pulsed-liquid chemistry and on-line phenyl-thiohydantoin amino acid identification. Each protein is subjected to 10–15 degradative cycles and the converted cleavage products from each cycle were analyzed by reverse-phase HPLC.

The nucleic acid sequence and the corresponding translation for the heavy and light chain variable regions of monoclonal antibody 1A7 is shown in FIGS. 1 and 2.

The heavy and light chain polynucleotide and amino acid sequences were compared using the BLAST algorithm at the National Center for Biotechnology Information with sequences available from the PDB, SwissProt, PIR, SPUpdate, GenPept, and GPUpdate databases. The comparison was performed on Dec. 16, 1995.

FIG. 12 shows the ten most closely matched polynucleotide sequences to the 1A7 light chain variable region encoding sequence. FIG. 13 shows the ten most closely matched polynucleotide sequences to the 1A7 heavy chain variable region encoding sequence.

FIG. 3 is a comparative depiction of the 1A7 light and heavy chain amino acid sequence with the 15 closest sequences found in the BLAST search. Panel (A) shows the light chain comparison. Panel (B) shows the heavy chain comparison. The database identifiers for the matched sequences are shown in Table 6:

### Table 6

<table>
<thead>
<tr>
<th>Light Chain Variable Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 gp</td>
</tr>
</tbody>
</table>
| 2 gp | L39941 | MU SIGK4G0_1 | Mouse rearranged immunoglobulin [...]
| 3 gp | Z22035 | MD EKKVAD_1 | Immunoglobulin variable region [Mus mu...]
| 4 gp | M32657 | MU SIGKCS1_1 | Mouse Ig rearranged kappa-chain mRNA |
| 5 gp | M34589 | MU SIGKABR1_1 | Mouse kappa-chain mRNA-V-J region |
| 6 gp | J04438 | MU SIGKCS1A_1 | Mouse Ig kappa-chain (PAC1) mRNA-V-J ...|
| 7 gp | M33127 | MUSIGKCS1_1 | IgM gene product [Mus musculus] |
| 8 gp | M32658 | MUSIGKCS1_1 | Mouse Ig kappa-chain mRNA |
| 9 gp | U29428 | MMU29428_1 | Anti-PC Ig kappa chain [Mus musculus] |
| 10 gp | N6770 | MMU06664_1 | IgM gene product [Mus musculus] |
| 11 gp | M35723 | MUSIGKDA2_1 | Immunoglobulin kappa-chain VK1 [Mus mu...]
| 12 pIR | B39276 | B39276 | Ig light chain precursor V-D-J region |
| 13 pIR | L14370 | MUSIGKVS1_1 | Immunoglobulin kappa chain [Mus mu...]
| 14 pIR | A31807 | A31807 | Ig kappa chain V region (PAC1) - m ...|
| 15 pIR | U29267 | MMU29267_1 | Ig L rearranged kappa chain V-J region |

<table>
<thead>
<tr>
<th>Heavy Chain Variable Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 gp</td>
</tr>
</tbody>
</table>
| 2 gp | U01185 | MU1185_1 | Immunoglobulin heavy chain [Mus mu...]
| 3 gp | P01819 | HY43_MOUSE | Ig HEAVY CHAIN PRECURSOR V REGION |
| 4 gp | M26985 | MUSIGHEP1_2 | Ig heavy gene product [Mus musculus] |
| 5 gp | M36217 | MUSIGHAEX_1 | Immunoglobulin heavy chain V-region |
| 6 gp | M36226 | MUSIGHEA_1 | Immunoglobulin heavy chain V-region |
| 7 gp | M3626 | MUSIGHEA_1 | Mouse Ig rearranged heavy chain (N...|
| 8 gp | A05515 | A05515_1 | Vector pSW2HPOI DNA sequence [...]
| 9 pDB | JFDL | H | IgG1 Fab Fragment (Anti-Lysozyme A...|
| 10 pIR | L43544 | MUSALCA_1 | Antibody [Mus musculus] |
| 11 pIR | A03907 | A03907_1 | Antibody DJ.3 V region (VDJ) [Homo...|
| 12 pIR | S38563 | S38563 | Ig heavy chain V region (ASWSI) |
| 13 pIR | A32456 | A32456 | Ig heavy chain precursor V region |
| 14 pIR | A05504 | A05504_1 | pSW1 protein [unidentified] [...|
| 15 pIR | L43544 | MUSALCA_3 | Mus musculus (clone pC.T.A1d1) ant...|

Amongst the 50 database sequences matched most closely to that of the 1A7 light chain variable region, none was identical. 1A7 differed from the five closest sequences by 2 substitution differences at residues 50 and 55, which are contained in the second complementary determining region (CDR2). The two differences at these positions were non-conservative substitutions, and persisted in comparisons with other light chain sequences.

Amongst the 50 database sequences matched most closely to that of the 1A7 heavy chain variable region, none was identical. The following summarizes the main points deduced from the comparison.

The closest match was with a heavy chain fragment beginning at residue 9 (designation gpM36221|MUSIGHAEB_1). There were 6 substitutions between residues 1 and 97 (before the VDJ junction), 6 substitution differences after residue 97, and 1A7 was shorter than the VDJ junction by 2 residues.

The closest match with a full length heavy chain variable region had the following features (designation...
gp100/118S [MMU1118S]: There were 10 substitution differences between residues 1 and 97, 6 substitutions after residue 97, and 1A7 was longer than the VDJ junction by 3 residues. 1A7 differed in length from all sequences but one, due to insertions or deletions of 1 to 8 residues about the VDJ junction. For the sequence of equal length (designation pif[S11106][S11106]), there were 18 substitution differences between residues 1 and 97, and 7 substitutions after residue 97.

All other comparisons showed at least 14 substitution differences between residues 1 and 97. All other comparisons showed at least 3 substitution differences after residue 97. All other comparisons showed a total of at least 20 insertions, deletions and substitution differences.

Differences appeared throughout the variable region. Amino acid consensus sequences of the 15 most closely matched Vκ and Vλ regions were designed, and compared with the 1A7 sequences. This is shown in Fig. 3C. Other than splicing differences about the VDJ junction, there appear to be 15 differences between 1A7 and the prototype sequences. Two of these differences are present in the light chain; 13 are present in the heavy chain. Seven occur in the CDRs, while nine occur in the variable region framework. The point differences likely have arisen from somatic mutation of germline variable region sequences.

EXAMPLE 3

1A7 as an Immunogen in Non-Human Primates

As a model more closely related to humans, we have investigated the effect of anti-id 1A7 on the induction of GD2-specific humoral responses in cynomolgus monkeys (Macaca fascicularis). The normal tissue distribution of GD2 in cynomolgus monkeys is very similar to that in human. As such, this primate model is ideal to gauge toxicities induced by these agents and to establish useful dosage for initial clinical trials.

Cynomolgus monkeys (two per group, 2–4 kg weight) received four intramuscular injections of purified Ab2 (2 mg) mixed with 100 μg of QS 21 as adjuvant. Control monkeys were immunized with unrelated Ab2, 3H1 mixed with QS 21 in the similar way. All injections were given at 2-week intervals. Monkeys were bled 10 days after each immunization.

The sera were analyzed for Ab3 responses by sandwich RIA and inhibition of Ab2 binding to Ab1. For these assays, the sera were pretreated with normal mouse immunoglobulin (500 μg/ml) to block anti-isotypic and anti-allotypic reactivities. 250 ng of 1A7 (Ab2) was coated into 96-well plate. After blocking, 50 μl of different concentrations of PRO#685 or PRO#778 (Ab3) was added and incubated 2 h at room temp with shaking. After washing, 90,000 cpm of 125I-1A7 was added to each well and incubated 1.5 h at room temp. The plate was then washed and bound radioactivity was measured.

Results are shown in Fig. 4. Ab3 sera from monkeys (PRO 685 and PRO 778) immunized with 1A7 bound specifically to the immunizing Ab2 (1A7) with minimal reactivity with unrelated Ab2 (3H1).

Fig. 5 shows that monkey Ab3 sera also inhibited the binding of radiolabeled Ab2 to Ab1 500 ng of 1A7 (Ab2) or 14G2a (Ab1) was coated into 96-well plate. After blocking, 50 μl of different concentrations of PRO#685 (Ab3) along with 50 μl of 125I-14G2a or 125I-1A7 (90,000 cpm) were added to each well. After 1.5 h incubation, plates were washed and bound radioactivity was counted. There was no inhibition with preimmune sera or sera obtained from monkeys (PRO 541 and PRO 667) immunized with the unrelated Ab2 3H1. These results indicate that monkey Ab3 sera share idiotypes with the Ab1.

To measure anti-GD2 reactivity in the serum of immunized monkeys, purified GD2 (250 ng/well) was absorbed into 96-well plates. After blocking wells with 1% BSA in PBS, test serum and Ab1 were diluted in same buffer and added to wells and incubated overnight at room temp. After washing, the bound antibodies were detected using alkaline phosphatase labeled anti-mouse or anti-human Ig reagents as second antibodies. Sera from both monkeys tested positively.

To determine whether 1A7 immunized monkey sera bound specifically to GD2-positive melanoma cells, the binding of monkey Ab3 sera to the melanoma cell line M21/P6 was tested. M21/P6 cells (2x10^4) were incubated with different concentrations of PRO#685, PRO#778 (Ab3) or 14G2a (Ab1), and the amount of bound antibody was determined.

Fig. 6 shows the sera collected after the fourth immunization, reacted with melanoma cells but not with the antigen-negative MCF-7 breast cancer cell line. The Ab3 sera also bound specifically to purified GD2 coated onto microtiter plates by ELISA. Control sera from preimmune monkeys or monkeys immunized with unrelated Ab2 (3H1) did not show appreciable binding to GD2. In parallel experiments, the same Ab3s from monkey PRO 685 were compared on a plate coated with CEA (an unrelated tumor-specific antigen) and were negative.

