Anti-Idiotype Monoclonal Antibody Carrying the Internal Image of Ganglioside GM3

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Murine anti-idiotype monoclonal antibodies were generated against a human IgM monoclonal antibody (L612) that recognizes ganglioside GM3 on human melanoma. Hybridomas secreting antibodies that bound specifically to L612 were selected by enzyme-linked immunosorbent assay using L612 and three negative control human IgMs, including monoclonal anti-GM2 and anti-GD2 antibodies, as well as purified serum IgM, as antigen sources. GM3-binding inhibition and cell-binding inhibition assays were used to identify seven anti-idiotype monoclonal antibodies that recognized determinants located within the antigen-combining sites of L612. To determine whether these anti-idiotype monoclonal antibodies possessed the internal image of the original antigen, we immunized syngeneic BALB/c mice with one of the anti-idiotype monoclonal antibodies, 4C10, coupled with keyhole limpet hemocyanin. Sera from the immunized mice reacted strongly with an antigen-positive M12 melanoma cell line and with purified GM3. Because L612 detects and kills melanoma tumor cells in vitro and in vivo in the presence of complement without affecting normal tissues, anti-idiotype monoclonal antibodies carrying the internal image of GM3 may be an effective tool for active specific immunotherapy in patients with melanoma. [J Natl Cancer Inst 82: 1757–1760, 1990]

Human malignant melanoma, a tumor of neural crest origin, synthesizes large amounts of several different ganglioside molecules (7) and has served as a useful model to assess the potential of ganglioside antigens as targets for passive, as well as active, specific immunotherapy. We have developed several human monoclonal antibodies to ganglioside antigens as targets for passive, as well as active, specific immunotherapy. We have developed several human monoclonal antibodies to ganglioside antigens that have strong cytotoxicity in vitro and in vivo (2). Recurrent cutaneous melanoma lesions have completely regressed following intralesional administration of these human monoclonal antibodies (3,4). The production of anti-ganglioside antibodies by purified gangliosides was associated with prolonged survival in patients with melanoma (5,6).

In the present study, we investigate the application of an anti-idiotype technology for the ganglioside-targeted immunotherapy of cancer. The use of anti-idiotype monoclonal antibodies for a ganglioside vaccine may have two advantages. First, the carbohydrate epitope of the ganglioside that is represented as a homologous peptide structure within the anti-idiotype monoclonal antibody may not only increase immunogenicity but also alter the nature of immunostimulation and regulation in patients with cancer, leading to activation of B cells producing IgG antibodies and induction of cytotoxic T cells. All the antiganglioside antibodies found in our patients with melanoma were IgM. IgG antibodies to these gangliosides were detected in patients only after hyperimmunization with tumor cell vaccine (7) or purified antigens (6). Cytotoxic T cells specifically targeted to gangliosides in patients with cancer have not been reported.

The second advantage of using anti-idiotype monoclonal antibodies involves the ability to produce them on a large scale. In most cases, tumor-associated gangliosides are present in nature only at low levels and are relatively difficult to isolate and purify in large amounts. The use of anti-idiotype monoclonal antibodies as surrogate ganglioside antigens is ideal because they are secreted from hybridoma cells in unlimited quantities. Furthermore, current genetic engineering technology, although not applicable to ganglioside epitopes, can be used to synthesize the anti-idiotype monoclonal antibody peptides. In the present study, we report the successful development of murine anti-idiotype monoclonal antibodies that have the ability to induce specific anti-ganglioside antibodies (Ab-3) in vivo.

Materials and Methods

Human Monoclonal Antibody

L612 human monoclonal antibody is an IgM-class antibody that has a strong binding affinity for GM3 on human melanoma; it was used as an immunogen to develop murine anti-idiotype monoclonal antibodies. The human B-cell line secreting L612 was established in culture from lymphocytes by the Epstein-Barr virus transformation technique described previously for our two other human monoclonal anti-ganglioside antibodies, L55 (anti-GM2) and L72 (anti-GD2) (8–10). Other human tumor biopsy specimens, including lung cancer, breast cancer, pancreatic cancer, colon cancer, and kidney cancer, also reacted to L612, although not as strongly as did melanomas. Normal tissue biopsy specimens tested included erythrocytes and lymphocytes from 40 different patients—nine skin, two kidney, three spleen, and three lung tissues. None were positive by absorption assays or immunohistochemical staining. The UCLASO-M12 melanoma cell line was identified as the most reactive cell line among the 24 lines tested and thus served as a target in the present study. The L612 cell line was initially grown in RPMI-1640 medium containing fetal bovine serum but was later adapted to a serum-free medium containing growth factor (SGF-6S in CEM 2000 from Scott Laboratories, Carson, Calif, or AIM medium from GIBCO Laboratories, Grand Island, NY). Human IgM in the spent medium was purified as previously described (II).
Gangliosides

GM2 and GD2 were isolated and purified from the M14 melanoma cell line (9). GM3 was obtained from two sources: the M12 melanoma cell line (12) and human tissues. The latter GM3 was prepared by K. G. Pallman, Munich, Federal Republic of Germany. CDH (asialo GM3) was purified from the neutral glycolipid fraction of the M12 cell line. [Abbreviations and nomenclature of gangliosides follow the system of Svennerholm (13) and IUPAC-IBU recommendations (14).]

