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Inhibition of Mannosidase in Hybridomas Yields Monoclonal Antibodies with Greater Capacity for Carbohydrate Labeling

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Labeling an antibody site specifically through its carbohydrate residues preserves more of its antigen-binding activity than does labeling through protein moieties. To boost the amount of immunoglobulin G carbohydrate capable of being labeled, we treated hybridoma cells with a mannosidase inhibitor, deoxymannojirimycin (dMM). Polyacrylamide gel electrophoresis showed formation of a glycoprotein with high mannose content, in that endo-β-*N*-acetylglucosaminidase H (EC 3.2.1.96) could digest the antibody from the dMM-treated cells, but not from control cultures. Carbohydrate analysis confirmed this conclusion, indicating that the antibody from the dMM-treated cells had twice as much mannose as did the control antibody. The glucosamine content of the treated-cells' antibodies was half that of the control, and no additional carbohydrate residues were detectable in the antibodies secreted by the dMM-treated cells. We conjugated both the dMM and control antibodies through their carbohydrate to a chelator. In labeling, the dMM antibody conjugate incorporated approximately threefold as much ^{111}In isotope as the control conjugate. The two labeled antibodies were injected into mice and showed similar organ distributions.

Additional Keyphrases: *deoxymannojirimycin · endo-(β-N-acetylglucosaminidase H · polyacrylamide gel electrophoresis · radioassay · tunicamycin*

With the advent of monoclonal antibodies (*1*), a great deal of research has been done to adapt these reagents to target cancer cells. Radioisotopes, anti-cancer drugs, and toxins have been coupled to monoclonal antibodies in the hope of using these antibody conjugates in cancer therapy (*2–4*). Most conventional methods involve random attachment of the agent to the antibody molecule. The activity of the antibody may be diminished if label is attached in the antigen-binding site (*5*).

Coupling an agent specifically through an antibody's carbohydrate region preserves the antibody's activity, given the lack of carbohydrate in the antigen-binding site (*5*). However, this type of coupling, through periodate oxidation and reductive amination of the carbohydrate residues, is limited by the amount of label that can be incorporated, immunoglobulin G (IgG) being only 2–3% carbohydrate (*6*).

Yet, cells that synthesize glycoproteins such as immunoglobulins start with a precursor containing more carbohydrate than the final product (for reviews, see *7, 8*). Before the antibody is secreted from the cell the asparagine-linked oligosaccharides are enzymatically trimmed. An intermediate in this process is a high-mannose form containing nine mannose and two glucosamine residues. This form of the carbohydrate has more available sites (vicinal hydroxyl groups) for periodate oxidation and therefore should couple with more labeling groups.

1-Deoxymannojirimycin (1,5-dideoxy-1,5-amino-D-mannitol; dMM), an inhibitor of mannosidase (EC 3.2.1.24) (*7*), blocks the conversion of the high-mannose to the complex oligosaccharide form.[2] Here we present the use of monoclonal antibodies from dMM-treated hybridomas as reagents that can be labeled to a higher specific activity by carbohydrate-directed methods than can normal antibodies.

Materials and Methods

Cell lines. The monoclonal antibody used in these studies, B72.3 (*9*), was produced by hybridoma cells of the same name obtained from the American Type Culture Collection (ATCC), Rockville, MD. This murine IgG1 antibody reacts with the antigen TAG-72, found on many human cancer cells (*10*). In testing its reactivity by enzyme-linked immunosorbent assay (ELISA), we used the human adenocarcinoma line LS174T (ATCC) (*11*), which expresses TAG-72 (*12*).

Endo-β-N-acetylglucosaminidase H (Endo H) treatment of normal and dMM-treated antibodies (13). We incubated B72.3 hybridoma cells (6×10^6 cells/mL) in CO_2-enriched atmosphere (50 mL/L) at 37 °C with 1 or 2 mmol of dMM (Boehringer Mannheim, Indianapolis, IN) or 4 mg of tunicamycin (TM; Sigma Chemical Co., St. Louis, MO) per liter in methionine-free minimal essential media, containing 50 mL of fetal-bovine serum and 50 g of L-glutamine (Gibco, Grand Island, NY) per liter. After a 1-h pre-incubation with inhibitor, we added [^{35}S]methionine (New England Nuclear, Boston, MA), 50 mCi/L, and incubated the cells for an additional hour. Supernates of the cell cultures were incubated with Endo-H (EC 3.2.1.96) (Boehringer Mannheim), 20 U/L, then were immunoprecipitated with *Staphylococcus aureus* coated with rabbit anti-mouse IgG (Litton Bionetics, Charleston, SC). The immunoprecipitated samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), with use of standard techniques (*14*). Autoradiograms of the dried gels was made by overlaying them with Kodak XAR-5 film (Eastman Kodak, Rochester, NY) in a cassette for three days at −70 °C.