The Ab3 antibodies were then purified from sera by absorption and elution from the affinity column made of Ab2-Sepharose 4B. The eluted antibody was then passed over an immunoabsorbent column consisting of normal mouse Ig coupled to Sepharose 4B to remove anti-isotypic and anti-allotypic reactivities. Antibody that passed through was concentrated and used as purified Ab3. The purified Ab3 from monkey sera were then compared with the reactivity of purified Ab1 (14G2a) in different assays. We recovered 2.6 mg of purified Ab3 from 10 ml of sera (i.e. about 260 μg of Ab3 per ml of serum) from monkey # PRO 685 and a little less from monkey # PRO 778.

Binding of Ab3 to M21/P6 cells was analyzed by flow cytometry. Target cells M21/P6 or control cells MOLT-4 (5x10^4 in PBS supplemented with 0.2% BSA) were incubated with different dilutions of Ab3 and Ab1 for 1 hr at 4° C. After washing with PBS, the staining was done with FITC labeled second antibody and analyzed on a FACScan flow cytometer.

Results are shown in Fig. 7. In Panel A, tumor cells (M21/P6) were reacted with preimmune sera and Ab3 sera (1:100 dilution) from monkeys immunized with 1A7 mixed with QS-21. The reaction was developed with goat anti-human (F(ab)2) IgG-FITC-labeled antibody. In Panel B, MOLT-4 cells that do not express GD2 were reacted with preimmune and immune monkey Ab3 sera raised against 1A7 plus QS-21. In Panel C, tumor cells (M21/P6) were reacted with PBS-BSA control and purified Ab1 (14G2a). The reaction was developed with goat-anti-mouse-F(ab)2 IgG-FITC-labeled antibody. The results show that Ab3 from immune but not pre-immune sera was specific for GD2-bearing M21/P6 cells.

Fig. 8 shows results from an experiment in which Ab3 was shown to bind directly to the GD2 target antigen in a
specific fashion. 250 ng of different gangliosides were coated into 96-well plate. After blocking, 50 μg of different concentrations of PRO#685 (Ab3) and 14G2a (Ab1) was added and incubated 4 h at room temp. Bound antibody was detected using alkaline phosphatase conjugated second antibody.

FIG. 9 shows the corresponding inhibition experiment. Different gangliosides (250 ng) were coated into 96-well plate as before. Different concentrations of Ab3 and Ab1 along with 90,000 cpm of 125I-14G2a were added. The plate was incubated 2 h at room temperature with shaking, washed and counted. Percent inhibition was calculated and plotted against concentration of Ab1 and Ab3.

Reactivity of immunized sera and purified Ab3 for anti-GD2 antibodies against various gangliosides was also measured by immunoblotting (FIG. 10). Purified gangliosides (2 μg each of GM3, GM2, GM1, GD3, GD2 and GT1b) were spotted on strips of PVDF cellulose membrane at 1 cm intervals. After blocking with 3% BSA in PBS, the strips were incubated with PRO#685 (Ab3) or PRO#788 (Ab3) or 14G2a (Ab1) or an unrelated monkey Ab3 which was raised against an unrelated Ab2, 11D10 and PBS control; each antibody was used at 10 μg/ml, 5 ml of total solution. The incubation was done for 4 h at room temperature with shaking. After washing, the strips were incubated with alkaline-phosphatase labeled second antibody (1:1000 dilutions) for 2 h at room temperature.

The results clearly demonstrate that 1A7-QS21 immunized monkey Ab3 antibody binds to the same antigen GD2 as Ab1.

The induction of Ab3 responses in monkeys did not cause any apparent side effects in animals despite the presence of GD2 in some normal tissues. Only mild local swelling and irritation were observed at the injection site as a result of multiple immunizations. The monkeys were routinely checked by physical examinations and weight measurements. They did not show any signs of abnormalities or neurological problems. Immunohistochemistry analysis of autopsy specimens obtained after 7 months indicated that there was no toxicity induced by the anti-id 1A7 plus QS-21 vaccine treatment.

EXAMPLE 4

Clinical Use of 1A7 Antibody Vaccines in Patients With High Tumor Burden

We have obtained 9.7 g of purified 1A7 antibody for clinical use. The antibody was purified by TSB BioServices under GMP-conditions. We have completed all the regulatory testing on the antibody preparation according to FDA guidelines. We have recently received approval from the FDA (BB-IND #6183) for use of 1A7 plus QS-21 in advanced melanoma patients.

The objectives of this study are to determine the effects of a first generation vaccine on various components of the immune response (both humoral and cellular), to determine the optimum immunomodulatory dose and toxicity of anti-id antibody, and to determine whether there is the benefit to clinical condition.

Eligible patients are those having metastatic melanoma that is confirmed as bearing the GD2 antigen. Patients must have a life expectancy greater than six months, adequate nutrition, non-pregnant, Southwest Oncology Group performance score 0, off all previous anti-cancer therapy for at least four weeks, no prior mAb therapy, no ongoing use of nonsteroidal anti-inflammatory agents or cimetidine or other H2 receptor antagonists, adequate blood count and the ability to sign an informed consent.

All patients in the study are immunized by the intramuscular route with either 1 mg, 2 mg, 4 mg or 8 mg of 1A7 mixed with 100 μg of QS-21 adjuvant. Patients are randomized to one of the four dose levels. The number of total patients is between about 12 and 32. Injections are given biweekly for four total doses. Therapy continues with monthly injections until tumor progression is found. Patients are monitored carefully for anaphylaxis, serum sickness, and other potential side effects.

Periodic blood samples are obtained to determine the effect on hematopoietic cells as well as renal and hepatic function. All patients entered into the study undergo leukapheresis prior to the first immunization (pre-therapy). In addition, blood samples are obtained prior to each injection of 1A7 to determine serum levels of Ab3 and Ab1 antibodies and cytotoxic T cell responses. The specificity of the humoral responses is confirmed by immune flow cytometry, radioimmunoassay, and Dot blot analysis. Antiglobulin responses to the murine antibody is tested by sandwich RIA. Sera are also be tested for the ability to inhibit the binding of anti-GD2 mAb to GD2 antigen. The immune profile of patients is further assessed by testing the proliferative response of patient’s lymphocytes to anti-id antibody, purified GD2 antigen, and irradiated tumor cells and the cytotoxicity of patient’s lymphocytes for GD2-positive ILA-matched cell lines or autologous tumor cells (where possible).

The development of humoral immunity induced by immunization with Ab2 is assessed by testing sera obtained from patients at different time points (pre-therapy, after 3 and 4 immunizations, and then once monthly before injection if therapy continues). The sera is initially tested for total human anti-murine antibody (HAMA) response (anti-isotype and anti-allotype) by sandwich RIA. Briefly, microtiter plates are coated with 1A7 and incubated with different dilutions of patients’ sera. After washing, the antigen-antibody reaction is developed using 125I-labeled anti-id reagent in a homogeneous sandwich RIA as above. Pre-treatment non-immune sera and sera from normal donors is used as control in these assays.

Sera from immunized patients with positive responses are then tested for the presence of anti-anti-idiotypic antibodies (Ab3). Sera are diluted with buffer continuing normal murine immunoglobulin to block human antibodies against isotypic and allotypic determinants, and then checked for the presence of anti-1A7 by RIA. Unrelated Ab2 is used as a control. After washing, the antigen-antibody reaction is developed using 125I-labeled anti-id reagent in a homogeneous sandwich RIA as above. Pre-treatment non-immune sera and sera from normal donors is used as control in these assays.

If antibody is detected against 1A7, the sera are checked for its ability to inhibit the binding of 125I-labeled 1A7 to 14G2a bound to microtiter plates, or inhibit the binding of radiolabeled 14G2a to 1A7 on the plate. An unrelated Ab1-Ab2 system is used as a control. This demonstrates whether Ab3 in patients’ sera share idiotopes with 14G2a (Ab1). This inhibition assay of Ab1-Ab2 binding by Ab3 sera also demonstrates whether Ab3 is a true anti-anti-idiotyp.

To assess humoral immune responses directed against native target antigens, patients’ Ab3 sera is tested for reactivity with cell lines known to express GD2 in a RIA, and also by FACS analysis, using anti-human IgG and IgM
tracer reagents. In addition, the sera is checked for reactivity against a solubilized purified preparation of GD2 antigen coated onto microtiter plates. The antigen-antibody reaction is detected by using $^{125}$I-labeled anti-human Ig reagents. Pre-immune sera is used as a control. Unrelated antigen is also used in the assay. Isotype of human Ab3 sera binding to GD2 antigen is determined by ELISA using anti-human isotype specific reagents.

To demonstrate that Ab3 and Ab1 bind to the same antigenic determinant, inhibition of 14G2a binding to an Ag positive tumor cell line or GD2 antigen by Ab3 sera is determined in an RIA. If Ab3 in patients’ sera bind specifically to tumor cells, the ability of Ab3 to lyse these cells in conjunction with ADCC effector cells or complement can be demonstrated in standard ADCC or complement-mediated cytotoxicity (CMC) assay

Quantitation of the Response:

Host Ab3 (anti-1A7 antibody) and Ab1 (anti-tumor antibody) reactivity are a key measurement in this example. The expression of anti-anti-id antibody (Ab3) in the patients’ sera is quantitated by RIA inhibition studies as follows. Briefly, microtiter plates are coated with 14G2a (Ab1) and reacted with a fixed amount of $^{125}$I-labeled Ab2. A standard inhibition curve is performed using 14G2a as inhibitors. Next, patients’ sera depleted of anti-iso-allotypic activity at different dilutions are checked for an ability to inhibit the Ab1-Ab2 reaction, and the amount of Ab1-like antibody in the sera is estimated from the standard inhibition curve. The induction and duration of Ab3 response are compared among different dose levels. If there is no statistical difference between Ab3 responses or duration at a number of doses, the titers of specific anti-Ab3 type and specific anti-tumor antibody response (Ab1) in the sera by ELISA assay against purified GD2 antigen coated plates is compared among different dose levels.

