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(54) Title: CHIMERIC MURINE/HUMAN ANTI-IDIOTYPE MONOCLONAL ANTIBODIES

(57) Abstract

A murine/human chimeric anti-idiotype monoclonal antibody is provided which has murine complementarity determining regions fused to human constant regions. The antidoby preferentially binds gangliosides present on tumors, and when introduced into a human, elicits an anti-ganglioside response causing regression of cancer cells bearing the gangliosides. Further provided are a transfectoma producing the antibody, compositions containing the antibody, and cancer treating methods that involve administering the compositions to subjects.

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CHIMERIC MURINE/HUMAN ANTI-IDIOTYPE MONOCLONAL ANTIBODIES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to anti-idiotype monoclonal antibodies and their use as surrogate antigens, immunomodulators, immunosuppressants and immunodiagnostic agents. More particularly, the present invention involves chimeric human monoclonal anti-idiotype antibodies which are developed against a human monoclonal antibody reactive to cancer cells. The present invention further involves use of the anti-idiotype antibodies for treating and diagnosing cancer, the cell lines which produce the anti-idiotype antibodies, and vectors for producing these cell lines.

2. Description of Related Art

The publications and other reference materials referred to herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference. For convenience, the reference materials are numerically referenced and grouped in the appended bibliography.

The possibility that the variable regions of immunoglobulins could act as external antigens was first recognized by Jerne in his idiotype network theory (1). According to this theory, recognition of idiotypes on the antigen-combining site, or on the framework of AB1, results in the production of anti-idiotypes (anti-ids or AB2) beta and alpha, respectively. Such "internal image" anti-idiotypes, by virtue of their complementarity with the original antigen binding site, mimic the original antigen and often behave in a similar biological manner. The concept of internal image refers to the fact that some AB2 molecules can act as surrogate antigens and their administration can lead to the

production of anti-anti-idiotype antibodies displaying similar characteristics of AB1.

Immunization using anti-ids as surrogate antigens has generated much interest among researchers, many of whom have experimented with AB2 vaccines for active specific immunization against viruses, bacteria, and other pathogens (2,3). This approach is useful when a conventional vaccine or antibodies are not available, or when they are difficult to produce or when the corresponding antigen is not a suitable product for genetic engineering. In addition, anti-ids can be used as immunomodulators for up-regulating immunity against cancer, and as immunosuppressants to prevent rejection of transplanted organs and to prevent the progression of auto-immune disease.

Gangliosides are glycospingolipids that are fundamental membrane components on human tissues. Gangliosides undergo characteristic changes during malignant transformation of normal cells and thus are desirable target antigens for immunotherapy of cancer. Unlike proteins, ganglioside antigens cannot be made using genetic engineering techniques and, accordingly, are not available in abundance. There is therefore no obvious way at this time to produce these important substances in large quantities. It would be desirable if ganglioside antigens, especially those associated with cancer cells, could be mimicked by proteins, which, unlike gangliosides, can be produced in abundance with genetic engineering techniques.

Melanoma synthesizes a large number of gangliosides and thus has served as a useful model to assess the potential of gangliosides as immunotherapy targets. A number of tumor-associated gangliosides of human melanoma and their respective immunogenicity have been defined (12-29). In addition, it has been shown that active immunization with ganglioside antigens results in prolonged survival of melanoma patients (4,5).

Nevertheless, this technique suffers in many areas, namely that the ganglioside antigens are frequently rare or in short supply.

Tumor-associated antigens, in most cases, are present in nature only at low levels and are relatively difficult to purify in large amounts. In contrast, anti-ids can be secreted from hybridoma cells at low cost over long periods of time. Furthermore, current genetic engineering technology, while not applicable to ganglioside epitopes, can be used to synthesize the anti-id peptides. Anti-ids previously developed for active specific immunotherapy of human cancer have used murine monoclonal antibodies (MuMabs) as the immunogens (6-11).

Murine monoclonal antibodies have been employed to define and characterize many antigenic molecules on human cancer cells. Murine monoclonal antibodies have a strong affinity for tumor antigens and are secreted at high rates by hybridoma ascites.

Although murine antibodies are valuable in therapy of human diseases, their effectiveness is limited because rodent monoclonal antibodies have a short survival time in humans and induce an immune response that neutralizes their therapeutic effect. Furthermore, the responses induced by murine antibodies are limited because they only weakly recruit human effector elements and are relatively ineffective as cytocidal agents.

To get around these difficulties, genetically engineered antibodies have been produced that combine the murine variable or hypervariable regions with the human constant or constant and variable framework regions (31-35). The goal of generating such humanized antibodies (HuMabs) is the reduction of their immunogenicity as compared to their murine counterparts.

The development of HuMAbs that react with ganglioside antigens on human cancer cells and the demonstration of their anti-tumor effect at the clinical

level has been reported (12, 23). Patients with recurrent melanoma received intratumor injections of HuMAb to ganglioside GD2 or GM2, and partial or complete regression was observed in about 70% of the patients. In those melanoma patients in whom the immunotherapy was ineffective, the target antigen GD2 or GM2, was not expressed on the tumor cells.

Because the quantity and quality of gangliosides on human melanoma are widely heterogeneous between different cancer patients, it is desirable to avoid unnecessary administration of HuMAb by examination of a pre-treatment biopsy to identify which gangliosides dominate on each patient's tumor cells.

Although human monoclonal antibodies are desirable over murine monoclonal antibodies for therapeutic use, researchers encounter persistent problems with them, including low affinity, low clonal frequency, low antibody production, and clonal instability.

Furthermore, researchers are limited to producing human monoclonal antibodies from human B cells only if they can obtain B cells from a human who happens to be making antibodies against a desired protein. Attempts have been made to develop techniques for in-vitro immunization of human lymphocytes, but the range of antigens is quite limited (36). Attempts to produce human monoclonal antibodies by reconstituting mice with human antibody-producing cells have met with limited success, as well (37). The responding human B cells make extremely poor primary antibody responses, and were not good candidates for immunization and subsequent production of human hybridomas for the production of human monoclonal antibodies.

Accordingly, there is a need for cells that produce or secrete monoclonal antibodies at high rates from which humanized monoclonal antibodies can be easily recovered and purified.

As is apparent from the above background, there presently is a need to provide additional types of anti-idiotype antibodies which can be used as surrogate antigens in treating tumors. There is a further need to provide these anti-idiotype antibodies in a form that does not elicit strong, pathogenic immune reactions reducing their effectiveness.

SUMMARY OF THE INVENTION

In accordance with the present invention, a murine/human chimeric anti-idiotype monoclonal antibody is provided. This monoclonal anti-id antibody is comprised of complementarity determining regions comprising variable regions substantially derived from murine variable regions fused to human constant regions.

The murine variable regions, both V_H and V_L, are derived from DNA sequences encoding an anti-idiotype antibody raised against a human monoclonal anti-ganglioside antibody identified as L612. The V_H and V_L regions are sufficiently juxtaposed in the murine/human chimeric monoclonal anti-idiotype antibody of the present invention so that the antibodies preferentially bind at least one antigenic determinant of the L612 human monoclonal anti-ganglioside antibody.

The complementarity determining region of the chimeric antibody of the present invention further includes an antigenic determinant site which mimics a sialic acid galactose residue of gangliosides present on tumors. When introduced into a human subject, the chimeric antibody of the present invention elicits an anti-ganglioside response. This response includes the production of antibodies immuno-reactive with gangliosides associated with the presence of cancer cells. This results in the cytoxic destruction of cancer cells bearing those gangliosides. In particular, the anti-ganglioside response is an anti-GM3 response.

The murine human chimeric anti-id monoclonal antibody of the present invention is produced by recombinant means. The recombinant means comprises an in-vivo recombinant gene expression system which expresses DNA sequences sufficient to code for murine V_H and V_L regions and human IgG gamma 1 and kappa constant regions. The DNA sequences encoding and expressing the murine V_H and V_L regions are derived from hybridoma cell line 4C10 (ATCC No. HB10722). These DNA sequences are sufficient to encode at least a portion of an immunoglobulin molecule. The DNA sequences encoding the human constant regions are derived in part from human IgG1 heavy chain constant region genes (38). The chimeric monoclonal antibody provided by the present invention is a β-type anti-idiotype antibody.

The present invention further provides a transfectoma which produces the chimeric murine/human monoclonal anti-idiotype antibody of the present invention.

As another feature of the present invention, a method is provided for treating a tumor present in a mammal. The method involves administering a pharmacologically effective amount of the chimeric monoclonal anti-idiotype antibody of the present invention in association with a pharmaceutically-effective carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows SDS-polyacrylamide gradient gel electrophoresis and Western blot analysis of the purified chimeric mouse-human anti-id monoclonal antibody.

Fig. 2 shows binding reactivity patterns of TVE1 and 4C10 with anti-GM3 L612, L72 (human monoclonal antibody to ganglioside GD2), and human polyclonal IgM.

Fig. 3 shows ELISA plates coated with L612, comparing the affinities of TVE1 and 4C10 with L612.

