Establishment of Monoclonal Antibodies Against Cancer associated Antigens and their Application to Cancer Diagnosis

癌関連抗原に対するモノクローナル抗体の作成と癌診断への応用

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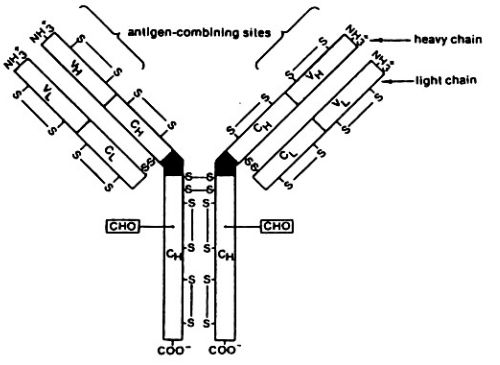

Abstract:

Novel monoclonal antibodies against cancer-associatied antigens were successfully prepared and characterized in order to study their usefulness for cancer diagnosis.

One was anti-galactosyltransfrase isoenezym II (GT-II) mouse monoclonal antibody designated MAb 3872IgG1, which was proved to be non-crossreactive to normal galactosyltransferase (GT-I) by western blotting transfered from native PAGE. A sensitive immunoassay was developed to measure the concentration of GT-II in human serum. Serum GT-II levels among normal controls, patients with various tumors, and patients benign diseases were compared. The mean values for normal were 110±50mU/ml and 2% were positive over 200mU/ml. GT-II levels in patients were elevated significantly in all tumor types: bladder (58%), cervical (59%), colorectal (46%), esophagus (64%), lung (50%), ovarian (50%), pancrea (77%), prostate (65%), and stomach (53%). These levels were distinguished from matched benign diseases. The other monoclonal antibodies were against the Hanganutziu Deicher (HD) antigen. NZB mice were immunized with highly purified gangliosides containing N-glycolyl neuraminic acid (NeuGc) on the non-reduced terminal. Seven hybridoma clones were isolated and examined about their epitopes on recognized glycosphingolipids.

Each monoclonal antibody showed different specificity on enzyme-linked immunosolvent assay (ELISA), and thin layer chromatography (TLC) enzyme-immunostaining. All monoclonal antibodies were reactive to GM2 (NeuGc) and mouse erythrocytes. This result suggests that GM2 (NeuGc) might be the anti-erythrocyte autoantigen in autoimmune hemolytic anenia of NZB mice.

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1 Introduction

Atempts have been made for many years to utilize tumor markers as an aid in cancer diagnosis. However, no tumor markers have sufficient potential in both sensitivity and specificity in cancer screening in early stages.

Moreover, tumor markers are still a subject in cancer research not only in their use in diagnosis, but in their basic cancer characteristics. Recent interest in tumor markers (cancer-associated antigens) has focused on aberrent glycosylation of glycoproteins and glycolipids. The carbohydrate structure in glycoprotein and glycolipid on the cell surface might be highly related to cell-cell recognition and regulation. Some unique carbohydrate structures on cancer cells (not seen on normal cells) have been found and characterized. The Hanganutziu Deicher (HD) antigen has been detected in some human tumor tissues, and its antigenic structure was determined to be N-glycolyl neuraminic acid (NeuGc). NeuGc is widely distributed among vetebrates but is hardly detectable in human and chicken. The mechanism of these aberrent carbohydrate structures in cancer is supposed not to be directly coded on oncogenes, but to be correlated to glycosyltransferases. Galactosyltransferase isoenzyme II(GT-II) has been reported as a tumor marker, but its role in aberrent glycosylation is still unclear. On the other hand, monoclonal antibodies have become a significant tool in the above areas. In fact, monoclonal antibodies against cell surface antigens could make clear the difference of cell subtypes which polyclonal antibodies have failed to do. We present here the preparation and the characterization of monoclonal antibodies against GT-II and gangliosides containing NeuGc, along with their usefulness in serological cancer diagnosis.

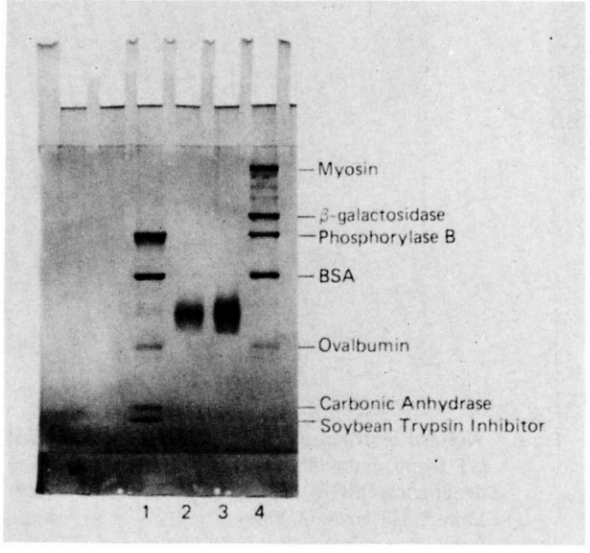


Fig.1
SDS-PAGE: Lane 1. Mr. marker
Lane 2. Purified GT from normal plazma
Lane 3. Purified GT from tumor effusion fluids
Lane 4. Mr. marker