If anti-GD2 cannot be detected in patient sera positive for anti-1A7, it may be because the Ab1’ are bound to patients’ tumor cells, or are of low affinity. To determine this, the patients’ PBMC are stimulated in vitro with antigen or Ab2 for the induction of tumor specific antibody. PBMC obtained from blood collected before therapy and then one month after the fourth immunization are cultured with various concentrations of 1A7 or unrelated Ab2 (10 ng to 100 ng) or GD2 antigen in a modified Mischell-Dutton culture. Culture supernatants are harvested and checked first for the presence of specific human immunoglobulin by ELISA assay and for binding to insolubilized preparation of 1A7 by radioimmunoassay. In addition, the supernatants are tested for the content of idiotope bearing molecule by their ability to inhibit the reaction between the $^{125}$I-labeled 14G2a to Ab2. Furthermore, the supernatants are checked for their reactivity with 14G2a Ag-positive human melanoma cells and Ag-negative carcinoma cells in a binding assay with $^{125}$I-labeled anti-human Ig reagents by RIA or ELISA assay (sensitivity=1 ng). The specificity of the effect is monitored by incubating PBMC with unrelated Ab2 of the same isotype. Since only Ab3 positive patients is included in this in vitro study, PBMC stimulated with 1A7 should secrete antibodies binding to 1A7 and serve as a positive control.

According to network hypothesis, patients immunized with 1A7 may eventually also induce an Ab4 response (anti-anti-anti-id) which may mimic the specificity of the anti-idiotype 1A7. To study this possibility, Ab3 positive patients’ sera (depleted of anti-isotype and anti-allotype antibodies) are measured for anti-idiotype activity by reacting with 14G2a (Ab1) by ELISA and negative controls are included as described for the Ab3 assay. Sera for this assay are obtained three months after the last therapy.

Cell-Mediated Immunity

Whether a specific T Cell response to the tumor associated glycolipid GD2 is generated in the 1A7 treated melanoma patients is tested by the following criteria: (1) if a T cell response is present which targets GD2 on the tumor cells, and (2) whether this response increases with repeated immunizations. Analysis proceeds in 2 phases. The first is to determine whether T Cell from all PBMC samples received can be expanded following in vitro immunizations against the 1A7. If this occurs, the next step is to determine whether these T cells, can lyse or release cytokines against autologous GD2 bearing melanoma tumor cells or allogeneic GD2 expressing melanoma cells sharing a single HLA antigen in common with the autologous CTL.

Patients entered into the study are leukopheresed prior to the first immunization and Ficoll Hypaque™ separated peripheral blood mononuclear cells (PBMC) are prepared and cryopreserved for future studies. These PBMC (1) provide antigen presenting feeder cells for subsequent studies (2) serve as baseline for T cell responses and (3) are used to generate an autologous EBV cell line which is a transfection target for immunological studies. In addition, following each round using primary and secondary PBMC, 1A7 is drawn, Ficoll Hypaque™ separated and cryopreserved for the determination of T cell responses. The T cell responses to be studied are generation of specific cytokotatic T cells, cytokine producing T cells, and proliferation of the T cell cultures in response to the antigens. When available, cutaneous tumor biopsies are obtained from the patients to provide a source of tumor infiltrating lymphocytes (TIL). Similar studies are run using TIL to determine if tumor biopsies become a source of GD2 specific cells. Also, tumor biopsies provide a source of tumor cells to serve as critical autologous targets for cytotoxicity assays, cytokine production, and proliferation assays.

In-Vitro Functional activity of T cells is measured as follows: Ficoll Hypaque™ separated PBMC (1 x 10$^5$) are incubated in the presence of medium alone, IL-2 (10 Cetus units/ml), on 0.1 to 100 ng/ml anti-id 1A7 antibody. The cell culture medium includes Iscoves medium supplemented with 10% human AB serum, gentamycin, sodium pyruvate, non-essential amino acids, L-glutamine and 10 Cetus units/ml recombinant IL-2. Every 7 days the cultures are stimulated with irradiated autologous PBMC-stimulated with the appropriate antigen used at day 0. The method of in vitro sensitization is similar to that recently described by Rivoltini et al., (1995) J. Immunol. 154:2257-2265. Beginning day 21 and on a weekly basis, proliferating cells are assessed for cell surface phenotype and cytokotoxic cytokine producing potential. Initially, all T cells are tested for their ability to recognize and lyse from autologous and allogeneic EBV cells transfected with the cDNA containing the sequence for the 1A7 anti-id molecule. Cultures lysing 1A7 transfected autologous EBV cells>10% are further tested against the NK sensitive line K562, the LAK sensitive line Daudi, autologous tumor if available and other HLA matched and mismatched GD2 bearing melanoma tumor cells. Preferably, a panel of over 40 well characterized melanoma tumor cell lines each expressing both class I and class II antigens is used. In addition, GM-CSF assays are run to determine if there is specific release of cytokines in addition to or in place of specific cytotoxicity. Proliferation of the cultures to the agents is determined by increases in cell numbers following in vitro stimulations.

The possible outcome of these studies following up to 6 rounds of in vitro stimulation is a kinetic increase in T cell cytotoxicity against both 1A7 transfected EBV cells, and
autologous tumor and/or HLA matched allogeneic melanoma tumor cell lines. This indicates a successful immunization of the patients against their own tumor cells using the anti-id IAT molecule. Studies are then done to determine if the antigen recognized is GD2 on the tumor cells and identify the possible mechanisms of recognition.

Objectives of this study include: (1) determination of an optimal dose to elicit an immune response against GD2 in the various arms of the immune system; a T cell response being particularly desirable; (2) ideally, remission or palliation of the cancer.

At the completion of the study, a second study is designed using the optimum immunomodulatory dose. The total number of patients in the second study is between 16 to 25 based on a statistical model.

EXAMPLE 5

Clinical Use of IAT Antibody Vaccines in the Adjuvant Setting

The objectives of this study comprise ascertaining the effects of the IAT in patients who have been treated for a GD2-associated cancer and have no clinical manifestations of the disease. Ideally, IAT given at an optimal dose lessens the risk or rate of recurrence.

Eligible patients are those with GD2-positive small cell lung cancer. All of the patients must have entered a complete clinical remission following standard chemotherapy, and be within 6 weeks of completion of chemotherapy and radiation therapy. Patients must have a life expectancy greater than six months, adequate nutrition, non-pregnant, Southwest Oncology Group performance status 0, no history of monoclonal antibody therapy, no ongoing use of nonsteroidal anti-inflammatory agents or cyclosporine or other H2 receptor antagonists, adequate blood count, and the ability to sign an informed consent.

Various dose levels of IAT are combined with a suitable adjuvant. One candidate is QS-21. The QS-21 molecule consists of a triterpene glycoside with the general structure of a quilliate acid 3,28-O-bis glycoside. It consists of two structural isomers designated V-1 and V-2 at a typical ratio of about 2:1. Both isomers have adjuvant activity. QS-21 has been shown to promote a response to both T-dependent antigens and unconjugated T-independent antigens. QS-21 also augments the induction of major histocompatibility complex-restricted class I cytotoxic T lymphocytes to subunit antigen vaccines, as well as antigen specific T-cell proliferation. 100 μg QS-21 has been established as the optimal amount of adjuvant per dose in a trial conducted at the Memorial Sloan-Kettering Cancer Center, NY. The other candidate is RibiPC, which is obtained from Ribi Immunochem Research Inc., Hamilton, Mont., and used according to manufacturer’s directions.

Patients receive one intramuscular injection of IAT every two weeks for a total of four injections. Patients are immunized with either 1 mg, 2 mg, or 4 mg of IAT per injection mixed with 100 μg QS-21. Therapy continues on a monthly basis for 24 months, then every 3 months until tumor progression is detected. The total number of patients is between 9 and 24.

Blood samples are obtained monthly prior to each treatment. Serum levels of Ab3 (anti-IAT), Ab1 (anti-GD2) and human anti-mouse antibody (HAMA) are measured by standard immunoassay. The specificity of these responses is confirmed by indirect immunoprecipitation of radiolabeled cells and SDS-PAGE. Sera is also tested for the ability to inhibit the binding of labeled IAT to M21/P6 cells or purified GD2.

To determine if the vaccine is inducing T-cell immunity, the buffy coat of one unit of blood is centrifuged on Ficoll Hypaque™. The peripheral blood mononuclear cells (PBMC) are removed, washed, and the lymphocyte precursor frequency is determined. Immunostaining for CD3, CD28, and CD45R markers is used to identify and sort cytotoxic T cells from suppressor T cells, using three-color flow cytometry. The proliferative response to IAT antibody and purified tumor antigen is determined. Cytotoxicity assays are conducted using HLA-matched colon cancer cell lines or autologous tumor cells. Suppressor cell function of CD8+CD28+CD45R+ cells is measured as the suppression of B cell immunoglobulin secretion.

The clinical features of each recipient are monitored regularly, and compared with the control population to determine the efficacy of treatment. The dose and frequency of IAT treatment is adjusted if necessary as more patients enter the study. A dose of IAT is identified that significantly increases the risk or rate of progression compared with controls.

EXAMPLE 6

Construction of a Recombinant Vaccinia Vector Encoding a IAT Polyepitope Fragment

Recombinant vaccinia virus (rVV) can provide a powerful agent for overcoming immune tolerance. Vaccinia virus is highly immunogenic and stimulates both humoral and cell-mediated immune responses. Vaccinia is also capable of representing TAA along with the cellular MHC complex.