Fig. 4 is an ELISA assay showing the specificity of chimeric TVE1. The plates were coated with GM3 positive M12 melanoma cells and tested for L612 binding inhibition with TVE1 and 4C10 antibodies.

Fig. 5 shows the PCR generation of V_L gene cloned into a drug marked expression vector. The heavy chain vector DNA was linearized at the Pvu I site and used to transfect into non-producing myeloma cell lines.

Fig. 6 shows the PCR generation of V_H gene cloned into a drug marked expression vector. The heavy chain vector DNA was linearized at the Pvu I site and used simultaneously with the light chain vector shown in Fig. 5 to transfect into non-producing myeloma cell lines.

DETAILED DESCRIPTION OF THE INVENTION

The chimeric murine/human anti-idiotype monoclonal antibodies of the present invention, or functional equivalents thereof, are comprised of complementarity determining region comprising variable regions substantially derived from murine V_H and V_L regions fused to human constant regions.

The variable regions are derived from a murine anti-idiotype monoclonal antibody raised against a human monoclonal antibody identified as L612. The L612 antibody is secreted by a human B-cell line also identified as L612 and which is maintained at the Division of Surgical Oncology at the University of California at Los Angeles School of Medicine. The L612 cell line is deposited at the American Type Culture Collection (ATCC) under ATCC Accession No. CRL10724.

The L612 cell line was established in culture from lymphocytes by the Epstein-Barr virus transformation technique used to produce two other human monoclonal anti-ganglioside antibodies, L55 (anti-GM2) and L72 (anti-GD2) (26-27). The L612 monoclonal antibody reacts strongly with human melanoma tumor biopsies. The L612 antibody also reacts less strongly with human tumor

biopsies from lung cancer, breast cancer, pancreatic cancer, colon cancer, and kidney cancer. The UCLASO-M12 melanoma cell line has been identified as the most reactive cell line among the lines tested with the L612 monoclonal antibody. The UCLASO-M12 cell line is maintained at the Division of Surgical Oncology at the University of California at Los Angeles School of Medicine.

Methods for preparing hybridoma cells, and in particular, the 4C10 hybridoma, that produce the anti-idiotype antibodies against human monoclonal anti-ganglioside antibody L612 are disclosed in U.S. Patent Application Serial No. 07/609,255.

The preferential binding to HuMAb L612 and immunogenic usefulness of the chimeric murine/human anti-idiotype monoclonal antibody of the present invention, comprising the V_H and V_L of the 4C10 anti-idiotype antibody, derives from the preferential binding and immunogenic usefulness of the complementarity determining region comprising homologous V_H and V_L regions of the 4C10 anti-id antibody.

The complementarity determining regions (CDRs) correspond to the hypervariable regions of the variable regions. The hypervariable regions comprise highly divergent stretches of amino acids. In an intact immunoglobulin, the hypervariable regions of each light chain and of each heavy chain can be brought together in three-dimensional space to form an antigen-binding surface. Because these sequences are thought to form a surface complementary to the three-dimensional surface of a bound antigen, the hypervariable regions are also called complementarity-determining regions (CDRs). The CDRs determine antigen-binding specificity, the residue in the CDRs often making contact with the antigen.

The preferential binding of the 4C10 anti-idiotype antibody to at least one antigenic determinant of human monoclonal anti-ganglioside antibody (HuMAb) L612 and

the immunogenic usefulness of the beta type anti-id 4C10 have been demonstrated as follows:

The 4C10 cloned hybridoma cell line was selected and grown in accordance with the methods of U.S. Patent Application Serial No. 07/609,255. The 4C10 hybridoma was selected from 40 hybridomas secreting antibodies with distinct reactivity to L612 HuMAb but no reactivity to three other control human IgMs and two unrelated serum protein antigens.

To determine whether these anti-L12 antibodies were beta-type directed against the antigen combining site of L612, or were alpha antibodies bound to peptide regions outside the antigen-combining site of L612, the inhibitory activity of these anti-L612 antibodies against L612 binding to GM3 positive target cells lines or to the purified antigen, ganglioside GM3, was tested.

Ganglioside GM3 includes a terminal sugar having NeuAc alpha 2,3 galactose residue. The three assay systems were: IA inhibition, cell-ELISA inhibition, and GM3-ELISA inhibition. Of the 40 antibodies tested, seven inhibited L612 binding to an antigen positive target melanoma cell line (UCLASO-M12), and to GM3 treater than 50% in the assays, while 12 others had weak or no inhibitory activity.

Of the seven inhibitory anti-ids, one identified as 4C10 was selected for cloning as the preferred beta-type anti-id for use in treating tumors. 4C10 was tested with isotype antiglobulins and found to be of the IgG1 class and contain kappa light chains.

The 4C10 cloned hybridoma cell lines were grown in FCS-containing RP MI 1640 medium and secreted 5-10 ug/ml of antibody into culture supernatants. Titers of the anti-ids in these culture supernatants against L612 by ELISA ranged between 1:200 to $1:1000/10^6$ hybridoma. Anti-id 4C10 demonstrated strong binding inhibition of HuMab L612 to target cells in the IA assay (100%) and to ganglioside GM3 in the ELISA assay (100%). As a control

assay, 4C10 failed to inhibit the binding of an unrelated antigen system, HuMAb L72, to M14 target cells, or to GD2 antigen. The specific binding inhibition of 4C10 indicates its binding location to be within or near the antigen combining site.

The hybridoma cell line which secretes the 4C10 anti-id is maintained at the Division of Surgical Oncology at the University of California at Los Angeles School of Medicine. The 4C10 hybridoma cell line was deposited at the American Type Culture Collection under ATCC No. HB10723.

The 4C10 anti-id and other beta-type anti-ids can be used alone or in combination with other agents to treat tumors. Using recombinant technology, the variable and hypervariable regions of the chimeric anti-id may be fused to proteins having properties including the biological activity of growth factors or cytokines. These growth factors include insulin like growth factor (IgF1 or IgF2), and transferrin. The cytokines include interleukin 2 or 4, and tumor necrosis factor.

The 4C10 anti-id and other beta-type anti-ids are preferred for use in treating melanoma tumors. These beta-type anti-ids may also be used as an immunomodulator to enhance anti-cancer immunity, suppress organ transplant rejection and suppress autoimmune disease.

The immunogenic usefulness of the chimeric murine/human anti-idiotype monoclonal antibody of the present invention is based, in part, upon the demonstration that murine anti-idiotype antibody 4C10, comprising homologous V_H and V_L regions, stimulated the production of antibodies which were immunoreactive with melanoma tumors. This was demonstrated as follows:

Five syngeneic Balb/c mice were immunized with purified 4C10-KLH. As controls, four mice were immunized with mouse IgG1-KLH and one mouse with KLH alone. The immunized sera were monitored by ELISA using

purified GM3 as the antigen source and by the IA assay using the antigen positive M12 melanoma cell line. In the ELISA, peroxidase conjugated goat anti-mouse IgM + IgG (Boeringer Mannheim) was used as a second antibody. Measurable antibody (AB3) was produced in three of the five immunizations with 100 ug 4C10-KLH. The immunized sera bound to GM3 but not to CDH (asialo-GM3). Sera from the control mice immunized with IgG-KLH or KLH alone gave no response to either glycolipid. In further analysis to determine the Ig class of the AB3 (ELISA and TLC immunostaining), the majority of the reactivity was identified as IgM.

In order to exclude the species specific natural antibodies that might react to M12 cells in the IA assay, the immunized murine sera were pre-absorbed by human red blood cells at 4°C overnight. An IA score of 4+ was obtained at 1:10 dilution of the absorbed sera. Control sera gave no reactivity even at 1:2 dilution. To confirm that the positive reactivity was directed against GM3 antigen on the cell surface, IA inhibition was performed using GM3 (10 ug), CDH (10 ug), 4C10 (10 ug) and unrelated IgG1 (10 ug) purified from Balb/c hybridoma ascites. While reactivity was completely inhibited by GM3 or purified 4C10, no inhibition was obtained with CDH or unrelated IgG1.

The above example demonstrated that the murine 4C10 beta-type anti-id AB3 antibodies are immunoreactive with melanoma tumors. As presented below in "Characterization of the Structure and Specificity of Chimeric Mouse/Human Antibody," the inventors found that the anti-id specificity property of the chimeric mouse/human anti-id monoclonal antibody of the present invention was virtually identical with the original mouse 4C10 monoclonal antibody. Based upon these findings and the effectiveness of the 4C10 anti-id in stimulating anti-melanoma response, the beta-type anti-ids of the present invention are considered

effective as an immunization agent in the treatment of melanoma.

In particular, the chimeric murine/human anti-idiotype monoclonal antibody, which incorporates the variable regions of the 4C10 anti-id antibody, is expected to be effective as an immunization agent in the treatment of melanoma. The chimeric antibody has a further advantage in not eliciting an immune response against the murine constant regions. These murine constant regions are present in the 4C10 antibody but absent in the chimeric anti-id antibody of the present invention.