Immunization Protocols and Hybridoma Production

BALB/c mice were immunized by subcutaneous (SC) injection of 100 μg of purified L612 emulsified in complete Freund's adjuvant. Animals were given a booster injection 14 days after the primary immunization and another 2 weeks thereafter with 100 μg of the same immunogen in incomplete Freund's adjuvant. The animals were killed 3 days after the last intraperitoneal injection with the same amount of antigen in saline, and their splenocytes were fused with nonsecreting SP 2/O murine plasmacytoma cells in the presence of polyethylene glycol 4000 by standard hybridoma procedures.

Selection and Screening of Anti-idiotype Monoclonal Antibodies

Culture supernatants from the hybridomas, mouse ascites, and mouse sera were screened by enzyme-linked immunosorbent assay (ELISA) for the presence of anti-idiotype monoclonal antibodies. Briefly, L612 human monoclonal antibody or control human IgM was diluted to 50 μg/mL in carbonate buffer at pH 8.6 and added to polyvinyl chloride flat-bottomed ELISA plates (50 μL/well) by overnight incubation at 4°C. ELISA was then carried out with each of the hybridoma culture supernatants using peroxidase-conjugated goat anti-mouse IgM + IgG (Boehringer Mannheim Biochemicals, Indianapolis, Ind) to detect anti-idiotype monoclonal antibodies.

Inhibition Assays

The ability of anti-idiotype monoclonal antibodies to bind the antigen-binding sites of L612 was determined by three different inhibition assays: the immune adherence inhibition, cell-ELISA inhibition, and GM3-ELISA inhibition. In these assays, the optimal dilution of L612 to M12 cells or to GM3 was determined first in the absence of anti-idiotype monoclonal antibodies. In the immune adherence inhibition assay, 100 μL of the mixture of L612 and murine anti-idiotype monoclonal antibody was incubated with 2.5 x 10⁴ M12 cells for 90 minutes at 37°C. The immune adherence assay was performed as previously described (8).

Melanoma cell-ELISA (12) inhibition assays were performed using 1 x 10⁴ M12 cells in each well of a 96-well U-bottomed culture plate after 24 hours of incubation. The cells were fixed with 0.25% glutaraldehyde in phosphate-buffered saline (PBS) for 10 minutes. L612 was mixed with anti-idiotype monoclonal antibody, and 100 μL of the mixture was added to the well; ELISA was performed using peroxidase-conjugated goat anti-human IgM antibody.

In the GM3-ELISA (12) inhibition assay, L612 mixed with anti-idiotype monoclonal antibody was incubated overnight at 4°C, and 200 μL of the mixture was transferred onto GM3-coated (4 μg/well) flat-bottomed ELISA plates containing 50 μL of 1% gelatin-PBS and incubated for 1 hour at 37°C. ELISA was performed with peroxidase-conjugated anti-human IgM antibody.

Induction of Antibodies Against Anti-idiotype Monoclonal Antibodies (Ab-3)

A murine anti-idiotype monoclonal antibody, 4C10, was produced in BALB/c ascites and purified using a protein A Sepharose CL4B column. Purified anti-idiotype monoclonal antibody was coupled to keyhole limpet hemocyanin KLH with glutaraldehyde (15). KLH-conjugated anti-idiotype monoclonal antibody (100 μg) was emulsified with Freund's complete adjuvant and injected SC into mice. Every 2 weeks the mice were given a booster SC of the same antigen dose in incomplete Freund's adjuvant. For serum antibody measurement, the mice were bled every 8 to 10 days after the last injection. Sera from mice immunized with normal mouse IgG-KLH were used as negative controls.

Results

We screened hybridoma culture supernatants for the presence of anti-idiotype monoclonal antibodies using ELISA by selecting for antibodies binding to L612 but not reactive with human IgM monoclonal anti-GM2 and anti-GD2 antibodies or normal human serum IgM. Forty clones secreting antibodies that showed differential reactivity to L612 were obtained. The amount of immunoglobulin in each supernatant ranged between 0.4 and 1.8 μg/mL. Antibody titers ranged between 1:4 (132A; 1.2 μg/mL) and 1:2,048 (4C10; 1.1 μg/mL). None of these supernatants had reactivity to L55 or L72 human monoclonal antibodies or human serum IgM.

To determine whether the antibodies selected as described above were directed against the paratope of L612, we performed cell-immune adherence inhibition, cell-ELISA inhibition, and GM3-ELISA inhibition assays. Of the
A-4C10, anti-4C10 antiserum; A-IgG, anti-IgG antiserum; 4+, 50% = 2+, and 0 if no adherence was observed. 

The adherence of one or more of these were also inhibitory in the immune adherence inhibition assay. A former two assays were found to inhibit antibody binding to GM3 in ELISA. The control human monoclonal antibodies, L55 (anti-GM2) and L72 (anti-GD2), had only weak reactivity to M12 cells. Therefore, in this experiment, we used GM2- and GD2-rich M14 melanoma cells (8-10). 4C10 had no inhibitory activity against L55 or L72 binding to M14 melanoma cells.