This example describes plasmid construction and production of recombinant vaccinia viruses. The scheme for construction of a general vaccinia vector (rVV) is shown in FIG. 11. We retrieved the complete sequence of TK gene of the wild type WR strain of vaccinia virus (GenBank, accession number J02425) from the National Center for Biotechnology Information (NCBI) by the BLAST program. Aitsch et al. (1990) J. Mol. Biol. 215:403–410. From the sequence data, forward and reverse PCR primers 5'CAGAATGGAAGGGCCACAC (SEQ ID NO:52) and 5'GATTGATGCAATCATTACC were synthesized, corresponding to nucleotides 22–39 and 727–708 respectively of the TK sequence Hruby et al. (1983) Proc. Natl. Acad. Sci. USA 80:3411–3415. An Apa I site (underlined) was introduced into the forward primer and a Nsi I site (underlined) in the reverse primer for insertion into the plasmid pGEM-7Zf (+) (Promega). DNA from the wild type WR strain of vaccinia was isolated and TK gene was amplified by PCR. A DNA fragment of expected size (about 700 bp) was obtained by PCR. This DNA was separated by electrophoresis in low melting point agarose and purified by digestion with Gelase (Epitentre Tech.). The TK DNA fragment was ligated to the pGEM-7Zf (+) after digestion with Apa I plus Nsi I. The resulting plasmid (pGEM-TK) was amplified by standard transformation techniques. Insertion was verified by restriction mapping.

Promoter 7.5K was amplified from wild type vaccinia virus by PCR using the forward primer 5'-TTGTCCTGCAATTTAGTCTCTTCT (SEQ ID NO:53) and the reverse primer 5'-TTGTCCTGCAATTTAGTCTCTTCTT (SEQ ID NO:55), corresponding to nucleotides 69–88 and 335–312 of the 7.5K promoter sequence. Cochran et al. (1985) J. Virol. 54:360–377. A Cla I site (forward) and a Pst I site (reverse) were included in the primers. The amplified DNA fragment was digested with Pst I. A polynucleotide adapter was synthesized with the smaller oligonucleotide

59,773,516
being phosphorylated at the 5'-end by poly nucleotide kinase. The
1 5
hemi-phosphorylated adapter was ligated to PstI 1 digested PCR amplified 7.5K promoter DNA fragment. The product was digested with ClaI/EcoRI digested pGEM-TK.

A cDNA insert encoding a 1A7 polypeptide is inserted between the Neo I and Xmal (SmaI) sites of pVV. This plasmid also contains the leader sequence of the Vβ4 at the 5' end of the scFv cDNA. If desired, a vaccinia control plasmid can be constructed containing cDNA for E.coli β-galactosidase.

Construction of rvv: Rws are constructed by homologous recombination of vaccinia plasmids and wild-type WR strain of vaccinia virus according to the procedure of Mackett al. (DNA Cloning, Vol. 11, D. M. Glover, ed., IRL Press 1985) using CV-1 cells. Recombinant viral clones expressing β-galactosidase are selected by growth on TK 143B cells in the presence of 5' bromodeoxyuridine and 5'-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). Blue recombinant viruses are picked by pasteur pipettes and plaque purified. As a second step in clone selection, Southern blot of extracted DNA is performed, using 1A7 cDNA as a probe. Further selection of rvv is made by assay of culture supernatant of the virally infected CV-1 or any other Eukaryotic cells by ELISA. If cell-associated 1A7 polypeptide is in the rvv (i.e., if the leader sequence is deleted), cell lysis is assayed. Western blotting with 14G2a as probe is also performed. Biological activity of the 1A7 polypeptide synthesized by the vaccinia virus is determined by cell binding inhibition assay, as described above. Rvv clones containing 1A7 polynucleotides are selected by staining with 0.1% neutral red and plaque purified as above. Viral clones are grown into a high-titer lysate using standard techniques (Mackett et al. 1982) Proc. Natl. Acad. Sci. USA 79:7415–7419). Typically a clone producing the highest amount of 1A7 polypeptide is selected for further studies.

Assay of 1A7 Polypeptides (Foreign Proteins) Expressed By Recombinant Vaccinia Virus: CV-1 cells are propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 100 units of penicillin and 100μg of streptomycin per ml in 25-cm² flasks or 6-well Cluster flasks. Cells are inoculated with rvv at a MOI of 30. The virus is allowed to adsorb for 2 hours at 37°C in a tissue culture incubator, following which the inoculum is replaced with the culture medium and the incubation was continued. Supernatant is removed after incubation for indicated time and the 1A7 polypeptide secreted is assayed. As a control, supernatant from mock infected cells is used. Assay of 1A7 polypeptides can be performed by testing for binding to 14G2a as described elsewhere in this disclosure. β-D-galactopyranoside produced by rvv-lacZ is assayed according to Miller (Experiments in Molecular Genetics, Cold Spring Harbor Pines 1972) with pH-nitro-β-D-galactopyranoside as the substrate. Culture supernatant from virus infected cells is treated with β-propion in toate to inactivate the virus before assay (Corcoran et al. 1988) J. Parasit. 74:763). Incorporation of 3H-thymidine by NFS60 cells is used as a measure of cell proliferation (Jaffe et al. (1993) Cancer Res. 53:2221–2222). Radioactivity due to 3H-thymidine incorporation in the presence of supernatant from mock infected CV-1 cells is subtracted as background. As positive control and for standards of biological activity, intact 1A7 is used. Alternatively, standard solutions of GM-CSF can be used as described in Qin and Chatterjee (1996) Gene Therapy.

Testing vaccinia 1A7 vaccines: For administration of vaccinia, a virus titer of 10⁶ to 10⁷ pfu is injected into a mouse. Injections can be subcutaneous, intramuscular, intradermal or interperitoneal. Immunizations are performed weekly. Mice are bled 7 days after every immunization for determination of Ab3 (including Ab1). Testing for development of T cell immunity is performed 10 days after the booster immunization. Mice can also be tested by tumor challenge, in which survival after injection with tumor cells is monitored.

For administration of vaccina virus virally infected tumor cells, autologous tumor cells are maintained in Eagles medium containing 10% (vol/vol) fetal calf serum, 2 mM glutamine and gentamicin. A monolayer of confluent cells in a 75-cm² flask is inoculated with (3x10⁶) plaque forming units (pfu) of rvv. After 2 hours at 37°C, the inoculum is replaced by DMEM and the incubation was continued for another 24 hours. After examination under microscope, cells are collected by scraping and washed two times with PBS and resuspended in PBS at a desired concentration. (10³ to 10²/200 μl). Female C57BL/6 mice, 6–8 weeks old are purchased from Harlan Bioproducts for Science Inc., (IN). Animals are injected subcutaneously with the cellular vaccine in the rear left flank and two weeks later tumor cells are injected at the rear right flank for challenge. Survival of mice following tumor challenge and the presence of tumor is monitored daily. If the tumor is measurable, tumors are measured weekly by a caliper in two dimensions and the volumes are calculated using the formula (width+length)²/2. Tumors which are palpable but too small for measuring the volume accurately are recorded as zero volume, but tumor incidence is recorded as positive. Tumor volumes are averaged only for those that actually develop tumors over the observation period of 120 days. Zero values are included for those mice that eventually develop tumors but were tumor-free at a given time point.

Statistical evaluation is done using SigmaStat software (Jandel, Inc. San Rafael, Calif., USA). A P value of <.05 is considered to indicate statistical significance.

EXAMPLE 7

Expression and Characterization of a 1A7 Single
Chain Variable Region Molecule

Based on the sequence data listed elsewhere in this application, a cDNA construct is prepared for the single chain 1A7 derivative Vβ4(GGGGS)₆-Vβ₄ (SEQ ID NO:56) by standard recombinant cloning techniques. The construct is incorporated into the pET-22b(+ ) plasmid vector (Novagen, Madison, Wis.) and expressed in E. coli. Sequence analysis is performed to confirm the plasmid construct, which contains the carboxy end of Vβ₄, linked to the framework of Vβ₄ and not the leader region. pET-22b(+) contains a nickel ion binding domain consisting of 6 sequential histidine residues (His₆) (SEQ ID NO:57). The His₆ domain has a high affinity for nickel, which is used for the purification of the recombinant 1A7 scFv.

A cell binding competition assay is performed to investigate whether the 1A7 scFv retains the antigen mimicry shown by intact 1A7. GD2-positive M21/P6 cells (1x10⁶ cells/well in 50 μl volume) are placed in a 96-well plate. The cells are incubated for 2 hours at room temperature with [³¹¹]I 14G2a (Ab1), 100,000 cpm, in the absence and presence of increasing concentrations of 1A7 or the 1A7 scFv fragment. Percent inhibition is calculated according to standard formulae.

Candidate 1A7 polynucleotide vaccines are tested for immunogenicity as follows. Two groups of 10–15 female C57BL/6 mice (6–8 weeks old) are immunized intramuscu-
larly with doses of 50–100 µg purified plasmid which is optionally coupled to KLH. Various routes of administration are compared, such as intramuscular, intradermal, subcutaneous and interperitoneal.

Mice are bled 7 days after every immunization for determination of Ab3 (including Ab1) production as described above. Three mice are sacrificed from each group for isolation of spleens for the T cell proliferation assay 10 days after a booster immunization. To determine whether effects are specific, the following controls are used: (a) plasmid without 1A7 polynucleotide insert; (b) plasmid with 1A7 polynucleotide insert in the opposite orientation; and (c) plasmid containing a polynucleotide encoding an unrelated Ab2.