2 Experiment and Result

1. Monoclonal antibodies against GT-II

1.1 Purification and characterization of GT-II.

A rapid three step purification scheme was developed to isolate highly purified GT. Representative purification of GT from human ovarian effusion fluid is summerized (Table-1). The SDS-PAGE of purified GT isolated from either tumor effusion fluid or normal plazma showed a single broad protein band (silver staining) that migrated with a molecular weiht centered at 48kd (Fig-1). No additional bands corresponding to IgG or high molecular weight GT were observed, thus suggesting the absence of contaminating protein in the final GT preparation. The non-denaturing PAGE of purified GT from normal human serum showed one major protein band corresponding to GT-I.

GT prepared from tumor effusion fluid showed two bands, with the GT-II band being a major protein band (Fig-2). In addition, several discrete slower migrationg bands were consistently observed in preparation of tumor GT. Elution of GT from parallel, non-denaturing PAGE and assay for GT activity showed that GT was associated with all of the silver staining bands (Fig-3).

Table-1

GT Purification
From ovarian and renal carcinoma effusion fluid.

	Vol. (mL)	Protein (mg)	GT Sp.*1 (U/mg)	Purification (factor)	Yield (%)
1.Starting material	3000	195,000	3.5	1	100
2.1st Lact Seph*2	300	156	4300	1230	90
3.2nd Lact Seph	240	2.4	380,000	110,000	78
4.Anti-IgG Sepharose	300	1.5	N/D*3	N/D	N/D
5.Concentrated	1.6	1.2	N/D	N/D	N/D

*1 GT specific activity Unit; nMol/hr
*2 α-lactalbumin sepharose
*3 not determined

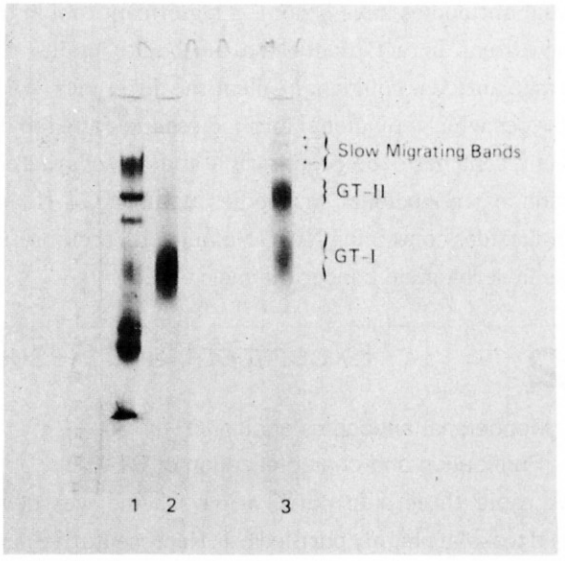


Fig.2 Non denaturing PAGE:
Lane 1. Mr. marker
Lane 2. Purified GT from normal plazma
Lane 3. Purified GT from tumor effusion fluids

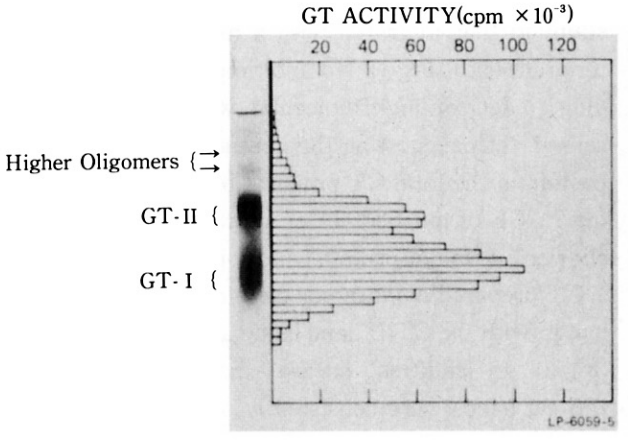


Fig.3 Non denaturing PAGE and GT activity assay:
Two samples (3ug) of purified GT from tumor effusion fluids were applied to paralleled twol-lanes on non-denaturing PAGE. one lane (on left) was stained for protein using silver stain.
the other lane was sliced in 2.5mm sections, eluted and assayed for GT activity.