The chimeric murine/human anti-idiotype monoclonal antibodies of the present invention, comprising the variable regions derived from the 4C10 beta anti-ids, may be administered by any of the conventional procedures used to introduce antibodies into patients. These procedures include subcutaneous, intravenous or intratumor injection. The chimeric beta-type anti-ids are preferably conjugated with KLH and emulsified in a suitable carrier typically used for administration of antibodies. The particular doses used for the chimeric beta-type anti-ids will vary depending upon the tumor being treated and numerous other factors. The dosage levels are established by the known techniques and principles generally recognized and utilized in treating patients with antigen immunization agents or monoclonal antibodies.

Recombinant Production of the Chimeric Murine/Human Anti-Idiotype Monoclonal Antibody

The chimeric murine/human anti-idiotype monoclonal antibodies of the present invention were produced by recombinant means. An in-vivo recombinant gene expression system was constructed so as to express DNA sequences sufficient to code for murine complementarity determining regions comprising V_H and V_L regions. The V_H

and V_L regions were derived, at least in part from hybridoma cell line ATCC No. 4C10. The transfectoma so constructed also express DNA sequences that code for human immunoglobulin constant region, including human $\gamma 1$ constant regions. Derivation of human IgG1 constant region sequences is well known in the art (38).

The transfectoma referred to above which secretes the chimeric murine/human anti-idiotype anticlonal antibody of the present invention was produced with light chain and heavy chain vectors. Preparation of the vectors is described below.

A light chain vector was prepared which contained a cloned DNA sequence encoding the variable region of the light chain anti-id monoclonal antibody expressed by the 4C10 hybridoma. This DNA sequence was prepared from mRNA which had been prepared from the 4C10 mouse myeloma cell line and reverse transcribed and amplified with the PCR amplification method. A heavy chain vector comprising a DNA sequence encoding the variable region of the heavy chain of the anti-idiotype monoclonal antibody raised against human monoclonal anti-ganglioside antibody L612 was similarly prepared.

Heavy and light chain vectors were used to simultaneously transfect non-producing myeloma cells. From these transformed cells, surviving clones were selected which secreted both heavy and light chains having the appropriate specificity.

Cloning of Variable Region cDNA sequences from 4C10

Preparation of RNA. RNA was prepared from the 4C10 mouse myeloma cell line using guanidinium thiocyanate and the polyA containing fraction isolated using oligodT cellulose (Boehringer Mannheim, Indianapolils, IN). Direct mRNA sequencing with a murine C_κ primer indicated that the light chain used Jκ1. From the sequence of framing region FR3, it was found that the light chain was in the V_κIII group of Kabat (45). Many members of

that group share similar or identical leader sequences. Therefore, a consensus leader primer was synthesized (ATGGAGACAGACACACTC) and in conjunction with a $J_\kappa 1$ primer was used to amplify the mRNA which had been reverse transcribed using a C_κ primer.

One V_K clone was identified and analyzed. Sequencing of this clone demonstrated that it was identical to an aberrant light chain transcript initially described by Walfield et al. (39) and demonstrated by Carroll (40) to be present in the MOPC-21 derived myeloma cell lines routinely used for producing hybridomas. The leader sequence of the aberrant transcript is identical to the leader sequence useful for priming; the aberrant transcript utilizes $J_\kappa 2$ instead of $J_\kappa 1$, however, over the extent of our primer, there is only one base mismatch between $J_\kappa 1$ and $J_\kappa 2$. Accordingly, our primer would effectively prime for $J_\kappa 2$.

PCR Amplification. One μg of poly A+ mRNA was mixed with 100 ng of 3′ primer. dNTPs were added to a final concentration of 200 μM, $MgCl_2$ to 1.5 mM, KCl to 50 mM, Tris-Cl pH 8.3 to 10 mM, and galatin to 0.01%. The reaction mix was heated to 70° C, cooled, after which 20 U of reverse transcriptase (Life Sciences, St. Petersburg, FLA) was added and incubated for 1 hour at 37°. 100 ng of the 5′ primer was then added and amplification continued for 25 cycles. The primers used included the following:

Heavy Chain Leader

CATAGGATATCCACCATGGGATGGAGCTGGATC

This contains an EcoRV site (underlined) to facilitate cloning into the promoter.

Heavy chain J region:

CTTGGTGCTAGCTGCAGAGACAGTGACCAG

This contains an NheI site (underlined) for cloning into $C_H 1$ of IgG.

Light Chain Leader:

CATAGGATATCCACCATGGAGACAGACACACTC

This contains an Eco RV site (underlined) to facilitate cloning into the promoter.

Light chain J region:

GGAAGTCGACTTACGTTTGATTTCCAGCTTGGAG

This contains a Sal I site (underlined) for cloning into the intron.

The strategy employed for constructing the light-chain expression vector and heavy chain expression vector of the present invention are schematically presented in Figs. 5 and 6, respectively. After PCR amplification, the products were digested with the appropriate restriction endonucleases: EcoRV and NhEI for heavy chain and EcoRV and Sal I for light chain. The heavy variable region was cloned into Bluescript containing an Nhe I site that had been produced by ligating Nhe I linkers into the Sma I site. The light chain was cloned into EcoRV and Sal I cut Bluescript. The variable regions were initially sequenced in Bluescript to verify that they encode a functional domain; they were then cloned into the expression vectors and resequenced. The nucleotide and deduced amino acid sequences of the light chain variable regions of 4C10 are shown in SEQ ID NO:1 and SEQ ID NO:2, respectively.

Sequencing of V_κ. Primers were constructed and the mRNA of the light chain sequenced up to the ATG initiation codon. This sequence analysis showed that the leader sequence predicted from the fact that the light chain was a member of the $V_{2\kappa}$ III family had been correct. The PCR reaction was repeated using the same primers as were originally used. From this reaction, the rearranged V_κ was cloned into both Bluescript and the expression vector, and sequenced. The sequence is shown in SEQ ID NO: 1. As noted in feature information for

SEQ ID NO:1, PCR amplification introduced a substitution at nucleotide position number 152. The substitution changed the codon from ACT in the original hybridoma to AGT in the PCR substituted nucleic acid. Accordingly, a serine was substituted for threonine at amino acid position number 31 in the polypeptide expressed from the PCR-substituted nucleic acid. It was determined that this substitution did not influence the function of the chimeric antibody of the present invention.

Sequencing of V_H. The sequence of the entire V_H mRNA was determined using a mouse primer and a set of intermediate primers. The construction of these primers was based on partial sequence information. Using the appropriate PCR primers, the V_H was amplified, cloned into both Bluescript and into the expression vector. The nucleotide and inferred amino acid sequences of the V_H are shown in SEQ ID NO:3 and SEQ ID NO:4, respectively.

The results showed that V_H uses J_H3. However, for the first residue, the T normally present is replaced by a G leading to a Trp to Gly replacement at amino acid position number 101. The sequence between the end of V_H and the beginning of JH (beginning at nucleotide number 349) is GGCGAAGGTCACGCGTGG.

Transfection

Vectors were linearized at the PvuI site. For transfection, 1.1×10^7 P3 X 63.Ag8.653 non-producing myeloma cells were suspended in 1 ml of PBS containing 10 μg of each vector into which the VL and VH regions from 4C10 cells were cloned. Accordingly, heavy and light chain vector DNA linearized at the PvuI site were simultaneously transfected into non-producing myeloma cell lines as follows. Cells were electroporated at 200

V, 960 microF using a Gene Pulser (BioRad, CITY, STATE), diluted to 2.2 x 10^6/ml with Iscoves modification of Dulbeccos Medium (GIBCO, Grand Island, NY) supplemented with 10% iron supplemented calf serum (Hyclone, CITY, STATE) and plated into 96 well microliter dishes, 125 µl per well. After 48 hours, Histodinol (Sigma, St. Louis, MO) as added to 10 mM and mycophenolic acid to 3 µg/ml. To screen for producing clones, ELISA plates were coated with an anti-human kappa chain antiserum (Sigma, St. Louis, MO. After adding culture supernatants and washing off unbound antibodies, the plates were developed with alkaline phosphatase labeled anti-human gamma chain (Sigma, St. Louis, MO). The frequency of surviving clones was 1.7 x 10^5; the frequency of clones secreting both heavy and light chains was 6.2 x 10^{-6}, calculated from the original number of transfected P3 cells.

The PCR generated V_L and V_H were cloned into separate drug marked expression vectors, respectively shown in Figs. 5 and 6. Heavy and light chain vector DNA linearized at the Pvu I site were simultaneously transfected into non-producing myeloma cell lines by electroporation and cells selected by mycophenolic acid and histidinol.

Transfectomas producing both chimeric heavy and light chains were identified, and one clone, TVE1, was amplified for further analysis. Transfectoma TVE1 has been deposited at the American Type Tissue Culture, designated number ATCC CRL 10867.