EXAMPLE 8

Development of an Animal Model for Testing Vaccines Comprising a 1A7 Polynucleotide or Polypeptide Derivative

A number of different fragments, constructs, plasmids, and fusion proteins are contemplated in this invention as a second generation vaccine for GD2-associated tumors. Animals have been established in the examples given so far as suitable for testing whether a candidate vaccine can elicit an immune response. Ultimately, the objective is to use the vaccine in tumor therapy, and it is desirable to have an animal model that can be used to screen candidate vaccines for this end-point.

Accordingly, we have developed a (57BL/6) mouse EL4 lymphoma model. Cheung et al. (1993, Int. J. Cancer 54:499–505) reported that murine lymphoma EL4 cells express GD2 at high density. We first wanted to see if MAb 14G2a binds to EL4 cells. Essentially 100% of the EL4 cells (a gift from Dr. Suzanne Rosenberg, Univ. of Maryland) bound at high fluorescence intensity with 14G2a by FACS analysis. The cell binding inhibition curve showed that 1A7 can effectively inhibit the binding of 125-labeled 14G2a to EL4 cells. Immunization of CS7BL/6 mice with anti-Id 1A7 plus QS-21 induced anti-GD2 antibodies which bind to EL4 cells and kill EL4 cells in in vitro ADCC assay. Also, spleen cells from immunized CS7BL/6 mice showed in vitro T cell proliferation in presence of irradiated EL4 cells. Thus, murine EL4 lymphoma in syngeneic immunocompetent CS7BL/6 mice can serve as a suitable animal model for evaluating the efficacy of anti-Id A7-based vaccines. 100, 000 EL4 cells are injected subcutaneously (s.c.) into CS7BL/6 mice. This is lethal to the host, and the median survival time is approximately four weeks. Since median survival time is dependent on the inoculum of tumor cells, a dose related time curve is generated using 103, 104, 105, 106, and 107 tumor cells/mouse, injected s.c., with 10 mice per group. A dose is determined that develops into a slow growing or rapidly growing tumor. The subcutaneous tumor model permits us to more closely monitor tumor growth and host immune response without sacrificing the mouse. EL4 cells also can be injected p.t. from vaccinated mice which grow very rapidly with intra-abdominal metastasis. The intraperitoneal model can be adapted to study the immune response to tumors which develop systemically.

Five different forms of vaccines are explored and compared for efficacy. The vaccines are:

(i) 1A7-QS21 (as our standard control)
(ii) Irradiated EL4 cells (Positive Control)
(iii) GD2-KLH plus QS-21 (Antigen Vaccine)

(iv) 1A7-plasmid
(v) 1A7-Vaccinia Construct

The serum levels of anti-anti-Id (Ab3) and anti-GD2 antibodies is measured as described elsewhere in this disclosure. Typically, blood samples are obtained before vaccination and ten days after each immunization and assayed for anti-GD2 antibodies. The time course is determined over which the immune response develops, the intensity of the immune response, the effect of multiple injections of vaccine (boosting), duration of the humoral response and variability of the humoral response between animals. Comparing the anti-GD2 titers with survival of tumor challenge establishes whether there is any correlation between the level of humoral response and tumor protection. The specificity of the immune response is examined by ELISA, RIA, Dot Blot analysis and FACS analysis. The serum Ab3 is also studied by in vitro ADCC or CMC assays. The target cells are EL4. The isotypes of anti-GD2 antibodies in the serum of mice are determined by ELISA using isotype specific reagents.

The cellular immune response is assayed using a T cell proliferation assay and a cytotoxic T lymphocyte (CTL) assay. The proliferation assay is used to assess the proliferative response of cells from mice vaccinated mice to various stimuli. Spelen cells are harvested from animals immunized with an experimental vaccine or control vaccine and placed into in vitro cultures. The splenocytes are then stimulated with either media alone, phytohemagglutinin (PHA), irradiated EL4 cells, purified GD2, anti-Id 1A7 or an irrelevant Ab2. Cell proliferation is measured after 5 days of culture and then stimulated for 18 hours by [3H]-thymidine incorporation. Stimulation of the spleen cells with PHA shows the maximum proliferation of T cells, and culturing with media alone measures baseline proliferation. Splenocyte cultures are of mixed cell type, including antigen presenting cells. Pulsing cells with the above stimulants results in antigen processing by these cells and a reactive T lymphocyte proliferation. By comparing the proliferative response of the differently vaccinated mice, it is possible to determine if there is an advantage of any particular vaccine form over others.

In order to determine which subset of T-cells are being induced, the proliferating cells are phenotyped by flow cytometry. Alternatively, splenic T-cells are depleted of either CD4+or CD8+cells before proliferation assay by incubation with monoclonal antibody RL172 (anti-CD4+) or mAb 168 (anti-CD8+) and complement.

One of the effector mechanism thought to be important for tumor protection is antigen specific CTL killing. EL4 or GD2 specific CTL activity will be assayed to determine if the vaccines induce this type of cellular response. Splenocytes are harvested from vaccinated mice and co-cultured with irradiated EL4 tumor cells and IL-2 for five days to expand Ag specific CTL. The splenocytes are then mixed with 51Cr-labeled target cells at various effector to target ratios, and then assayed for released radioactivity. The target cells are EL4 cells and MC38 cells. MC38 is a murine colon tumor cell line which was derived from the same mouse strain and thus shares the same H-2 haplotype but has different surface antigen markers. Both of these tumor cells take up 51Cr and have a low spontaneous release. Vaccination of mice should stimulate the production of CTL which specifically lyse EL4 cells and not MC38 cells.

By comparing the CTL activity in differently vaccinated mice, the particular type of vaccine stimulates different levels of CTL response is determined. Also determined is whether different adjuvants or cytokines are needed to influence this type of immune response generated.
EXAMPLE 9

Evaluating the Efficacy of Derivative Vaccines

Efficacy of the different vaccinations is measured by the degree of tumor protection caused by vaccines using the animal model outlined in the previous example. Tumor protection is compared with other measurements of the immune response to determine if there is any correlation between tumor protection and level of humoral or cellular response.

To evaluate the ability of vaccinations to prevent tumor growth, vaccinated mice are challenged with a lethal number of tumor cells. The immunization schedule is determined by evaluating the humoral and cellular immune response as described above. It is likely that the schedule and number of immunizations necessary to induce a significant immune response varies between the different types of vaccines. Fifteen mice are used in each vaccination group including one group of mice immunized with an irrelevant Ab2. Blood samples are obtained after 7 days of each immunization to monitor the humoral response. Once immunized, 10 of the mice are challenged with a previously established lethal number of EL4 cells injected s.c. The survival of the different vaccination groups is compared using Kaplan-Meier analysis. The other five mice in each vaccination group are used for CTL assays. These studies give information about the immune status at the time of tumor challenge and protection from tumor growth. Different types of vaccines are also compared (anti-id protein, cells, GD2-QLH or DNA) for their ability to stimulate a protective immune response.

The level of tumor protection is evaluated by the number of mice that survive a tumor challenge and the dose of tumor cells immunized mice can survive. The level of tumor protection is measured for each of the vaccines which stimulate tumor protecting immune responses.

Specificity of tumor protection is evaluated using an unrelated tumor cell such as MC38 which grown subcutaneously in C57BL/6 mice. Groups of mice are vaccinated with different vaccines and then challenged with either EL4 or MC38 cells. The mice are observed daily and survival comparison between two groups of mice is made using Kaplan-Meier analysis.

To evaluate the effectiveness of vaccine therapy in the treatment of mice with established tumor, C57BL/6 mice are injected with appropriate number of EL4 cells subcutaneously and then 24 hours later immunized with different vaccines or unrelated Ab2 vaccine. The mice are reimmunized every week and followed for tumor growth and survival.

Experiments are conducted to determine the immune effector arm involved in protective immunity against syngeneic GD2 antigen bearing tumors. Adoptive transfer of immune Ab3 serum (containing Ab1) or immune T-lymphocyte subsets (CD4+ or CD8+) or NK cells is done into naive recipient C57BL/6 mice previously treated with sublethal dose of radiation to allow for cell expansion. The recipient mice and previously vaccine immunized positive control mice are then injected with a lethal number of tumor cells and followed for tumor growth and survival.

EXAMPLE 10

Clinical Use of Derivative Vaccines

Promising results using plasmids in the animal tumor model of the previous example lead to development of plasmid vectors for human use. The pcDNA3 vector is modified according to Coury et al. for incorporation of the appropriate Ab2β fragment. This modification involves deletion of the neomycin gene and replacing it with Tn903 kanamycin resistance gene. Ampicillin resistance gene and viral sequences corresponding to nucleotide 5004-208 are also deleted. The plasmid is prepared on a large scale under Good Manufacturing Practice (GMP) conditions. Purified plasmid is stored at ~70°C in aliquots of 5 mg/ml.

Vaccinia constructs are made in Wyeth-calf adapted strain of vaccinia. This strain of vaccinia has been used for smallpox vaccination and has been shown to be safe in cancer patients. Clones of rvw are picked and plaque purified. Rvw are grown under GMP conditions using certified strains of eukaryotic cells. Virus is concentrated by sucrose gradient centrifugation.

An initial study is done in six advanced melanoma patients. Depending on results, anti-id based DNA vaccines is combined with the standard control 1A7 plus QS-21 vaccines for augmenting tumor specific immunity in vaccinated patients.