Treatment with dMM and large-scale purification. To perform activity assays, carbohydrate analyses, and in vitro and in vivo binding studies, we needed milligram quantities of B72.3 antibody from both control and dMM-treated cells. We incubated about 5×10^6 B72.3 cells per milliliter for 24 h at 37 °C in Dulbecco's media containing dMM, 1 mmol/L; NCTC-109 media, 50 mL/L; L-glutamine, 50 g/L; and gamma-globulin-free fetal bovine serum (Gibco), 50 mL/L. A control culture was set up under the same conditions but

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[2] Nonstandard abbreviations: dMM, 1-deoxymannojirimycin; ATCC, American Type Culture Collection; ELISA, enzyme-linked immunosorbent assay; endo-H, endo-β-*N*-acetylglucosaminidase H; TM, tunicamycin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, pH 6; GYK-DTPA, glycyl-tyrosyl-lysyl-diethylenetriamine pentaacetic acid.

without inhibitor. The cells in the two cultures looked morphologically similar and grew at similar rates. We collected supernates from each culture by centrifugation at 500 × *g* for 10 min. Both supernates were concentrated to about 50 mL by ultrafiltration through a YM-30 membrane (Amicon. Danvers, MA). We purified the antibody from these concentrated supernates by passage through a 5-mL column of Protein-A–agarose (Bio-Rad, Richmond, CA), equilibrated with 25 mL of "binding buffer" (Bio-Rad), a proprietary formulation containing a high salt concentration and having a pH of 9.0. The culture fluid samples were diluted twofold with binding buffer and applied to the Protein A column.

The column was washed with 75 mL of binding buffer and the IgG eluted with citrate buffer (0.1 mol/L, pH 5.6). An additional wash with citrate buffer (0.1 mol/L, pH 3.0) did not elute any more material. The purified antibody samples were dialyzed against phosphate-buffered saline (PBS: 150 mmol of NaCl and 10 mmol of phosphate per liter, pH 6.0). The yields of antibody from the two cultures were similar.

ELISA of B72.3 activity. The activities of the B72.3 antibody from normal and dMM-treated cells were compared by using a standard ELISA. For this assay we plated LS174T cells, 1×10^5 cells/well, onto a 96-well microtiter plate. The cells were fixed with formaldehyde and the ELISA was performed by standard techniques (*15*) with a horseradish peroxidase-labeled goat anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD).

Carbohydrate analysis. Dr. Fulvio Perini of the University of Michigan Medical School performed these analyses for us. The hydrolysis conditions for each type of analysis were as follows: amino sugars were treated with 6 mol/L hydrochloric acid at 100 °C for 3 h; neutral sugars with 4 mol/L trifluoroacetic acid at 100 °C, 2 h; and sialic acid with 20 mmol/L sulfuric acid at 80 °C, 1 h. Neutral sugars were converted to amino forms by reductive amination before analysis. Analysis was by cation-exchange HPLC, with an *o*-phthaldehyde reactor and a fluorescence detector.

Preparation of B72.3-GYK-DTPA conjugates. Purified samples of B72.3 antibody from both normal and dMM-treated cells were adjusted to a concentration of 1 g/L in PBS. This carbohydrate was oxidized to aldehyde groups by incubation with sodium meta-periodate (Sigma), 10 mmol/L, on ice, for 1 h. Free periodate was removed by passing the antibody samples through a column of Sephadex G-50 (Pharmacia, Piscataway, NJ) equilibrated with PBS. The linker, chelator glycyl-tyrosyl-lysyl-diethylenetriamine pentaacetic acid (GYK-DTPA) (*5*), was dissolved in PBS and adjusted to pH 6.0 with 5 mol/L NaOH solution. The oxidized antibody was incubated with a 2000-fold molar excess of GYK-DTPA in the presence of sodium cyanoborohydride (Sigma), 10 mmol/L. The solution was incubated overnight in the dark at room temperature. The free GYK-DTPA was removed by chromatography on a 1 × 30 cm column of Superose-12 (Pharmacia).