A plot of the log Mr. vs relative migration (assuming a monomer Mr. of 48kd and each observed band as a oligomer of the monomer) yielded a straight line (Fig-4).

This result strongly suggests that the slower migrating bands ard oligomers of GT.

1.2 Preparation and characterization of anti-GT-II monoclonal antibodies

Balb/c mice were immunized with 30ug of purified GT in complete Freund's adjuvant. Splenocytes from

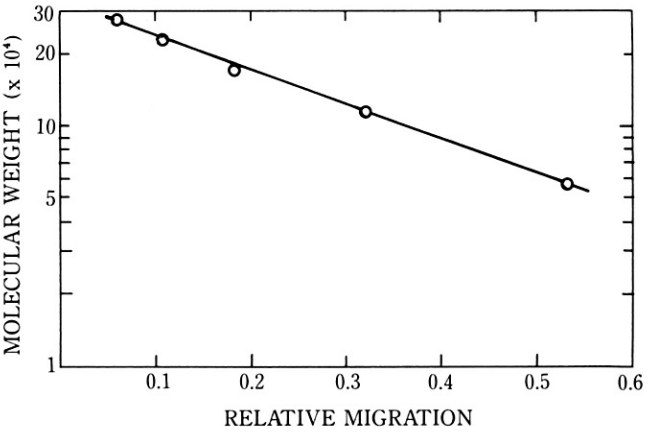


Fig.4 Semi-log graph of molecular weight of tumor derived GT oligomers vs. relative migration (Rf), assuming 48kd as the monomer molecular weight. Linear regression analysis yielded a correlation coefficient of -0.998.

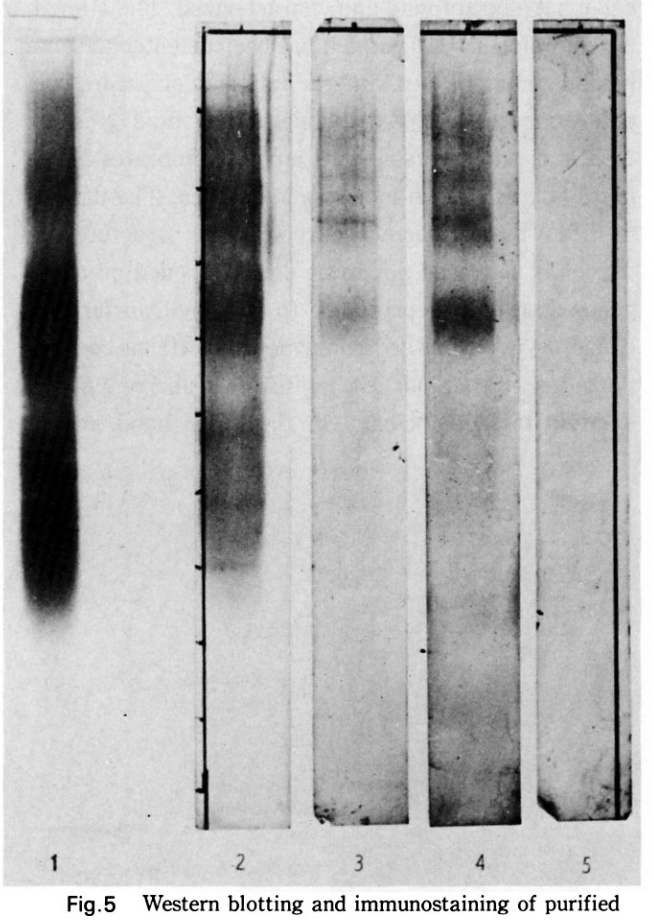


Fig.5 Western blotting and immunostaining of purified GT from tumor effusion fluids subjected to non-denaturing PAGE.
Lane 1. GT protein stained with silver procedure
Lane 2. immunostained with mouse anti-GT serum 1:1000 dilution
Lane 3. immunostained with MAb 3872 5 ug/ml
Lane 4. immunostained with MAb 3872 50 ug/ml
Lane 5. human serum immunostained with MAb 3872 50 ug/ml

immunized animals were fused with mouse myeloma sp2/0-Ag14 cells as usual. Positive clones against GT in ELISA were harvested as mouse asciety, and monoclonal antibodies were purified by Fast Protein Liquid Chromatography (FPLC).

One of these monoclonal antibodies, designated MAb3872 (IgG_1), was characterized by immunostaining of western blotting prepared from non-denaturing PAGE of purified GT (Fig-5). The GT preparation (purified from ovarian tumor effusion fluid)

contained GT-I, GT-II, and the higher oligomer GTs. All of these GT isoenzymes were stained by whole mouse anti-GT serum.