Additional Cited References:

Gangliosides as Tumor Antigens


Cheres, D. A., et al. Disialogangliosides G3 and G2 are involved in the attachment of human melanoma and
Plasmid and vaccinia vaccines
Anti-idiotypes as inducers of anti-tumor immune response

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..447

(x) FEATURE:
(A) NAME/KEY: met_peptide
(B) LOCATION: 59

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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-19 -15 -10 -5
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Ser Ser Asp Asp Val Phe Met Thr Gln Thr Pro Leu Ser Leu Pro Val
1 5 10
AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT
Ser Leu Gly Asp Gin Ala Ser Ile Ser Cys Arg Ser Ser Gin Ser Ile
15 20 25
GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGC TAC GTC CAG AAA CCA
Val His Ser Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gin Lys Pro
30 35 40 45
GCC CAG TCT CCA AAC CTC CTG ATC TAC TTT GGT TCC AAG CTA TCT
Gly Gin Pro Pro Asn Leu Leu Ile Tyr Phe Val Ser Ser Arg Phe Ser
50 55 60
GGG GTC CCA GAC ACC TCC AGG ATC GGC AGT GGA TCA GGG ACA GAT TCC ACA
Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
65 70 75
CTC AGC ATC AGC AGA GTG GAG CTT GAT CAG GAT GTC GAA GTC TAT
Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys
90 95 100
TGT CAA GGT TCA CAT GTT CCG TCG TCG GAT CTT GGT GGA GCC ACC AAG GTG
Phe Gin Ser His Val Pro Thr Phe Gly Gly Gly Thr Lys Leu
105 110 115
GAA ATC AAA CGG GCT GAT GCT GGA CCA ACT GTA ATC TCC CCA CCA
Glu Ile Arg Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro
120 125
TCC AGT AAG CTT GGG
Ser Ser Lys Leu Gly
130
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 149 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(x) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Lys Leu Pro Val Arg Leu Leu Val Leu Met Phe Trp Ile Pro Ala
-19 -15 -10 -5
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1 5 10
Ser Leu Gly Asp Gin Ala Ser Ile Ser Cys Arg Ser Ser Gin Ser Ile
15 20 25
Val His Ser Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gin Lys Pro
30 35 40 45
Gly Gin Ser Pro Asn Leu Leu Ile Tyr Phe Val Ser Asn Arg Phe Ser
50 55 60
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Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
65 70 75
Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys
80 85 90
Phe Gln Gly Ser His Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu
95 100 105
Glu Ile Lys Arg Ala Asp Ala Ala Ala Pro Thr Val Ser Ile Phe Pro Pro
110 115 120 125
Ser Ser Lys Leu Gly
130

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 458 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..456

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 58

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GTC TCG TTC CAG GTG CAG GTG AAG GAG TCA GCA CCT TCC GTG GCG
96
Val Leu Ser Glu Val Glu Val Lys Glu Ser Gly Pro Phe Leu Val Pro
1 5 10

CCC TCA CAG AGC CTG TCC ATC ACA TGC ACT GTC TCA GGG TCC TCA TTA
144
Pro Ser Glu Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser Leu
15 20 25

ACC ACC TAT GGT GTA AGC TGG ATT CGC CAG CCT CCA GGA AAG GGT CTC
192
Thr Thr Tyr Gly Ser Arg Thr Ile Arg Glu Pro Pro Gly Lys Gly Leu
30 35 40 45

GAG TCG GTG GGA GCA ATT TGG GGT GAC ACC ACA AAT TAT CAT TCA
240
Glu Trp Leu Gly Ala Ile Trp Gly Asp Gly Thr Asn Ser Tyr His Ser
50 55 60

GCT CTC ATA TCC AGA CTG AGC ATC AGC AGA GAT AAC TCC AGG AGC CAA
288
Ala Leu Ser Arg Leu Ser Ile Ser Lys Asn Ser Ser Lys Ser Gln
65 70 75

GTT TTC TTA AAA CTG AAC GTG CAA ACT GAT GAC GCC AGC TAC
336
Val Phe Leu Lys Leu Asn Ser Leu Glu Thr Asp Asp Thr Ala Thr Tyr
80 85 90

TAC TGT GCC AGA CTG GAT AAC TAC GAT GCT GTC GAG TAC TGG GGT CAA
384
Tyr Cys Ala Lys Gly Asn Tyr Asp Ala Leu Asp Tyr Trp Gly Glu
95 100 105

GGA ACC TCA GTC ACC GTC TCC TCA GCC AAA AGC ACA CCC CCA CCC GTC
432
Gly Thr Ser Val Thr Val Ser Ser Ala Lys Thr Thr Thr Thr Pro Pro Val
110 115 120 125

TAT CCA TTG GTC CCT GGA AGC TGG GG
458
Tyr Pro Leu Val Pro Leu Ser Leu
130

(2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 152 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
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-19  -15 -10  -5
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  1   5  10
Pro Ser Gln Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser Leu
 15  20  25
Thr Thr Tyr Gly Val Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu
 30  35  40  45
Glu Trp Leu Gly Ala Ile Trp Gly Asp Gly Thr Asn Tyr His Ser
 50  55  60
Ala Leu Ile Ser Arg Leu Ser Ile Ser Lys Asp Asn Ser Lys Ser Gln
 65  70  75
Val Phe Leu Lys Leu Asn Ser Leu Gln Thr Asp Asp Thr Ala Thr Tyr
 80  85  90
Tyr Cys Ala Lys Leu Gly Asn Tyr Asp Ala Leu Asp Tyr Trp Gly Gln
 95 100 105
Gly Thr Ser Val Thr Val Ser Ser Ala Lys Thr Thr Pro Pro Pro Val
110 115 120 125
Tyr Pro Leu Val Pro Gly Ser Leu
130

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 98 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
Gln Val Gln Val Lys Gln Ser Gly Pro Phe Leu Val Pro Pro Ser Gln
  1  5 10 15
Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser Leu Thr Thr Tyr
 20  25 30
Gly Val Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Leu
 35  40  45
Gly Ala Ile Trp Gly Asp Gly Thr Asn Tyr His Ser Ala Leu Ile
 50  55  60
Ser Arg Leu Ser Ile Ser Lys Asp Asn Ser Lys Ser Gln Val Phe Leu
 65  70  75  80
Lys Leu Asn Ser Leu Gln Thr Asp Asp Thr Ala Thr Tyr Cys Ala
 85  90  95
Lys Leu

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
Gly Asn Tyr Asp Ala Leu Asp Trp Gly Gln Gly Thr Ser Val Thr

Val Ser Ser

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRAND: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
Tyr Asp Tyr Glu Xaa Xaa Xaa Xaa Xaa
1  5

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRAND: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1  5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRAND: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
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1  5 10

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRAND: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
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(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRAND: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
Xaa Xaa Xaa Xaa
1

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
Arg Asp Tyr Arg

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
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(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 112 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
Asp Val Leu Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
1  5  10  15
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Ile Val His Ser
20 25 30
Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Gly Gln Ser
35 40 45
Pro Lys Leu Leu Ile Tyr Phe Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Cys Phe Gly Gly
85 90 95
Ser His Val Pro Trp Thr Phe Gly Gly Gly Thr Leu Glu Ile Lys
100 105 110

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Gln Val Gln Leu Lys Glu Ser Gly Pro Gly Leu Val Ala Pro Ser Gln 1 5 10 15
Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser Leu Thr Ser Tyr 20 25 30
Gly Val His Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Leu 35 40 45
Gly Val Ile Trp Gly Asp Gly Ser Thr Asn Tyr Asn Ser Ala Leu Lys 50 55 60
Ser Arg Leu Ser Ile Ser Lys Asp Ser Ser Lys Ser Gln Val Phe Leu 65 70 75 80
Lys Met Asn Ser Leu Gln Thr Asp Thr Ala Arg Tyr Tyr Cys Ala 85 90 95
Arg Glu Xaa Xaa Xaa Xaa Tyr Tyr Ala Met Asp Tyr Trp Gly Gin Gly 100 105 110
Thr Ser Val Thr Val Ser Ser 115

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 base pairs
(B) TYPE: nucleic acid
(C) STRANDINESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GATTTTTG GTAACCAACAC TCCACTTCCC CGTGCCTTTCA GTCCGAGAA TCAAGCTTCC 60
ATCTCTTCA GATCTAGCTA GAAGCAGTTA CATAGTATGU GAACACCTA TTTAGAATGG 120
TACCTCGAGA AACAGGGCCA GTCTCCAAGG CTCTGTATCT ACAAGTGTCC CACCCCATT 180
TCTGCGGTCC CAGAACGTTT CAGGCGAGTT GATCGAGCTA CAGATTTTCA ACTCAAGATC 240
AGCAGACTG AGCGTGAAGAA TCGGAGGTTT TATACCTGCT TCTCAAGGTT ACATGTTCCG 300
TGGACGTTCC GGTGAGGCTCAC CAAGCTGGAATCTAA 336

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 base pairs
(B) TYPE: nucleic acid
(C) STRANDINESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GATTTTTG GTAACCAACAC TCCACTTCCC CGTGCCTTTCA GTCCGAGAA TCAAGCTTCC 60
ATCTCTTCA GATCTAGCTA GAAGCAGTTA CATAGTATGU GAACACCTA TTTAGAATGG 120
TACCTCGAGA AACAGGGCCA GTCTCCAAGG CTCTGTATCT ACAAGTGTCC CACCCCATT 180
TCTGCGGTCC CAGAACGTTT CAGGCGAGTT GATCGAGCTA CAGATTTTCA ACTCAAGATC 240
AGCAGACTG AGCGTGAAGAA TCGGAGGTTT TATACCTGCT TCTCAAGGTT ACATGTTCCG 300
TGGACGTTCC GGTGAGGCTCAC CAAGCTGGAATCTAA 336
(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:
GATTTTTGTA TGACCCCAAAC TCACACCTCC CTCCCTTGCA GTCTTGAGGA TCAAGCTTCC 60
ATCTCTTCTA GATCTATTCA GAGCATTTA CATGTAATG GAAACACCTTA TTGAGAATGG 120
TACCTCGAGA AACCAGGCCA GTCCTCAAGG CTCTCTATCT ACAAAGTTTC CAACCGATT 180
TCTGGGTTCC CAGACAGGTT CATGCGACT GTATCGAGGA CAGATTTTCA ACTCAAGRTC 240
AGGAGATGAG AGGCTGAGGA TCTGGAAGTT TTTACTGCT TTTCAAGGTTTC AAGATTTCCG 300
TGGGAGTTCC GTGGAGGCAC CAAGCTGGA AATCAA 336