Labeling of the B72.3–GYK-DTPA conjugates with ^{111}In. We mixed 1 mCi of $^{111}InCl_3$ (Amersham, Arlington Heights, IL) with an equal volume of sodium acetate (0.5 mol/L, pH 6.0), and added this to 100 μg of the B72.3–GYK-DTPA conjugate in a final volume of 500 μL. The samples were incubated at 37 °C for 1 h and then passed through 0.5 g of Chelex-100 (Bio-Rad). To remove free ^{111}In from the conjugate, we chromatographed the samples on a 7.8 × 300 mm column of TSK-G-3000 SW (Phenomenex, Rancho Palos Verdes, CA) in PBS.

In vivo study of ^{111}In-labeled antibodies. A biodistribution study was performed comparing monoclonal B72.3 antibodies from normal and dMM-treated hybridoma cells. Approximately 10^6 LS174T tumor cells were injected subcutaneously into the flank of athymic female nude mice (Taconic Farms, Germantown, NY). After allowing the tumors to develop for two weeks, we injected the mice with 10 μg of the ^{111}In-labeled conjugates. As a control we also injected conjugates into nontumor-bearing mice. Five days after antibody injection, the mice were killed, the organs of interest were weighed, and their radioactivity was counted in a gamma counter.

Results

Figure 1 is the autoradiogram of the SDS-PAGE gel, showing the effect of Endo-H enzyme on B72.3 antibody from normal and dMM-treated hybridomas. This enzyme cleaves all but the first N-linked acetylglucosamine residue of high-mannose oligosaccharides, but does not act on complex oligosaccharides (*16*). The Endo-H-cleaved antibody will have heavy chains of a lower molecular mass, because of the loss of almost all of the carbohydrate. The autoradiogram showing the heavy chains clearly indicates that the dMM-treated material did yield a heavy chain of lower molecular mass after Endo-H treatment (*cf.* lanes 1 and 2 with 3, and lanes 4 and 5 with 6). The effect was seen with dMM concentrations of both 1 mmol/L (lanes 1–3) and 2 mmol/L (lanes 4–6), indicating that the 1 mmol/L concentration was sufficient. As a reference, a sample of B72.3 from cells treated with TM was also run on the SDS gel (lane 7). This inhibitor (*7*) blocks glycosylation of the protein, resulting in a heavy chain that should have about the same molecular mass as that of Endo-H-treated high-mannose antibody (*17*). This was the case in our study. Lane 8 contains Endo-H-treated antibody from control cultures (without dMM). On a separate gel this control antibody, with or without Endo-H treatment, migrated to the same position (data not shown).

Because the results from the Endo-H assay suggested that the dMM-treated cells were secreting antibody in a high-mannose form, we scaled up production of cell supernate to give enough antibody for direct measurement of the carbohydrate content. Figure 2 shows the results of carbohydrate

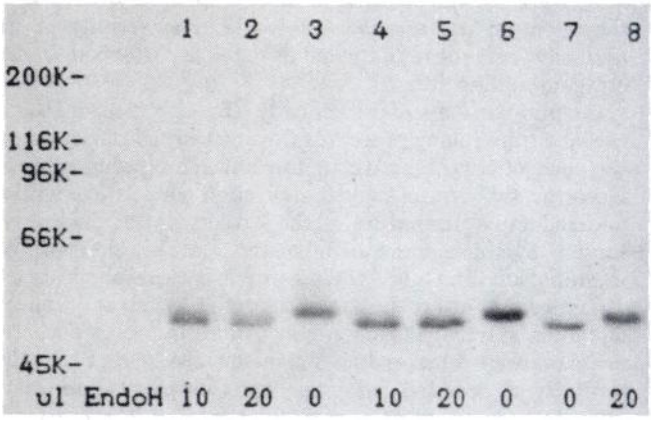

Fig. 1. Autoradiograms of reduced SDS-PAGE showing effect of 0–20 μL of Endo-H (1 U/μL) on antibody from B72.3 hybridoma cells treated with (from *left* to *right*): deoxymannojirimycin, 1 mmol/L (lanes *1–3*) or 2 mmol/L (lanes *4–6*); or tunicamycin, 0.40 μg/mL (lane *7*); or no inhibitor (lane *8*)

Only the heavy chains are depicted; the light chains were run off the gel to better display the differences between the treated vs control antibody. Molecular masses (k = kilo-Daltons) are shown at *left*