To initially characterize the chimeric protein, the transfectoma TVE1 was labeled by growth in ^{35}S-methionine, cytoplasmic was extracted, and secreted antibody was isolated, and Ig species precipitated with rabbit anti-human Fab and Staphylococcus protein A. The precipitates were analyzed by SDS-PAGE, both before and after reduction of the disulfide bonds (Fig. 4). The chimeric heavy and light chains were of the expected

55,000 daltons and 22,000 daltons molecular weights. The chimeric protein was secreted as a fully assembled H_2L_2 molecule.

Characterization of Structure and Specificity of Chimeric Antibody

To characterize the assembly, secretion, and molecular weight of the immunoglobulin, cells were labeled with ^{35}S-methionine and cytoplasmic lysates and secretions prepared. Antibody molecules were immunoprecipitated with polyclonal rabbit Ab against human Fc and Staphylococcus aureus protein A (IgGsorb, The Enzyme Center, Malden MA) and analyzed by SDS/PAGE with and without reduction of disulfide bonds. The chimeric protein was secreted as a fully assembled H_2L_2 molecule.

Figs. 1a and 1b show the SDS-polyacrylamide gradient (4-20%) gel electrophoresis profile of chimeric antibody TVE1 after purification by protein affinity chromatography from culture media. The results show that there was no significant difference in the size of intact IgG molecules of the chimeric TVE1 antibody, the original mouse 4C10 anti-id monoclonal antibody, polyclonal murine IgG, or polyclonal human IgG (Fig. 1a).

Figs. 1c, 1d, and 1e show the results of Western blotting analysis of the TVE1 chimeric antibody to test specificity. The chimeric antibody after blotting showed specific anti-id reactivity with human L612 monoclonal antibody like the original murine 4C10 anti-id (Fig. 1e). However, unlike 4C10, the chimeric antibody after blotting reacted with anti-human IgG, but not with anti-mouse IgG immunoglobulins (Figs. 1c and 1d).

The anti-idiotype specificity of the TVE1 chimeric antibody also was confirmed by ELISA. Fig. 2 shows binding reactivity patterns of TVE1 and 4C10 with anti-

GM3 L612, L72 (human monoclonal antibody to ganglioside GD2), and human polyclonal IgM. ELISA plates were coated with TVE1 or 4C10, then the reactivities of the three human IgM monoclonal and polyclonal antibodies were examined. TVE1 and 4C10 reacted only with L612 IgM but did not react with L72 or human polyclonal IgM. Fig. 3 shows the results of a reversed ELISA experiment to compare the affinities of TVE1 and 4C10 with L612. ELISA plates were coated with L612 and binding reactivity of TVE1 and 4C10 were tested alone or by competition. Both TVE1 and 4C10 exhibited the expected concentration dependent binding. In competitive assays, TVE1 and 4C10 displayed reciprocal inhibition with L612 at almost identical concentration, consistent with equivalent anti-id affinity.

The specificity of chimeric TVE1 was further examined by the cell ELISA inhibition assay using melanoma cell line UCLASO-M12, which mainly expresses ganglioside GM3 on the cell membrane at high density (Fig. 4). ELISA plates were coated with GM3 positive M12 melanoma cells and tested for L612 binding inhibition with TVE1 and 4C10 antibodies. Both chimeric TVE1 and 4C10 antibodies inhibited binding activity of L612 to ganglioside GM3 on the tumor cell membrane to a similar extent (Fig. 4). These results showed that the anti-id specificity property of the chimeric human antibody, TVE1, is virtually identical with the original mouse 4C10 monoclonal. Thus, by all the assays used, it appears that the chimeric TVE1 bears the internal image of ganglioside GM3. When injected into humans, the principal immune response should be directed against the variable region of the TVE1. Accordingly, the TVE1 antibody of the present invention has clinical usefulness as an idiotypic vaccine in cancer patients, inducing specific anti-GM3 immunity against human tumors expressing this ganglioside.

Purification of Chimeric Antibody

Antibody secreting transfectomas were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5% fetal calf serum (Gemini Bioproducts, Calabasas, CA) and a combination of antibiotics including penicillin, streptomycin, and Fungizone (Gibco) in humidified 5% CO_2/95% air at 37° C.

After four days in culture, 1/10 cells were transferred to fresh medium and maintained. The remaining cells were washed with serum free RPMI 1640 three times and sub-cultured in serum-free medium containing growth factor, AIM-V medium (Gibco), for an additional four days.

The serum-free spent supernatant was then obtained by centrifugation at 2000x g for 10 minutes and pelted cells were discarded. These transfectoma cells were freshly prepared each time from the seed culture flask and transferred into serum free medium.

The chimeric antibody in pooled serum-free spent medium was precipitated by slow addition of solid ammonia sulfate to 50% saturation at 22° C. The protein precipitate was obtained by centrifugation at 4000x g for 20 minutes. After resuspension and dialysis against phosphate buffered saline (PBS) at 4° C, the chimeric antibody was purified on an affinity column (5ml bed volume of protein G sepharose 4B Fast Flow, Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) equilibrated with PBS containing 0.05% Tween 20(TPBS).

The dialysate was applied repeatedly to the column at half bed volumes with protein G binding for 1 hour at 22° C for each sample. After washing with 10-bed volumes of TPBS, the chimeric antibody was eluted with 0.1 M glycine HCl buffer (pH 2.8) and neutralized immediately by adding a small amount of 1.5 M Tris-HCl (pH 8.8). The fractions containing the chimeric antibody were pooled, concentrated, and dialyzed against

PBS. One mg of the purified IgG/ml was calculated based on a standard value of 1.35 absorbance units at 280 nm.

Immunochemical analysis

SDS-polyacrylamide gradient (4-20%) gel electrophoresis (41) and Western blotting (42) were carried out as previously described. For the detection of human or mouse IgGs, anti-human or anti-mouse IgG antibodies conjugated with peroxidase were used. For the detection of reactivity with the human IgM monoclonal antibody L612, the blotted strip was incubated with 20 μg/ml of L612 in TPBS at 22° C for 1.5 hours. Then bound L612 was detected with peroxidase-conjugated anti-human IgM.

4-chloro-1-naphthol (0.05% in PBS) was used as the substrate for the peroxidase reaction. Enzyme-linked immunosorbent assay (ELISA) and cell-ELISA inhibition assays were used to test the specificity of the chimeric antibody as described in (43).

Anti-human IgG antiserum was obtained from Dako Corp., Carpenteria, CA. Other antisera were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN; human IgG and IgM were obtained from Sigma Chemicals, St. Louis, MO; mouse IgG was from Calbiochem Corp., La Jolla, CA. Human monoclonal antibodies L612 and L72 were purified as described in (44).

Having thus disclosed exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations, and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

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SEQUENCE LISTING

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Morrison, Sherie L.
Irie, Reiko F.

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(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(B) FILING DATE:
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 396 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(B) STRAIN: mouse
(G) CELL TYPE: Hybridoma
(H) CELL LINE: ATCC No. HB10722

(ix) FEATURE:
(A) NAME/KEY: sig peptide
(B) LOCATION: 1..60
(D) OTHER INFORMATION: /function= "region coding for cleavable leader sequence"

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(B) LOCATION: replace(151..153, "act")
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(ix) FEATURE:
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(B) LOCATION: 61..396

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(B) LOCATION: 1..396

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                65                  70                  75

CTC ACC ATT AAT CCT GTG GAG GCT GAT GAT GTT GCA ACC TAT TAT TGT      336
Leu Thr Ile Asn Pro Val Glu Ala Asp Asp Val Ala Thr Tyr Tyr Cys
            80                  85                  90

CAG CAA AGT AAT GAG GAT CCC ACG TGG ACG TTC GGT GGA GGC TCC AAG      384
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(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

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Gly Ile Pro Ala Arg Phe Ser Gly Ser Glu Ser Arg Thr Asp Phe Thr
                 65                  70                  75

Leu Thr Ile Asn Pro Val Glu Ala Asp Asp Val Ala Thr Tyr Tyr Cys
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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..414

(ix) FEATURE:
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(B) LOCATION: 1..57
(D) OTHER INFORMATION: /function= "region coding for cleavable leader sequence"

(ix) FEATURE:
(A) NAME/KEY: mat peptide

(B) LOCATION: 58..414

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

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ATC CAA GCA CAG ATC CAG TTG GTG CAG TCT GGG CCT GAG CTG AAG AAG        96
Ile Gln Ala Gln Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Lys
             1               5                  10

CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GCC TCT GGG TAT ACC TTC       144
Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
     15                  20                  25

ACA AAC TAT GGA ATG AAC TGG GTG AAG CAG GCT CCA GGA AAG GGT TTA       192
Thr Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu
 30                  35                  40                  45

AAG TGG ATG GGC TGG ATA AAC ACC AAC ACT GGA GAG CCA ACA TAT ACT       240
Lys Trp Met Gly Trp Ile Asn Thr Asn Thr Gly Glu Pro Thr Tyr Thr
                50                  55                  60

GAA GAG TTC AAG GGA CGG TTT GCC TTC TCT TTG GAA ACC TCT GCC AAC       288
Glu Glu Phe Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Asn
            65                  70                  75