Immunostaining with MAb3872, however, resulted only in staining of the GT-II and higher Mr. oligomers (Fig-4).

1.3 Immunoassay of GT-II and clinical evaluations

To measure the GT-II level in human serum, a sensitive immunoassay was developed.

Serum samples were incubated at 4°C overnight with MAb3872 immobilized on a support matrix (GF-2000). The bound GT-II enzyme activity was assayed using UDP-[^{3}H]Gal as donor and ovalbumin as acceptor (Fig-6). This GT-II immunoassay was linear with the amount of GT-II ranging 20 to 400mU/ml.

Assay of 29 samples obtained from normal controls for GT-II revealed a mean level of 85.3±30.9mU/ml for GT-II in normal human serum is considered to be the standard cut-off value. A series of coded serum samples derived from patients with well-characterized ovarian tumors were evaluated for GT-II levels, and these levels were compared with CA-125 levels, a commonly used marker for ovarian tumors (Table-2). In 32/35 ovarian cancer patients (91.4%), GT-II was elevated above 200mU/ml, ranging 216 to 8469mU/ml.

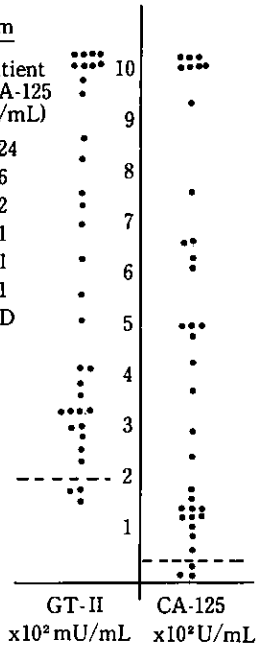
GT-II and GA-125 values were not necessarily correlated.

In a separate, blind study, coded serum samples representing ten tumor types with matched benign

Table-2

GT-II in Ovarian Tumor Patient Serum

Tumor Type	No. Patient with GT-II (>200mU/mL)	No. Patient with CA-125 (>35U/mL)
Serous	23/24	23/24
Endometrioma	5/6	5/6
Clear cell	2/2	2/2
Granulosa	1/1	0/1
Mucinous	0/1	1/1
Ovarian epithlial	1/1	1/1
Healthy control	0/29	N/D



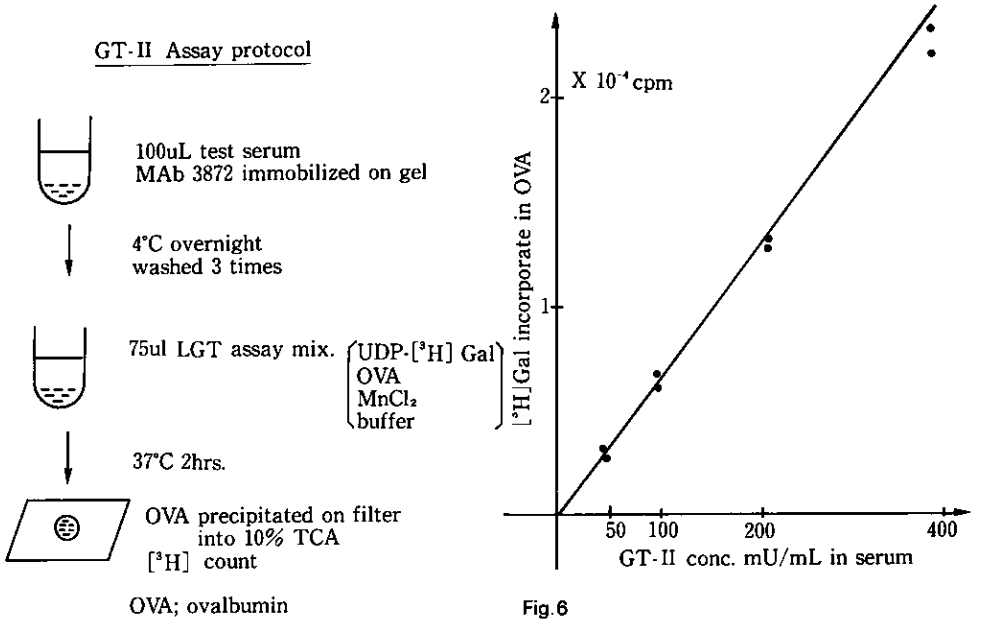


Fig.6

diseases and healthy normal controls were obtained from the National Cancer Institute (NCI) serum bank and assayed for GT-II (Fig-7).

The results suggest that GT-II is most effective in indentifying pancreatic, prostate, esophagus, cervical and bladder cancer.