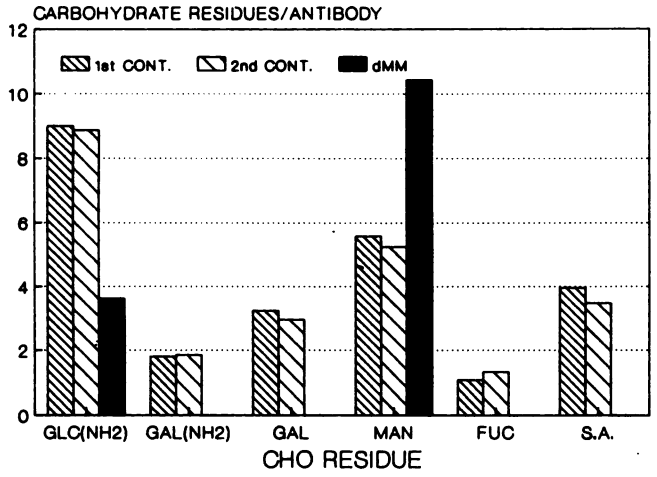

Fig. 2. Carbohydrate content analysis of B72.3 from control and dMM-treated cells: *GLC(NH_2)*, glucosamine; *GAL(NH_2)*, galactosamine; *GAL*, galactose; *MAN*, mannose; *FUC*, fucose; *S.A.*, sialic acid

The *first control* is a scale-up sample of B72.3. The *second control* is from a culture supernate. Trace (<0.8) amounts of *GAL(NH_2)* and *S.A.* were found in the antibody from the dMM-treated cells

analyses of antibody from dMM-treated and control cultures. As expected, the mannose content of the antibody from the treated cultures was much higher than that of the controls. Furthermore, the content of *N*-acetylglucosamine was reduced by half and other carbohydrate residues were absent.

Figure 3 shows the results of an ELISA comparing the immunoreactivity of B72.3 antibodies from control and dMM-treated cells. There appears to be no difference in their antigen-binding activities.

The ^{111}In labeling of the B72.3–GYK-DTPA samples from both control and dMM-treated cells is shown in Figure 4. In two separate experiments, the specific activity of the antibody from dMM-treated cells averaged 3.5 ± 0.2 times higher than that of the control.

The biodistribution of the ^{111}In-labeled antibodies, expressed as percent injected dose per organ, is presented in Figure 5. The distributions of the dMM and control antibodies were identical both in mice bearing LS174T tumors and in control mice not bearing tumors. Approximately 30% of the injected dose of both control and dMM-treated B72.3 accumulated in the tumor. The organ/blood ratio for the LS174T tumor was 11.5:1 for both antibodies (data not shown).

Discussion

Labeling of a monoclonal antibody at its carbohydrate is known as a method that minimizes interference with the antigen-binding sites (*5*). Conventional random attachment of agents risks inhibiting the antigen-binding site, which contains amino-acid residues reactive with the labeling reagent. The need to limit the number of such labels added to the antibody, to preserve immunoreactivity, has been well documented (*17*). Carbohydrate-labeling avoids this problem, but when very high labeling is desired, this method is limited by the fact that IgG is only 2–3% carbohydrate (*6*). Here we describe a biosynthetic method to increase the amount of labelable carbohydrate on a monoclonal antibody.

Other workers (*13, 18, 19*) interested in the biosynthesis and role of the oligosaccharides on IgG used various inhibitors of the enzymes that trim the larger oligosaccharides first attached to the protein. We used one of these inhibitors,

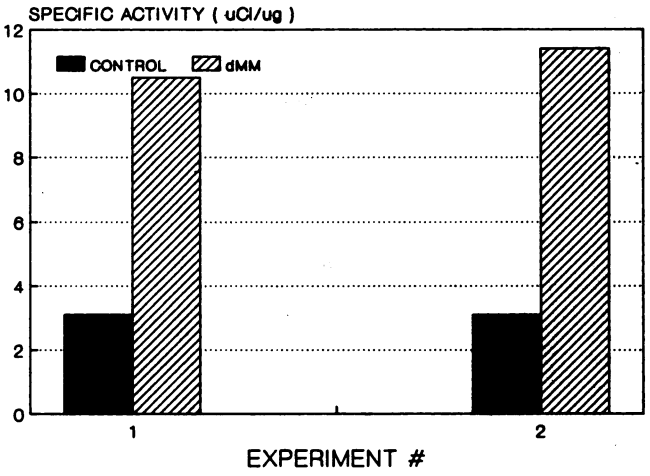

Fig. 4. ^{111}In-labeling of B72.3–GYK-DTPA from both control and dMM-treated cells (results of two separate experiments)