ACT GCC TAT TTG CTG ATC AAC AAC CTC AAA AAT GAG GAC ACG GCT ACA       336
Thr Ala Tyr Leu Leu Ile Asn Asn Leu Lys Asn Glu Asp Thr Ala Thr
         80                  85                  90

TAT TTC TGT GCA AGA GGG GAA GGT CAC GCG TGG GGG TTT GCT TAC TGG       384
Tyr Phe Cys Ala Arg Gly Glu Gly His Ala Trp Gly Phe Ala Tyr Trp
     95                  100                 105

GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA                              414
Gly Gln Gly Thr Leu Val Thr Val Ser Ala
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(2) INFORMATION FOR SEQ ID NO:4:

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(A) LENGTH: 138 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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              1               5                  10

Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
     15                  20                  25

Thr Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu
 30                  35                  40                  45

Lys Trp Met Gly Trp Ile Asn Thr Asn Thr Gly Glu Pro Thr Tyr Thr
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Glu Glu Phe Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Asn
             65                  70                  75

Thr Ala Tyr Leu Leu Ile Asn Asn Leu Lys Asn Glu Asp Thr Ala Thr
         80                  85                  90

Tyr Phe Cys Ala Arg Gly Glu Gly His Ala Trp Gly Phe Ala Tyr Trp
     95                  100                 105