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 333 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
GATTTTTGTA TGACCCCAAAC TCACACCTCC CTCCCTTGCA GTCTTGAGGA TCAAGCTTCC 60
ATCTCTTCTA GATCTATTCA GAGCATTTA CATGTAATG GAAACACCTTA TTGAGAATGG 120
TACCTCGAGA AACCAGGCCA GTCCTCAAGG CTCTCTATCT ACAAAGTTTC CAACCGATT 180
TCTGGGTTCC CAGACAGGTT CATGCGACT GTATCGAGGA CAGATTTTCA ACTCAAGRTC 240
AGGAGATGAG AGGCTGAGGA TCTGGAAGTT TTTACTGCT TTTCAAGGTTTC AAGATTTCCG 300
TGGGAGTTCC GTGGAGGCAC CAAGCTGGA AATCAA 333

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
GATTTTTGTA TGACCCCAAAC TCACACCTCC CTCCCTTGCA GTCTTGAGGA TCAAGCTTCC 60
ATCTCTTCTA GATCTATTCA GAGCATTTA CATGTAATG GAAACACCTTA TTGAGAATGG 120
TACCTCGAGA AACCAGGCCA GTCCTCAAGG CTCTCTATCT ACAAAGTTTC CAACCGATT 180
TCTGGGTTCC CAGACAGGTT CATGCGACT GTATCGAGGA CAGATTTTCA ACTCAAGRTC 240
AGGAGATGAG AGGCTGAGGA TCTGGAAGTT TTTACTGCT TTTCAAGGTTTC AAGATTTCCG 300
CGGAGGTTCC GTGGAGGCAC CAAGCTGGA AATCAA 336

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATUTTTGTA TGACCCCAAC TCCACTGCTCC CTGCTGTGCA GTCTTGGAGA TCAGGCCTCC 60
ATCCTCTGCA GATCTAGTCA GAGCTTTAGA CATAGTAATG GAAGACCTTA TTTAGAATGG 120
TCACCTGGAA AGGACGGGCA GTCTCCAAAG CTCTGTGATCT ACAAGGTTTCA CAAGCAGATT 180
TCTGCGGTCC CAGCAGAGTT CAGTGCGACT GATCAGGGGA CAGATTTGCAC ACTCAAGATC 240
AGGAGAGTGC AGGCTGTAGA TCTGGGAGTCT TATTACTGCT TCTCAAGGTTCC ACATGTTCCG 300
TGAGCCTTCG GTGGAAGGCAC CAAAATCGGAA ATCACA 336

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GATUTTTGTA TGACCCCAAC TCCACTGCTCC CTGCTGTGCA GTCTTGGAGA TCAGGCCTCC 60
ATCCTCTGCA GATCTAGTCA GAGCTTTAGA CATAGTAATG GAAGACCTTA TTTAGAATGG 120
TCACCTGGAA AGGACGGGCA GTCTCCAAAG CTCTGTGATCT ACAAGGTTTCA CAAGCAGATT 180
TCTGCGGTCC CAGCAGAGTT CAGTGCGACT GATCAGGGGA CAGATTTGCAC ACTCAAGATC 240
AGGAGAGTGC AGGCTGTAGA TCTGGGAGTCT TATTACTGCT TCTCAAGGTTCC ACATGTTCCG 300
TGAGCCTTCG GTGGAAGGCAC CAAAATCGGAA ATCACA 336

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 333 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GATUTTTGTA TGACCCCAAC TCCACTGCTCC CTGCTGTGCA GTCTTGGAGA TCAGGCCTCC 60
ATCCTCTGCA GATCTAGTCA GAGCTTTAGA CATAGTAATG GAAGACCTTA TTTAGAATGG 120
TCACCTGGAA AGGACGGGCA GTCTCCAAAG CTCTGTGATCT ACAAGGTTTCA CAAGCAGATT 180
TCTGCGGTCC CAGCAGAGTT CAGTGCGACT GATCAGGGGA CAGATTTGCAC ACTCAAGATC 240
AGGAGAGTGC AGGCTGTAGA TCTGGGAGTCT TATTACTGCT TCTCAAGGTTCC ACATGTTCCG 300
TGAGCCTTCG GTGGAAGGCAC CAAAATCGGAA ATCACA 333

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 336 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDINESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GATGGTTTGA TGACCCCAAC TCACACTCTCC CTGCTGTGCA GCTGGAAGA TCAAAGCTCC 60
ATCTCTCGCA GATCTGATCA GAACAGTGTGA CATGTAATGT GAACACCTTA TTGAGATGG 120
TACCAGCAGA AACGAGGCCA GCTCCTCAAG CTCTCTGATCT ACAAAATTCG CAACGGATTT 180
TCCTGGGTCC CAGCACGATGT CGTGGGAGGT GCACGAGGA CAGATTTTCA ACTCAAGATC 240
AGCAGAGGCG AGGCTGAGGGA TGGGAGATTT TATTACTGCT TCAAAGGTAC AGATUTTCCCC 300
TGAGCAGTCC GTGGAGGCCAC CAAGCTGGAA ATCAA 336

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 291 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDINESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CAGCTGGGACGC TGACAGGGAGT AAGACCTGGCC CTGCTGAGGG CCTCAGAGAG CCTGCTCATC 60
ACATGCGACT TACCAGGCTTT CTCTAATACC ACACTAGTGTA TAATCCTGGT TGGGACCTCT 120
CCAGGGAGG GGCTGGGATGG GCTGGGGAAT ATGAGGCTGT ACGAAACAC AATTATCATG 180
TCAGCTCATC TATCAGACTG GACGCAAGG AAGGATAACT CCAAGGGCCA AGTTTCCTTA 240
AAACTGACAG GCCTGGCAAC TGATGCAGCA GGGACGATG ACTGTGCCCC A 291

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 45 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDINESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCTAGGACT ACTGGGAGTC AGAAGACTCA GCACACTGCT CCTCA 45

(2) INFORMATION FOR SEQ ID NO:29:
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 292 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** DNA (genomic)

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO: 29:

CAGCTGCACG TGAGGAGAC AGGAACCTGGC CTGCTGGCCG CCTCACAGAG CCTGCATCTC 60
ACATTCAACG TTCTAGGGGT CTTATTAAACC AGCATATGTG TACACTGGGTT TGCCCAAGCT 120
CCAGGAAAG GTCTCGAGTG GCTGCAAGTG ATATAGGAGT ATGGAGACAC AAACATAAT 180
TCACGTCTCA AAACCAAGACT GAGCTAGACG AAGGACAACCT CCAAGAAGCCA AGTTTTCTTA 240
AAATTGACA GTCTCACCAC TGATGACACA GCCATUTACT ACTGTCGGCA GC 292

(2) **INFORMATION FOR SEQ ID NO: 30:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 57 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** DNA (genomic)

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO: 30:

GCTGACTACT ATGCTATGGA CTACTGGGTT CAAGGACCT CAGTCAACGCT CTCTCTCA 57

(2) **INFORMATION FOR SEQ ID NO: 31:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 336 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** DNA (genomic)

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO: 31:

CAGCTGCACG TGAGGAGAC AGGAACCTGGC CTGCTGGCCG CCTCACAGAG CCTGCATCTC 60
ACATTCAACG TTCTAGGGGT CTTATTAAACC AGCATATGTG TACACTGGGTT TGCCCAAGCT 120
CCAGGAAAG GTCTCGAGTG GCTGCAAGTG ATATAGGAGT ATGGAGACAC AAACATAAT 180
TCACGTCTCA AAACCAAGACT GAGCTAGACG AAGGACAACCT CCAAGAAGCCA AGTTTTCTTA 240
AAATTGACA GTCTCACCAC TGATGACACA GCCATUTACT ACTGTCGGCA GC 336

(2) **INFORMATION FOR SEQ ID NO: 32:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 394 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** DNA (genomic)

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO: 32:

CAGCTGCACG TGAGGAGAC AGGAACCTGGC CTGCTGGCCG CCTCACAGAG CCTGCATCTC 60
ACATTCAACG TTCTAGGGGT CTTATTAAACC AGCATATGTG TACACTGGGTT TGCCCAAGCT 120
CCAGGAAAG GTCTCGAGTG GCTGCAAGTG ATATAGGAGT ATGGAGACAC AAACATAAT 180
TCACGTCTCA AAACCAAGACT GAGCTAGACG AAGGACAACCT CCAAGAAGCCA AGTTTTCTTA 240
AAAATGACAG GCTCTGCAAC TGATGACACA GCCATGTACT ACTGTGCCAG AGGCAATTAC 300
TAGG
304

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:
CTACTAAGCT ATGGATCTG AGGTCAGAG AACCCTAAGT ACCGCTCC
49

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 292 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:
CAGGTGACAC TCAAGAGACT AGAACCTGCT CTCGTCGCC CTCACAAGAG CCTGCCCATC 60
ACTGCACTG TTCTGGGGTT TCTCAAACC AGCTAAGGTT TACACTGGGT TGGCCAAGCT 120
CCAGCAAGGC GTCTGGAGTG ATCTGGGCTG ATGGAAGCAG AAATTATAAT 180
TCGCTCTCA TCTGCAACT CAAGATCAC AGAAAGCGCA AGTCTTTTCTTA 240
AAAATGACAG GCTCTGCAAC TGATGACACA GCCATGTACT ACTGTGCCAG AC 292