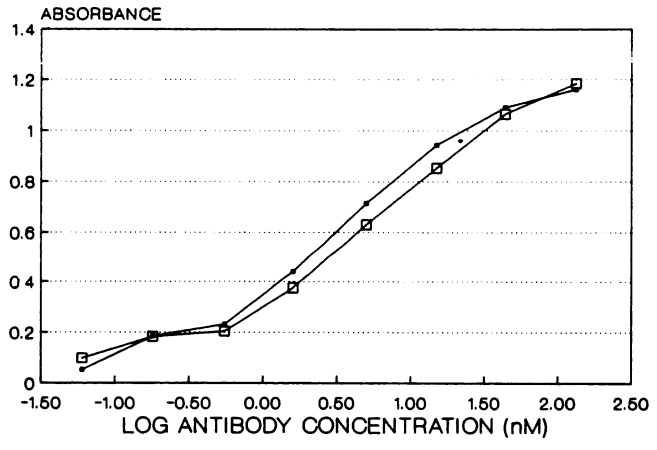

Fig. 3. ELISA measure of B72.3 activity on LS174T tumor cells, comparing control (■—■) and dMM-treated (□—□) antibody

Means of triplicate determinations are plotted. The standard deviation averaged 7.5% in the linear range of the graph (0.56 to 42.7 nmol/L, −0.25 to 1.63 on log plot)

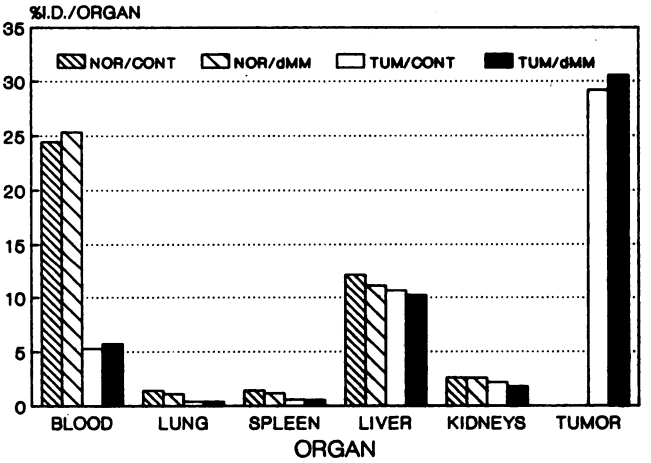

Fig. 5. Biodistribution of ^{111}In-labeled B72.3 from control (*CONT*) and dMM-treated cells in both normal (*NOR*), and LS174T tumor-bearing (*TUM*) athymic nude mice

All values are expressed as percent of injected dose (i.d.) per organ

dMM, on a murine hybridoma line that secretes an IgG1 that binds selectively to an antigen found in many cancer cells (*9*). This inhibitor of mannosidase effectively induced the cells to secrete antibody that was rich in mannose. The use of dMM reduced neither cell viability nor yield of antibody.

Several lines of evidence support the conclusion that the cells treated with dMM produced antibody in a high-mannose form. First, the antibody was susceptible to cleavage by the Endo-H enzyme, whereas the antibody from the control culture was not. Secondly, the carbohydrate analysis on the antibody from the dMM-treated cells showed that only glucosamine and mannose were present, as would be expected from a high-mannose form of glycoprotein. Finally, labeling the antibody with a carbohydrate-directed label showed that approximately three times as much label was incorporated into the antibody from the dMM-treated cells as from the control cells.

In spite of these changes in carbohydrate content, the high-mannose antibody showed no difference in its binding to tumor cells, either in vitro or in vivo. This supports the notion that modifications in carbohydrate regions of an antibody do not affect its binding to antigen (*5*).

The value of having antibody labeled to a higher specific activity is that more of the label can be delivered to the target on the same amount of antibody. This would enhance the efficiency of detection and treatment of tumor cells, especially for those tumors that express small amounts of antigen. Furthermore, if less antibody conjugate could be used in treating human patients, the intensity of side effects, e.g., the human anti-mouse IgG response (*20, 21*), could be reduced.

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