Gly Gln Gly Thr Leu Val Thr Val Ser Ala
110                 115
```

CLAIMS

What is claimed is:

1. A murine/human chimeric monoclonal anti-idiotype antibody, and functional equivalents thereof, comprised of

- complementarity determining regions comprising variable regions substantially derived from murine V_H and V_L regions derived from an anti-idiotype antibody raised against a human monoclonal anti-ganglioside antibody identified as L612;
- human constant regions fused to said murine V_H and V_L regions

wherein said murine/human chimeric monoclonal anti-idiotype antibody preferentially binds at least one antigenic determinant of said human monoclonal anti-ganglioside antibody identified as L612.

2. The murine/human chimeric monoclonal anti-idiotype antibody of claim 1 wherein the antibody is produced by recombinant means.

3. The murine/human chimeric monoclonal anti-idiotype antibody of claim 2 wherein said chimeric anti-idiotype antibody is a beta-type anti-idiotype antibody.

4. The murine/human chimeric monoclonal anti-idiotype antibody of claim 3 further including an antigenic determinant that mimics a sialic acid galactose residue of gangliosides present on tumors.

5. The monoclonal antibody of claim 3 which when introduced into a human subject elicits an immune

anti-ganglioside response, said anti-ganglioside response including the production of antibodies immunoreactive with gangliosides associated with the presence of cancer cells.

6. The monoclonal antibody of claim 5 wherein the anti-ganglioside response is an anti-GM3 response.

7. The monoclonal antibody of claim 5 wherein said cancer cells include melanoma cells, lung cancer cells, breast cancer cells, pancreatic cancer cells, colon cancer cells, and kidney cancer cells.

8. The murine/human chimeric monoclonal antibody of claim 3 wherein the recombinant means comprises an in vivo recombinant gene expression system, the gene expression system constructed so as to express DNA sequences sufficient to code for said murine V_H and V_L regions and said human constant regions, said DNA sequences encoding said murine V_H and V_L regions being derived at least in part from hybridoma cell line ATCC No. HB10722 and encoding at least a portion of an immunoglobulin molecule.

9. The murine/human chimeric monoclonal antibody of claim 3 wherein said murine/human chimeric monoclonal antibody is produced by a transfectoma hybridoma which is identified as TVE1 and which is deposited at the ATCC under ATCC accession number CRL 10867.

10. A method for treating a tumor present in a mammal comprising the step of administering to said mammal a pharmacologically effective amount of the murine/human chimeric monoclonal antibody of claim 3.