2. Monoclonal antibodies against HD antigens

2.1 Purification and immunization of HD antigens

Glycosphingolipids used in this study are listed in Fig-8 and were purified from the following sources according to the published methods.

GM_2 (NeuGc) and GM_3 (NeuGc) were prepared from bovine kidney and horse erythrocyte, NeuGc-nLcOse$_4$ Cer and NeuGc-nLcOse$_6$Cer from bovine erythrocyte, GM_2 (NeuAc) from human brain, GM_3 (NeuAc) from dog erythrocyte, and asialo-GM_2 from a formic acid treatment of GM_2

The purities of these gangliosides were comfirmed by means of high performance thin layer chromatography (HPTLC).

GM_2 (NeuGc)	GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 α2 NeuGc
GM_2 (NeuAc)	GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 2 αNeuAc
GM_3 (NeuGc)	Galβ1-4Glcβ1-1Cer 3 α2 NeuGc
GM_3 (NeuAc)	Galβ1-4Glcβ1-1Cer 3 α2 NeuAc
NeuGc-nLcOse$_4$ Cer	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Ger 3 α2 NeuGc
NeuGc-nLcOse$_6$ Cer	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1- 3 3Galβ1-4Glcβ1-1Cer α2 NeuGc
asialo GM_2	GalNAcβ1-4Galβ1-4Glcβ1-1Cer

Fig.8 Structures of glycosphingolipids used for analysis.

GT-II Serum Level in Cancer and Control Subject

Assay were performed on blind, coded serum samples obtained from NCI Serum Bank

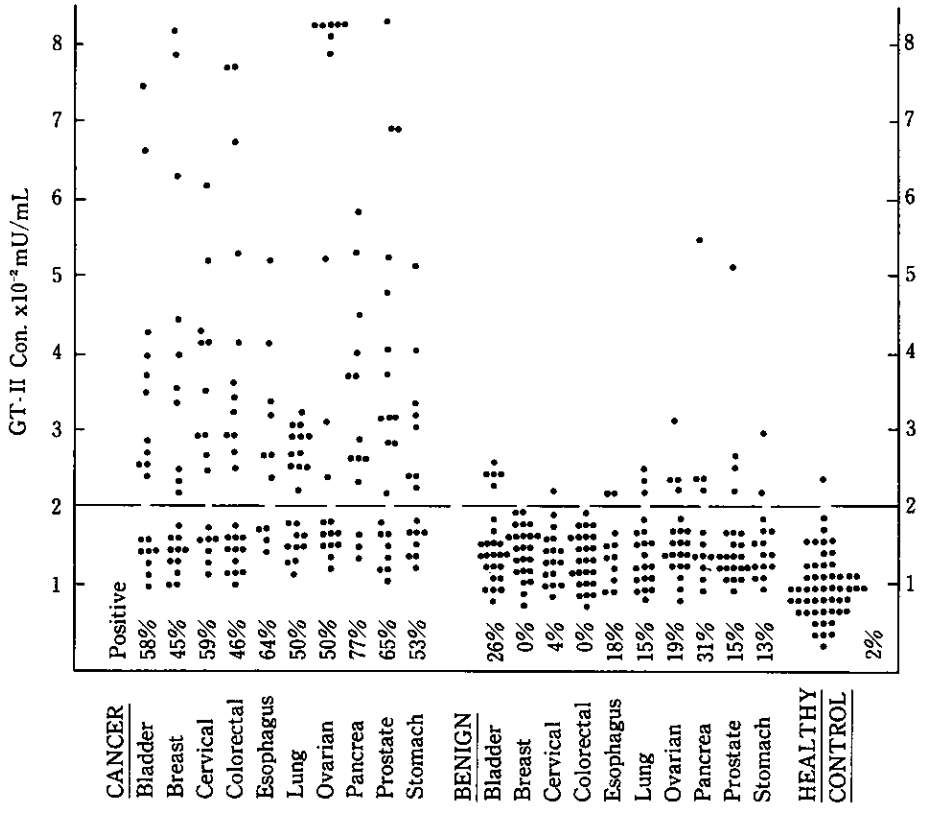


Fig.7

The ganglioside-containing liposomes were prepared by the mixture of ganglioside, egg phosphatidylcholine and cholesterol.

Female NZB mice were immunized with a membrane fruction of rabbit thymus in complete Freund's adjuvant and the ganglioside-containing liposomes (Fig-9).

2.2 Preparation and characterization of monoclonal antibodies against HD antigens

The splenocytes of immunized NZB mice and the myeloma cells p3-X63-Ag8. 653 were fused under the usual procedure.

Seven hybridomas obtained originally were cloned and more characterized. One clone (designated Y-2-HD-1) secreted the IgM antibody which strongly reacted with GM_2 (NeuGc).