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:
ACAATGCTGA GGACTACTGG GTCTGAAGGA CCTCAGTCA GUTCTCTCA 50

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 351 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:
CAGGTCAAGC TGAAGAGTGC AGAACCTGCG CTCGTCGCAC CCTCAACAGAG CCTGCCCATC 60
ACATCACTG TCTCTGGGTT TCTCAAACC AGATATAGTG TACACTGGGT TGCCCAAGCT 120
CCAGCAAGGC GTCTGGAGTG ATCTGGGCTG ATGGAAGCAG AAATTATAAT 180
TCGCTCTCA TAATCAAGCT GAAGCTACGC AAGCAACCAT CCAAGAAGCCA AGTCTTTTCTTA 240
AAAATGACAG GCTCTGCAAC TGATGACACA GCCATGTACT ACTGTGCCAG AGAATGTAC 300
(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 291 base pairs
(B) TYPE: nucleic acid
(C) STRANDINESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CAAGTTGAGCT CTAGACTA CTGGAAGCTA AGAAAGCTGCA CTCGACATGC CTCGACTCTC C 351

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDINESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TACTACTAGT TGAGCTAGTC GTGAGCTAGC ACCTGACATC CGCTCTGCTC 48

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 292 base pairs
(B) TYPE: nucleic acid
(C) STRANDINESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CACATGCAAG TAAAGAGCTG AGAACGCTAT CTCGACCGG GCTGACAGAC CTCGACCTTC 60

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDINESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TACTACTAGT TGAGCTAGTC GTGAGCTAGC ACCTGACATC CGCTCTGCTC A 51
(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 288 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CAGUGUGAGC CUGAAGGGTGG AAGGACCTGGC CTOGUGGCCG CCTCGACAGG CCTGUCGATC 60
ACTGGCAGCT TCTCTGGGTT TCGATTACG AGCTATGGTG TAGACTGGTG TGCCGAGCCT 120
CCAGGAAGG GTCTGGAGTG GTGCGGAGTA ATATGCGGTT GTGGAGCGAC NAATTATATAT 180
TCACCTCTCA TUTCCGACGT GCAGCAGCG ATACAGGAA CTCAAGAGGCA AGTTTTCTTA 240
AAAAAGACCA GTCTCGACCA TGATGACCA GCCAGTACT ACTGCGCC 280

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 57 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ACUGGUUUGT TACTATGCTA TGACTACGTG GGCTCAAGGA ACCTCCAGCA CGTGCTC 57

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 293 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CAGUGUGAC GUGAAGGGTGG AAGGACCTGGC CTOGUGGCCG CCTCGACAGG CCTGUCGATC 60
ACTGGCAGCT TCTCTGGGTT TCGATTACG AGCTATGGTG TAGACTGGTG TGCCGAGCCT 120
CCAGGAAGG GTCTGGAGTG GTGCGGAGTA ATATGCGGTT GTGGAGCGAC NAATTATATAT 180
TCACCTCTCA TUTCCGACGT GCAGCAGCG ATACAGGAA CTCAAGAGGCA AGTTTTCTTA 240
AAAAAGACCA GTCTCGACCA TGATGACCA GCCAGTACT ACTGCGCC 283

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 53 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(x) SEQUENCE DESCRIPTION: SEQ ID NO:44:

ACGACTATGC TGATGACTG TGCGGTCAGA GAACCTCAAG CCACGTCTCC TCA 53

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(x) SEQUENCE DESCRIPTION: SEQ ID NO:45:
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 30
(D) OTHER INFORMATION: /note= "N represents the Nucleotide Inosine(l)"

(x) SEQUENCE DESCRIPTION: SEQ ID NO:46:
CCCAAGCTTC CAGGURCCAR KQGATARRCN GRTUG
35

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x) SEQUENCE DESCRIPTION: SEQ ID NO:47:
ACTAUGCGAC ATGCGTGTCY TRGCTGCTG YCTCG
36

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x) SEQUENCE DESCRIPTION: SEQ ID NO:48:
TAATAGACT CACTAGGG
20

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x) SEQUENCE DESCRIPTION: SEQ ID NO:49:
GTTTCCCAG TCACGAGCT
19

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x) SEQUENCE DESCRIPTION: SEQ ID NO:50:
ACTATCGAC ATGAGGTGC CGTITAGCT GTGGTGGCT

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:
CCCAAGCTTA CTGAGTGGTG GGAAGATGGA

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:
CAGAGGGAAG GGCCTAAC

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:
GATTGATGCA TATCATATCC

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:
GTATCGATG TGTAATAGCC

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:
TAGCTGAGA TGAGTACTG TCT

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:
Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser
We claim:
1. Monoclonal antibody 1A7 in an amount sufficient to elicit an anti-GD2 immunological response in a human, wherein monoclonal antibody 1A7 has the light and heavy chain variable region sequences contained in SEQ. ID NO:2 and SEQ. ID NO:4, respectively.
2. A sterile composition, comprising the amount of monoclonal antibody 1A7 according to claim 1 sufficient to elicit an anti-GD2 immunological response in a human.
3. Antibody purified from an antibody producing cell deposited under ATCC Accession No. HB-11786, or the progeny thereof, in an amount sufficient to elicit an anti-GD2 immunological response in a human.
4. A sterile composition, comprising the amount of the purified antibody according to claim 3 sufficient to elicit an anti-GD2 immunological response in a human.
5. A polypeptide capable of eliciting an anti-GD2 immunological response in a mammal, comprising an immunoglobulin variable region containing the three light chain complementarity determining regions (CDRs) of monoclonal antibody 1A7, or an immunoglobulin variable region containing the three heavy chain CDRs of monoclonal antibody 1A7, wherein monoclonal antibody 1A7 has the light and heavy chain variable region sequences contained in SEQ. ID NO:2 and SEQ. ID NO:4, respectively.
6. A polypeptide according to claim 5, wherein the anti-GD2 immunological response comprises production of anti-GD2 antibody by the mammal.
7. A polypeptide according to claim 3, wherein the anti-GD2 immunological response comprises production of anti-GD2 reactive T cells by the mammal.
8. A polypeptide according to claim 5, comprising an immunoglobulin variable region containing the three light chain CDRs of monoclonal antibody 1A7.
9. A polypeptide according to claim 5, comprising an immunoglobulin variable region containing the three heavy chain CDRs of monoclonal antibody 1A7.
10. The polypeptide of claim 8, wherein the immunoglobulin variable region of the polypeptide is contained in SEQ. ID NO:2.
11. The polypeptide of claim 9, wherein the immunoglobulin variable region of the polypeptide is contained in SEQ. ID NO:4.
12. The polypeptide of claim 5, which is a fusion polypeptide.
13. The fusion polypeptide of claim 12, in which the variable region is a light chain variable region joined by a linker polypeptide of about 5 to 20 amino acids to a heavy chain variable region, or else the variable region is a heavy chain variable region joined by a linker polypeptide of about 5 to 20 amino acids to a light chain variable region.
14. The fusion polypeptide of claim 12, comprising a light chain variable region of monoclonal antibody 1A7 and a heavy chain variable region of monoclonal antibody 1A7.
15. The fusion polypeptide of claim 12, further comprising a cytokine.
16. The fusion polypeptide of claim 15, wherein the cytokine is granulocyte-macrophage colony stimulating factor (GM-CSF).
17. The fusion polypeptide of claim 15, wherein the cytokine is interleukin 2 (II-2).
18. The fusion polypeptide of claim 12, further comprising a heterologous immunoglobulin constant region.
19. The polypeptide of claim 5, wherein the immunoglobulin variable region is humanized.
20. The polypeptide of claim 5, comprising a plurality of said variable regions.
21. A polypeptide according to claim 5, comprising both an immunoglobulin variable region containing the three light chain CDRs of monoclonal antibody 1A7 and an immunoglobulin variable region containing the three heavy chain CDRs of monoclonal antibody 1A7.
22. The polypeptide of claim 21, wherein said immunoglobulin variable regions are linked by a disulfide bond.
23. The polypeptide of claim 21, wherein said immunoglobulin variable regions are part of the same polypeptide chain.
24. The polypeptide of claim 5 in an amount sufficient to elicit an anti-GD2 immunological response in a human.
25. A sterile composition, comprising the amount of polypeptide according to claim 24 sufficient to elicit an anti-GD2 immunological response in a human.
26. The composition of claim 2, comprising an adjuvant.
27. A kit for detection or quantitation of an anti-GD2 antibody in a sample, comprising the polypeptide of claim 5 in suitable packaging.
28. A method for determining anti-GD2 antibody in a sample, comprising the steps of:
   a. contacting antibody in the sample with the polypeptide of claim 5 under conditions that permit the formation of a stable complex between the antibody and the polypeptide;
   b. detecting the stable complex formed in step a), if any; and
   c. correlating the absence, presence or amount of stable complex detected in step b) with the absence, presence, or amount of anti-GD2 antibody in the sample.
29. A method for purifying anti-GD2 antibody from a sample, comprising the steps of forming a complex between the anti-GD2 antibody and the polypeptide of claim 5 to form an antibody-polypeptide complex, separating the complex from other components of the sample, and recovering the antibody from the complex.

30. A method for detecting the presence of an anti-GD2 antibody bound to a tumor cell, comprising contacting the tumor cell with a polypeptide according to claim 5 under conditions that permit the polypeptide to bind to the anti-GD2 antibody, and detecting any polypeptide that has bound.

31. A method of eliciting an anti-GD2 immune response in a human, comprising administering to the human an effective amount of the product of claim 1.

32. A method of eliciting an anti-GD2 immune response in a mammal, comprising administering to the individual an effective amount of the polypeptide of claim 5.

* * * * *
CERTIFICATE OF CORRECTION

PATENT NO. : 5,977,316
DATED : November 2, 1999
INVENTOR(S) : Malaya CHATTERJEE et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In column 95, Claim 7, please replace “3” with --5--.

Signed and Sealed this Tenth Day of April, 2001

Attest:

NICHOLAS P. GODICI

Attesting Officer Acting Director of the United States Patent and Trademark Office