11. The method for treating a tumor present in a mammal according to claim 10 wherein said murine/human chimeric monoclonal antibody is produced by a transfectoma which is identified as TVE1 and which is deposited at the ATCC under the ATCC accession number CRL 10867.

12. A composition for treating a tumor present in a mammal comprising providing to said mammal a therapeutically effective amount of the chimeric murine/human monoclonal antibody of claim 4 in association with a pharmaceutically acceptable carrier vehicle.

13. The murine/human chimeric monoclonal anti-idiotype antibody of claim 1 further wherein said human constant region is selected from the group of immunoglobulin constant regions consisting of IgG1, IgG2, IgG3, IgG4, IgM, and IgA.

14. The murine/human chimeric monoclonal anti-idiotype antibody of claim 1 further comprising a growth factor fused to said antibody.

15. The murine/human chimeric monoclonal anti-idiotype antibody of claim 14 wherein said growth factor is selected from the group consisting of insulin like growth factors and transferrin.

16. The murine/human chimeric monoclonal anti-idiotype antibody of claim 1 further comprising a cytokine fused to said antibody.

17. The murine/human chimeric monoclonal anti-idiotype antibody of claim 16 wherein said cytokine is selected from the group consisting of interleukins and tumor necrosis factor.

18. A transfectoma which is identified as TVE1 and which is deposited at the American Type Culture Collection under ATCC accession number CRL 10867.

19. The transfectoma of claim 18 which produces a murine/human chimeric anti-idiotype monoclonal antibody comprised of

- complementarity determining regions comprising variable regions substantially derived from murine V_H and V_L regions derived from an anti-idiotype antibody raised against a human monoclonal antiganglioside antibody identified as L612;
- human constant regions fused to said murine V_H and V_L regions

wherein said murine/human chimeric monoclonal anti-idiotype antibody preferentially binds at least one antigenic determinant of said human monoclonal anti-ganglioside antibody identified as L612.

FIG. 1

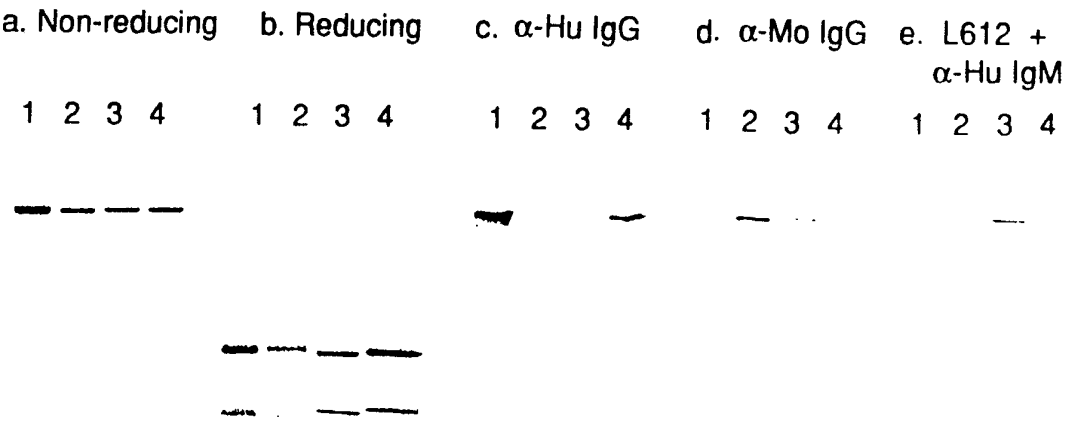

a. Non-reducing
b. Reducing
c. α-Hu IgG
d. α-Mo IgG
e. L612 + α-Hu IgM
1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4

FIG. 2b

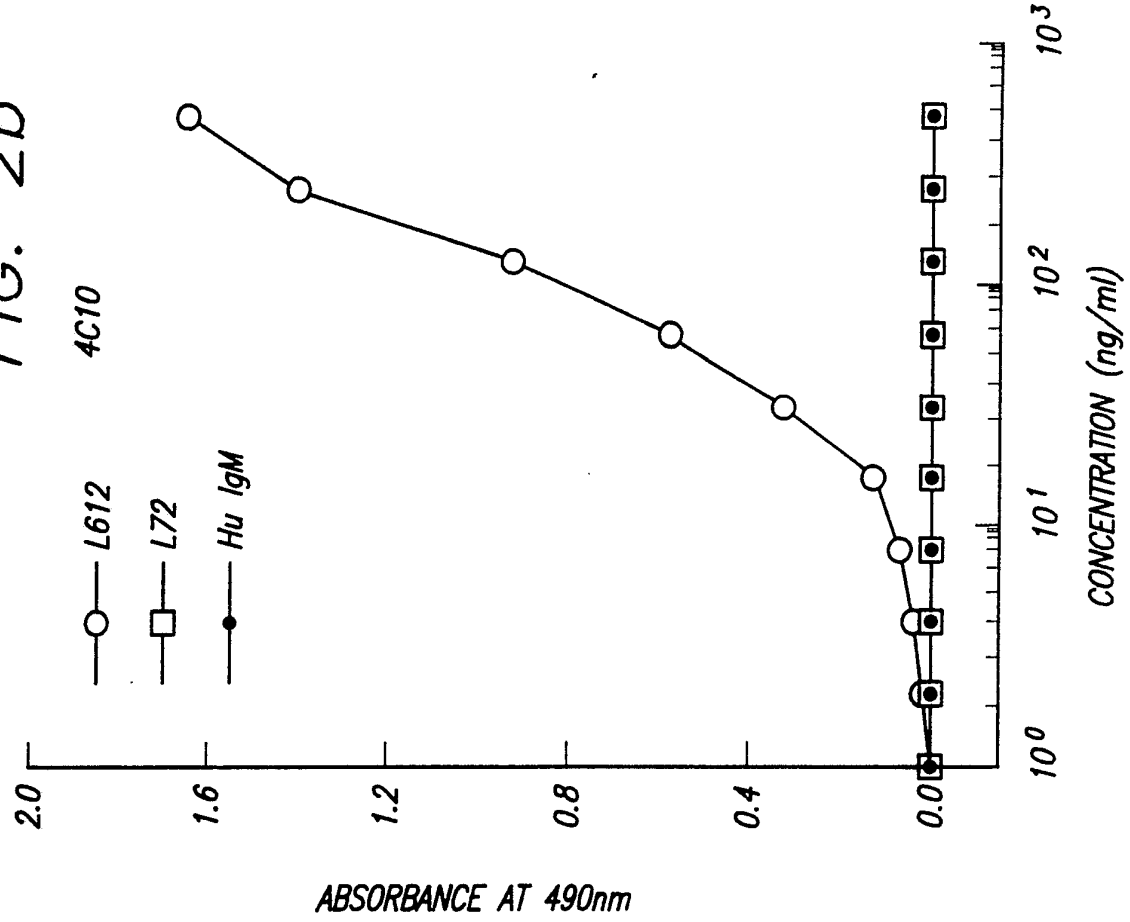

4C10
L612
L72
Hu IgM
2.0
1.6
1.2
0.8
0.4
0.0
10^0
10^1
10^2
10^3
CONCENTRATION (ng/ml)
ABSORBANCE AT 490nm

FIG. 2a

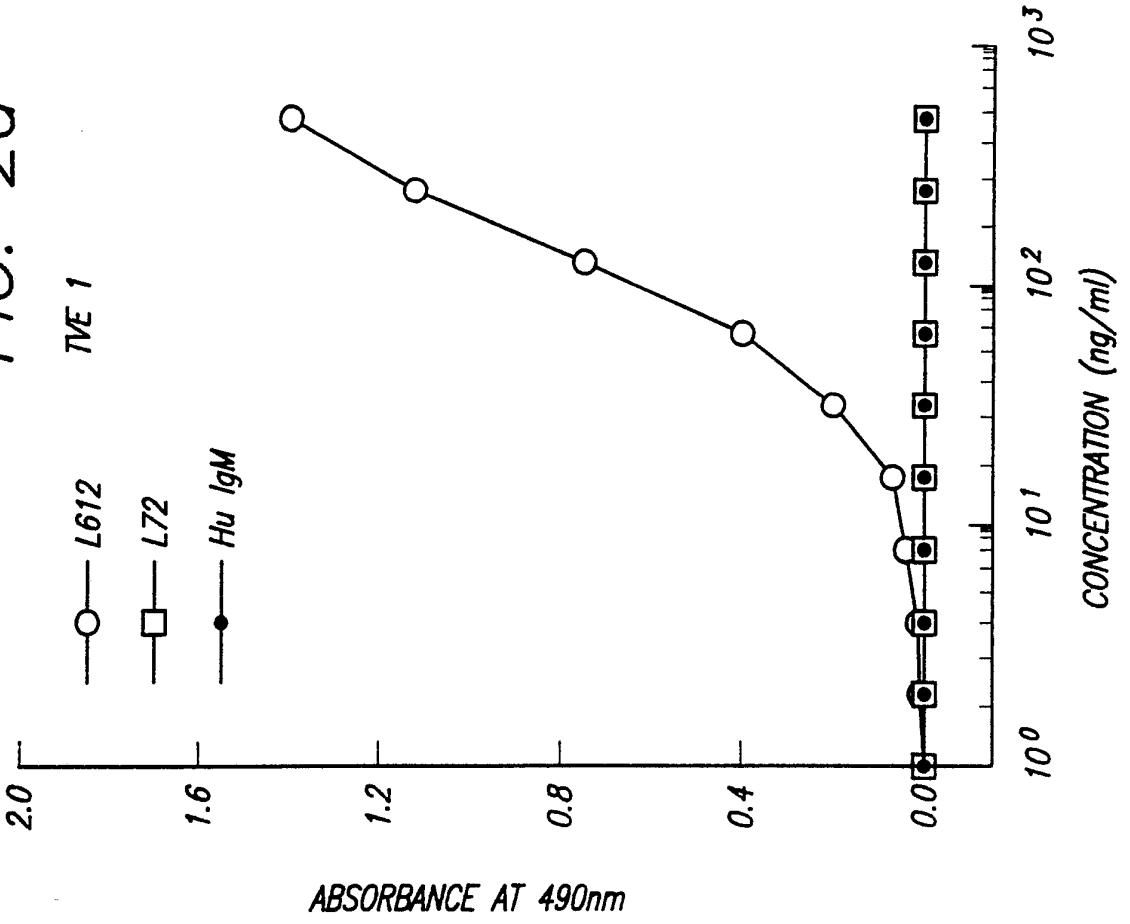

TVE 1
L612
L72
Hu IgM
2.0
1.6
1.2
0.8
0.4
0.0
10^0
10^1
10^2
10^3
CONCENTRATION (ng/ml)
ABSORBANCE AT 490nm

FIG. 3b

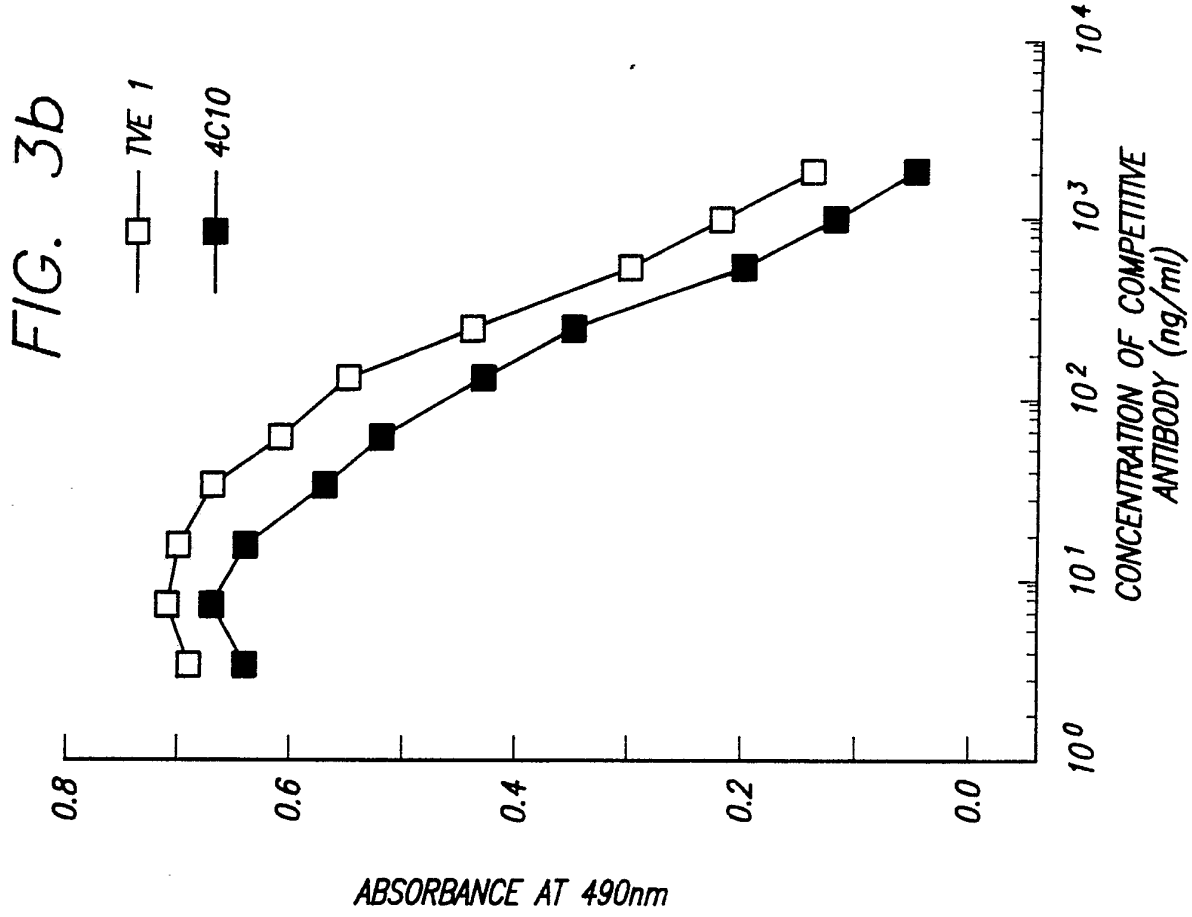
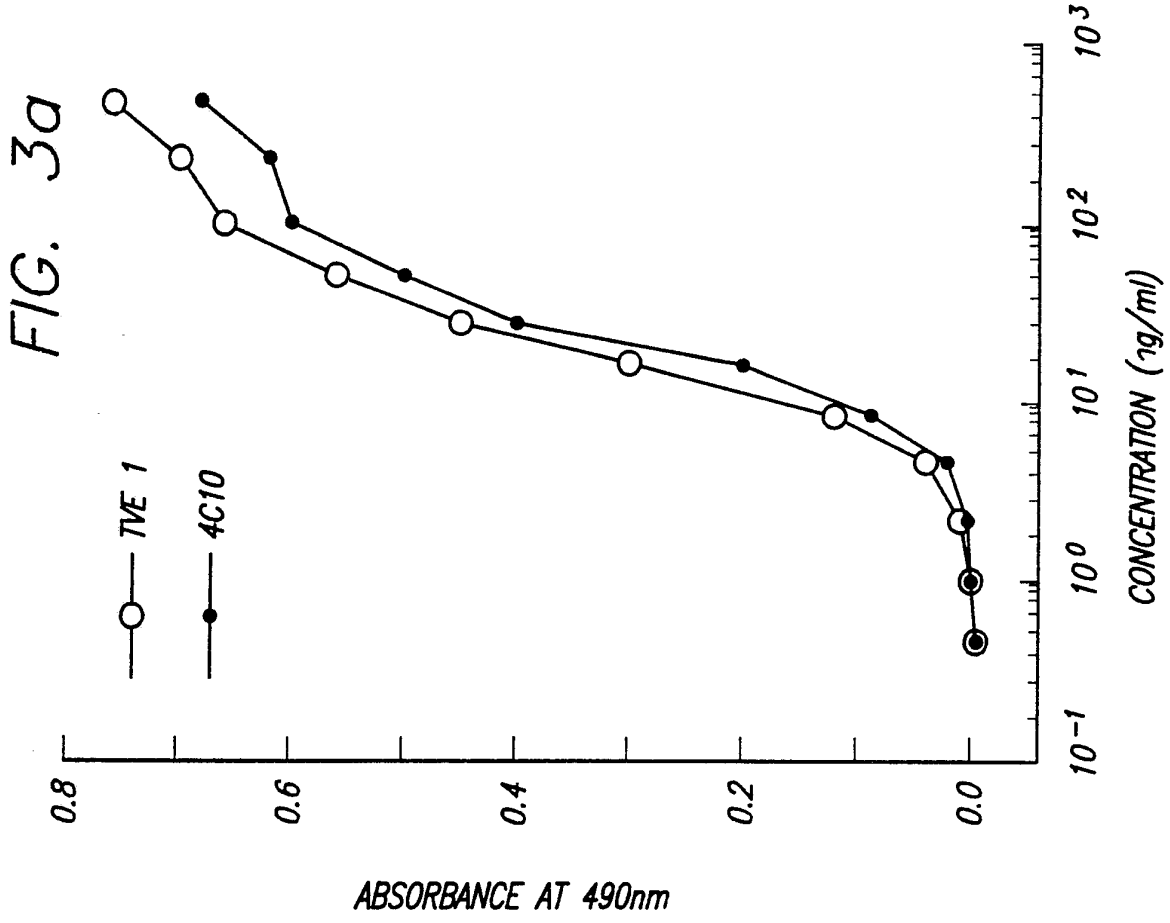

TVE 1
4C10
0.8
0.6
0.4
0.2
0.0
10^0
10^1
10^2
10^3
10^4
ABSORBANCE AT 490nm