When a panel of glycosphingolipids reacted with Y-2-HD-1 in a ELISA, the antibody detected possessed only a terminal GalNAc β1-4 [NeuGc α2-3] Gal-structure (Fig-10).

The specific binding of antibodies to GM_2 (NeuGc) were also assessed by TLC enzyme immunostaining (Fig-11).

Equal amounts of purified glycosphinolipids were subjected to TLC and then immunostained with Y-2-HD-1. Only GM_2 (NeuGc) was stained. This result

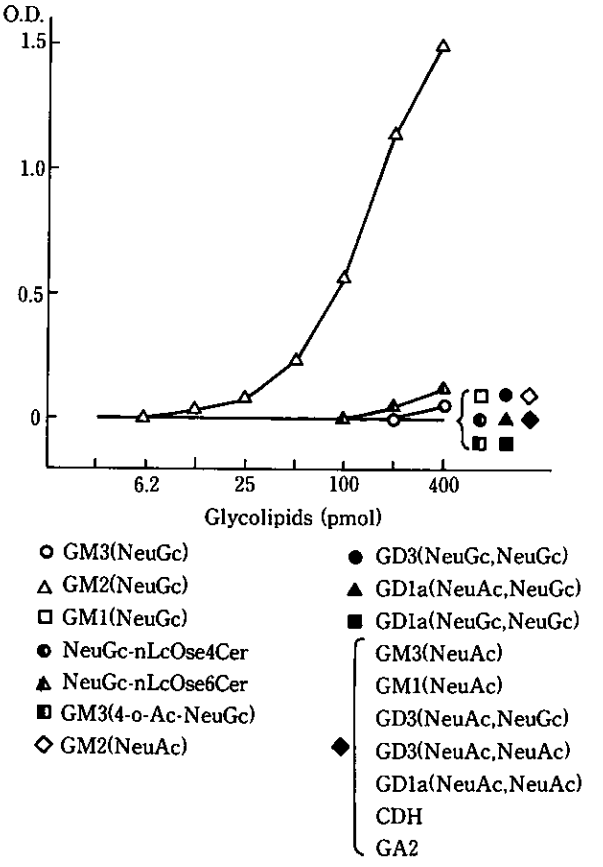


Fig.10 ELISA of purified glycosphingolipids with MAb Y-2-HD-1.Several amounts of gangliosides were used for coating antigen and undiluted hybridoma culture supernatant was used.

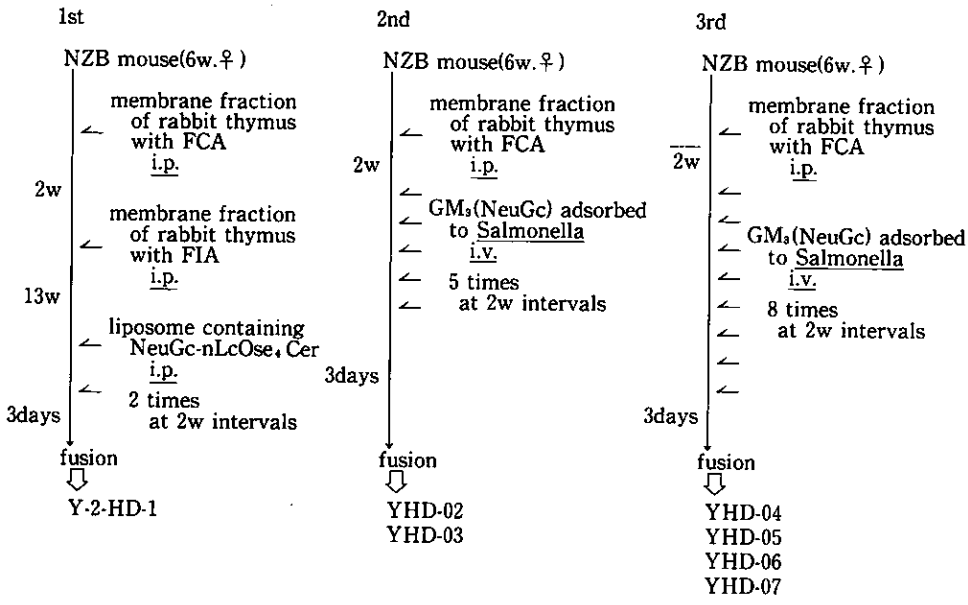
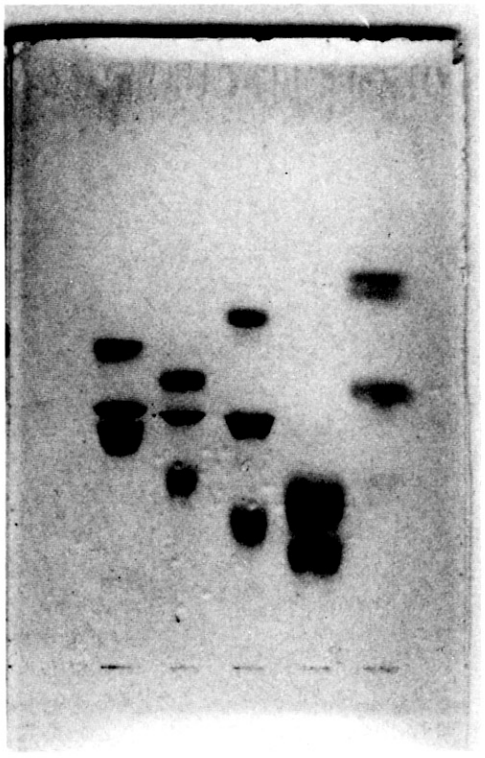