To determine whether the anti-idiotypic monoclonal antibodies identified above carried the internal image of the original antigen, we attempted to generate an anti-GM3 response by immunization of five syngeneic BALB/c mice with purified 4C10–KLH. As controls, four mice were immunized with mouse IgG1–KLH, and one mouse was immunized with KLH alone. The immunized sera were monitored by ELISA using purified GM3 as the antigen source and by the immune adherence assay using the antigen-positive M12 melanoma cell line. In the ELISA, peroxidase-conjugated goat anti-mouse IgG + IgM (Boehringer Mannheim) was used as a second antibody. Measurable antibody (Ab-3) was produced in three of the five mice after five immunizations with 100 μg 4C10–KLH. As shown in figure 3, the immunized sera bound to GM3 but not to CDH (asialo GM3). Sera from the five mice immunized with IgG–KLH or KLH alone gave no response to either glycolipid. In further analysis to determine the immunoglobulin class of the Ab-3 (ELISA and thin-layer chromatography immunostaining), the major class of the reactivity was identified as IgM. So that we could exclude the species-specific natural antibodies that might react to M12 cells in the immune adherence assay, the immunized murine sera were preabsorbed by human red blood cells at 4°C overnight. As shown in figure 4, an immune adherence inhibition score of 4+ was obtained at a 1:10 dilution of the absorbed sera. Control sera gave no reactivity, even at a 1:2 dilution. To confirm that the positive reactivity was directed against the GM3 antigen on the cell surface, we performed immune adherence inhibition using GM3 (10 μg), CDH (10 μg), 4C10 (10 μg), and unrelated IgG1 (10 μg) purified from BALB/c hybridoma ascites. Although reactivity was completely inhibited by GM3 or purified 4C10, no inhibition was obtained with CDH or unrelated IgG1.

Discussion

Forty murine monoclonal antibodies were generated against human monoclonal antibody L612, which specifies an epitope of the ganglioside GM3 on human melanoma. Using three inhibition assays, seven of these anti-idiotypic monoclonal antibodies were selected as those bound to idiotopes located within or near the antigen-combining site of L612. Subsequent immunization with one of these anti-idiotypic monoclonal antibodies (4C10) in syngeneic mice led to the induction of IgM Ab-3 with specificity for GM3. The results demonstrated that 4C10 is an anti-idiotypic monoclonal antibody beta type bearing an internal image of the original antigen. A delayed type hypersensitivity (DTH) test to determine whether mice immunized with 4C10 induced cellular responses was not performed, based on our assumption that gangliosides are unable to induce delayed hypersensitivity. In our ongoing tumor cell vaccination studies, none of the melanoma patient's skin sensitized with ganglioside, including GM3, ever displayed DTH.

Although GM3 is a major ganglioside distributed on many normal tissues, and L612 reacts with GM3 isolated from either normal tissues or cancer tissues, the clinical potential of these anti-idiotypic monoclonal antibodies for cancer treatment was indicated in our preliminary studies. First, immunohistochemical studies with L612 showed a distinct binding pattern of L612 to malignant tissues but not to adjacent normal tis-
Another important feature of L612 is its strong antitumor effect on melanomas in patients. In our ongoing clinical trials to assess the therapeutic value of human monoclonal antibodies, patients with recurrent cutaneous melanoma received intraslesional L612. Tumors expressing antigens for L612 were specifically killed without damage to adjacent noncancerous tissues (data not shown). These results provide evidence that GM3 epitopes detected by L612 are expressed in a unique configuration on human tumors.

Anti-idiotype monoclonal antibodies previously developed for active specific immunotherapy of human cancer have been developed using murine monoclonal Ab-1s as the immunogens (17-21). However, human tumor-associated antigens that activate the murine immune system to produce Ab-1 do not necessarily elicit the same Ab-1 response in humans. The Ab-3 in patients due to Ab-2 immunization may not be identical to Ab-1. In this study, we circumvented these problems by generating anti-anti-idiotype monoclonal antibodies against a human monoclonal antibody that defines an antigen epitope that elicits immune responses in cancer patients. The immunogenicity of GM3 in humans is evidenced by the fact that human monoclonal antibody is secreted from human B cells and that the autoantibody GM3 antibody is present in human sera. The ability of an Ab-2 carrying the internal image of ganglioside antigens to produce Ab-3 was suggested in our previous clinical study. A patient with recurrent cutaneous melanoma who received an intraslesional injection of human monoclonal antibody L72 (IgM anti-GD2 antibody) developed a high level of circulating IgG anti-GD2 (Ab-3) 81 days after the first Ab-1 administration (4). The patient's tumor regressed completely, and the subject has been free of disease for more than 3 years since antibody administration was begun.

Human monoclonal antibody L612 reacts strongly with 100% of melanoma tissues (16). Because the prevalence of this antigen is so high in these tumors, stimulation of the idiotypic cascade in cancer patients may provide protection against occult micrometastasis and possibly the tumor itself.

References

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