CONCENTRATION OF COMPETITIVE ANTIBODY (ng/ml)

FIG. 3a


TVE 1
4C10
0.8
0.6
0.4
0.2
0.0
10^{-1}
10^0
10^1
10^2
10^3
ABSORBANCE AT 490nm
CONCENTRATION (ng/ml)

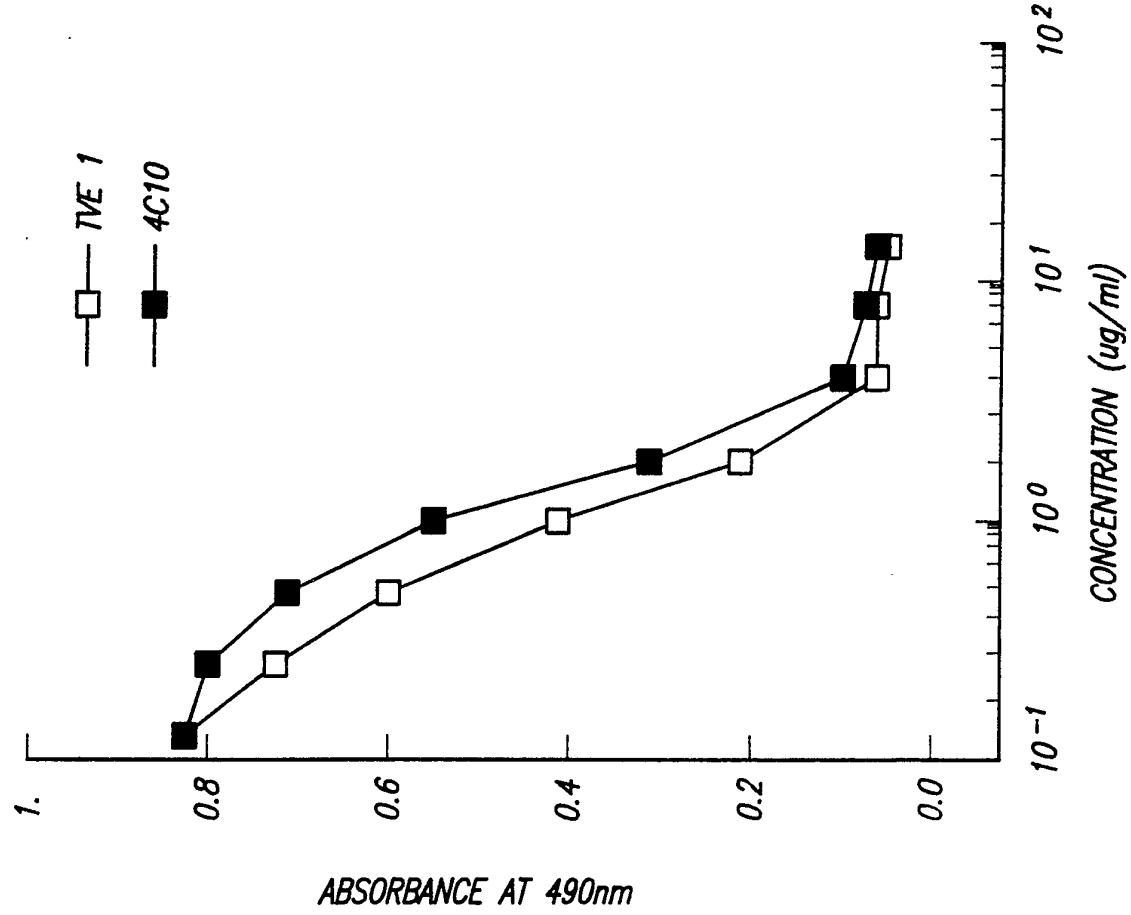

TVE 1
4C10
1.
0.8
0.6
0.4
0.2
0.0
ABSORBANCE AT 490nm
10^{-1}
10^{0}
10^{1}
10^{2}
CONCENTRATION (ug/ml)

FIG. 4

FIG. 5

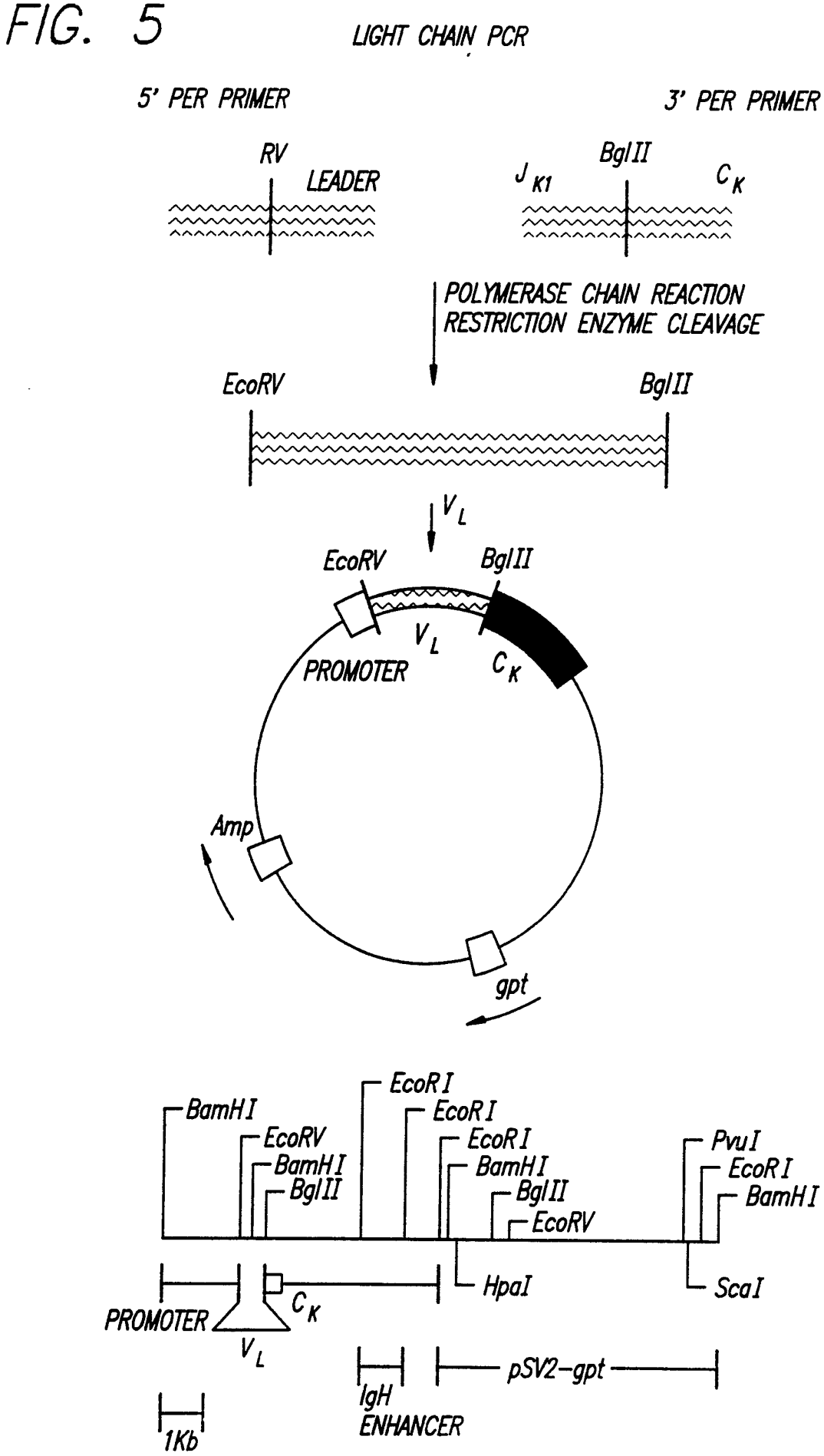
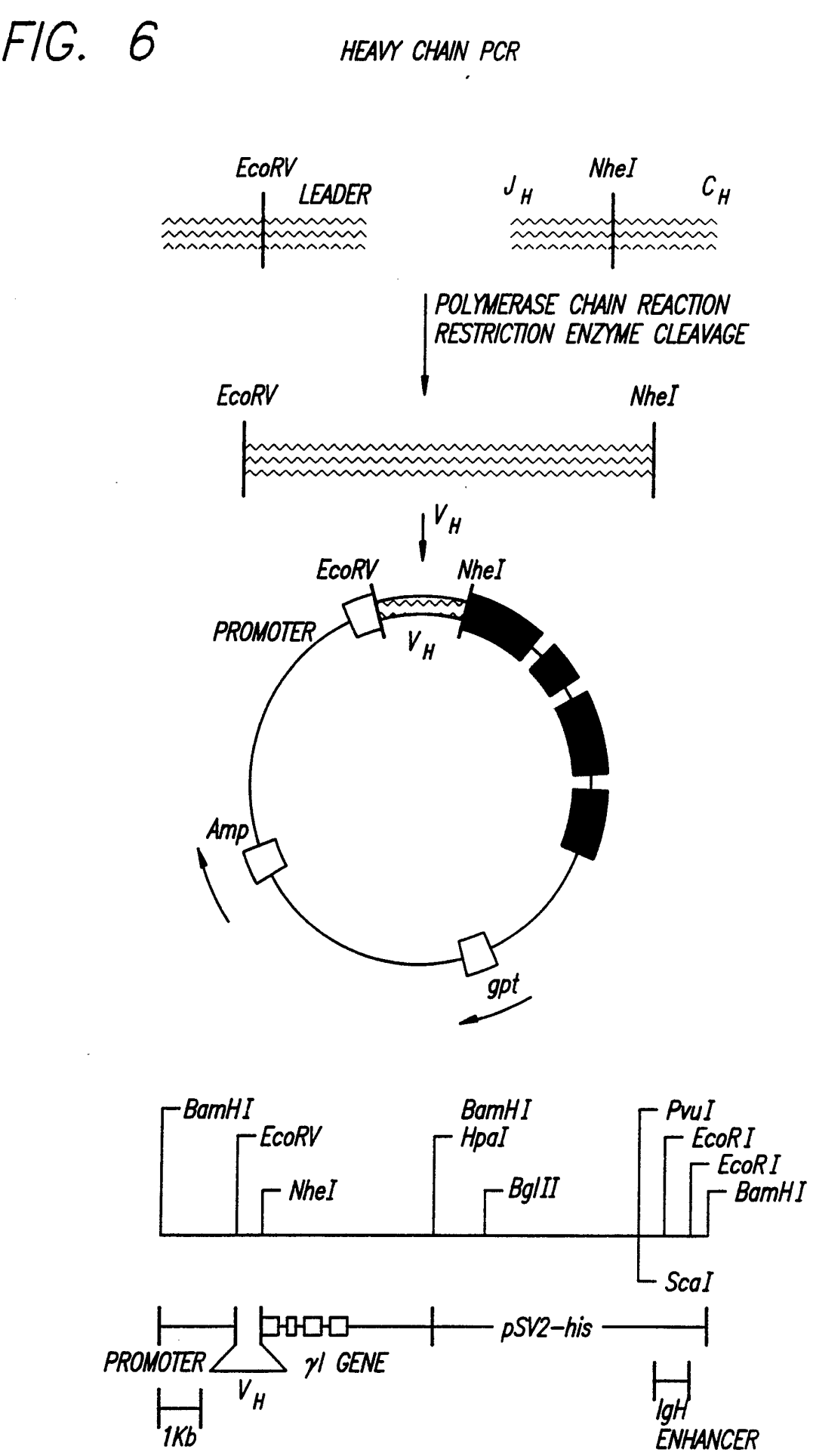

LIGHT CHAIN PCR
5' PER PRIMER
3' PER PRIMER
RV
LEADER
BglII
J K1
C K
POLYMERASE CHAIN REACTION
RESTRICTION ENZYME CLEAVAGE
EcoRV
BglII
V L
EcoRV
BglII
V L
PROMOTER
C K
Amp
gpt
BamHI
EcoRV
BamHI
BglII
EcoR I
EcoR I
EcoR I
BamHI
BglII
EcoRV
PvuI
EcoR I
BamHI
PROMOTER
C K
V L
HpaI
ScaI
pSV2-gpt
IgH
ENHANCER
1Kb

FIG. 6


HEAVY CHAIN PCR
EcoRV
LEADER
NheI
J H
C H
POLYMERASE CHAIN REACTION
RESTRICTION ENZYME CLEAVAGE
EcoRV
NheI
V H
EcoRV
NheI
PROMOTER
V H
Amp
gpt
BamHI
EcoRV
NheI
BamHI
HpaI
BglII
PvuI
EcoRI
EcoRI
BamHI
ScaI
PROMOTER
V H
γl GENE
pSV2-his
1Kb
IgH
ENHANCER

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/10166

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 5/12; C12P 21/02
US CL :530/387.2, 387.3, 387.5; 435/70.21, 240.27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.2, 387.3, 387.5; 435/70.21, 240.27

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

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

APS, Medline
search terms: Interleukin, tumor necrosis factor, growth factor, antibody

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Natl. Cancer Inst., Volume 82, Number 22, issued 21 November 1990, S. Yamamoto et al., "Anti-idiotype monoclonal antibody carring the internal image of ganglioside GM3", abstract.	1-19
Y	EP A, 0,239,400 (Winter) 30 September 1987, see entire document.	1-19
Y	Nucleic Acids Research, Volume 14, Number 8, issued 1986, Y. Furutani et al. "Complete nucleotide sequence of the gene for human interleukin I alpha", pages 3167-3179, see entire document.	1-19
Y	A.M. WU ET AL eds, "The Molecular Immunology of Complex Carbohydrates", published 1988 by Plenum Press (New York), see pages 437-464, entire document.	1-19
Y	Bona et al., "Monoclonal and Anti-Idiotypic Antibodies: Probes for Receptor Structure and Function", published 1984 by Alan R. Liss, Inc. (NY), see pages 141-149, entire document.	1-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
05 January 1993

Date of mailing of the international search report
02 FEB 1993

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/10166

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FEBS Letters, Volume 196, Number 1, issued February 1986, Y. L. Bouc, et al. "Complete characterization of the human IGF-I nucleotide sequence isolated from a newly constructed adult liver cDNA library", pages 108-112, see entire document.	1-19