Fig.9 Immunization protocol

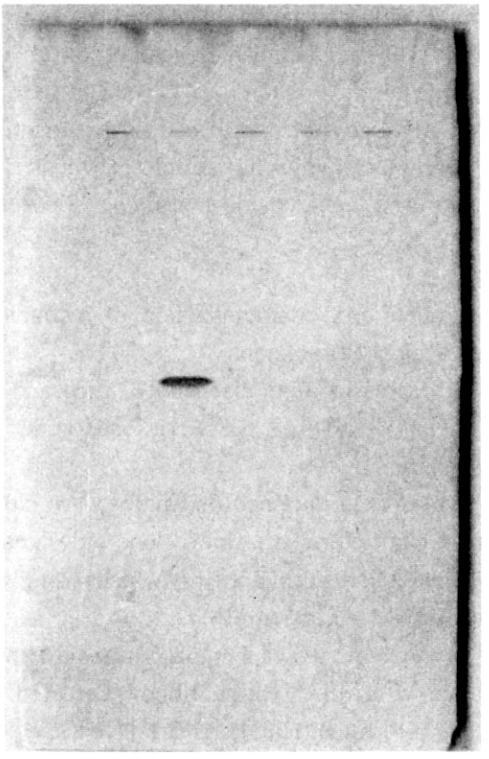


1 4 7 10 12
2 5 8 11 13
3 6 9

A: Stained with orcinol reagent.

1. GM_3(NeuAc)
2. GM_2(NeuAc)
3. GM_1(NeuAc)
4. GM_3(NeuGc)
5. GM_2(NeuGc)
6. GM_1(NeuGc)
7. GM_3(4-O-Ac-NeuGc)
8. NeuGc-nLcOse$_4$ Cer
9. NeuGc-nLcOse$_6$ Cer
10. GD_{1a}(NeuAc, NeuGc)
11. GD_{1a}(NeuGc, NeuGc)
12. CDH
13. GA_2

Fig.11a



B: Enzyme immunostaining with MAb Y-2-HD-1

Fig.11 b

Table 3 Specificity of monoclonal antibodies

		Y-2-HD-1	YHD-02	YHD-03	YHD-04	YHD-05	YHD-06	YHD-07
NeuGc	GM3	±	+++	+++	−	++	−	−
	GM2	+++	++	++	+++	+++	+++	+++
	GM1	−	−	+	++	−	−	+
	nLc4	−	++	+	−	++	−	−
	nLc6	±	++	−	−	++	−	−
	4-0-Ac-GM3	−	−	+	−	+	−	−
	GD3	−	−	+	−	−	−	−
	GD1a	−	+++	++	+	++	−	+
NeuAc	GM3	−	−	−	−	−	−	−
	GM2	−	−	−	−	−	+++	−
	GM1	−	−	−	−	−	−	−
	GD3	−	−	−	−	−	−	−
	GD2						−	
	GD1a	−	−	−	−	−	−	−
Ac/Gc	GD3	−	−	−	−	−	−	−
	GD1a	−	−	+	−	−	−	−
	CDH	−	−	−	−	−	−	−
	GA2	−	−	−	−	−	−	−

Table 4 Antigenic determinants of monoclonal antibodies

antibodies	structures		reactive GSLs
Y-2-HD-1	NeuGcα2-3Gal- 4 GalNAcβ1		GM2(NeuGc)
YHD-02	NeuGcα2-3Gal- R	R=(−), GalNAcβ1-4	GM3(NeuGc) nLc4(NeuGc) GM2(NeuGc) nLc6(NeuGc) GD1a(NeuGc, NeuGc)
YHD-03	(4-o-Ac-)NeuGcα2-3Gal- R_1 R_2	R_1=(−), NeuGcα2-8 R_2=(−), GalNAcβ1-4, Galβ1-3GalNAcβ1-4, siaα2-3Galβ1-3GalNAcβ1-4	GM3(NeuGc) GM3(4-o-Ac-NeuGc) GM2(NeuGc) GD1a(NeuGc, NeuGc) GM1(NeuGc) GD1a(NeuAc, NeuGc) nLc4(NeuGc) GD3(NeuGc, NeuGc)
YHD-04 YHD-07	NeuGcα2-3Gal- 4 R-GalNAcβ1	R=(−), Galβ1-3, NeuGcα2-3Galβ1-3	GM2(NeuGc) GM1(NeuGc) GD1a(NeuGc, NeuGc)
YHD-05	(4-o-Ac-)NeuGcα2-3Gal- R	R=(−), GalNAcβ1-4	GM3(NeuGc) GM3(4-o-Ac-NeuGc) GM2(NeuGc) GD1a(NeuGc, NeuGc) nLc4(NeuGc) nLc6(NeuGc)
YHD-06	(NeuGc or NeuAc)α2-3Gal- 4 GalNAcβ1		GM2(NeuGc) GM2(NeuAc)

also suggests that the epitope defined by Y-2-HD-1 was determined to be GalNAc β1-4 [NeuGc α2-3] Gal.

The other six monolonal antibodies were also characterized and summarized. Table-3 shows the specificities of those monoclonal antibodies to NeuGc containing gangliosides.

Antigenic determinats of seven monoclonal antibodies were predicted (Table-4).

Each monoclonal antibody exhibits a different epitope and a different specificity.

3 Discussion

Since the previous reports of a human serum cancer-associated with GT isoenzymes, reports have appeared on the identification of numerous GT isoenzymes through various methods. Our purified GT shows a single broard band on SDS-PAGE with a Mr. of 48kd. Non-denaturing PAGE of GT isolated from tumor effusion fluid showed major bands of GT-I and GT-II and several minor bands of high molecular weight which were characterized as being oligomers of GT.

Our interpretation of these results is that GT-II contains a structual element not found in GT-I that is responsible for the association of GT-II subunits of 48kd into GT isoenzymes of higher Mr..

As the very weak immunogenicity of GT has been previously noted, we experienced similar difficulties in immunizing Balb/c mice. However, we successfully isolated and characterized MAb3872 that is specific for the GT-II isoenzyme.

Immobilized MAb3872 was able to specifically remove GT activity from the solution in an enzymatically active form; elution of MAb3872 bound GT followed by non-denaturing PAGE further confirmed that the isoenzyme bound was GT-II.

Characterization of GT-II epitope bound by MAb3872 demonstrated that cyanogen bromide cleaved peptides of 8.4kd and 7.4kd were bound by MAb3872. Endoglycosidase removal of GT carbohydrate structures faciliated the binding of GT-II to MAb3872, demonstrating that the epitope does not involve the carbohydrate structure. The immunoassay for GT-II that we developed is also unique in that the enzyme activity of the antigen molecule is utilized for detection and quantitation; this gives the assay a very high sensitivity without having to use a second antibody. Using this GT-II assay, a significantly increased level of GT-II in the sera of various cancer patients was found, and this GT-II levels correlated with the clinical status of the tumor (data not shown).

Seven monoclonal antibodies against HD antigens were established and their specificities were examined.

Gangliosides containing NeuGc exist in such high quantities in the liver and other organs in mice that these glycolipids exhibit poor antigenicity. It is known that autoimmune animals produce autoantibodies against their own antigens.

Hence, we could enhance immunoresponse to gangliosides containing NeuGc by immunizing NZB mice (an autoimmune animal), and successfully prepare monoclonal antibodies which previously was extremely difficult. This method might be able to be applied to the preparation of monoclonal antibodies for other glycolipids. Though some of these monoclonal antibodies are neither reactive with GM_3(NeuGc) nor NeuGc-nLcOse$_4$Cer used as antigen, all of them are reactive with GM_2 (NeuGc). It was reported that a membrane fraction of rabbit thymus contains gangliosides containing NeuGc in high concentration, but no GM_2 (NeuGc) was proved to be detected by TLC enzyme immunostaining. On the other hand, GM_2 (NeuGc) exists in NZB mice erythrocyte, and NZB mice produce an anti-erythrocyte autoantibody. This information suggests that NZB spleen contains lymphoid cells which are able to produce antibodies for GM_2 (NeuGc), which is a ubiquitous autoantigen in mice.

Finally, these monoclonal antibodies against GT-II and HD antigens reported here should be useful in histological and serological studies, as well as in application to cancer diagnosis.

References

1. Uemura, M., Winant, R.,C., Sikic, B., Brandt, A.,E., Characterization and immunoassay of human tumor-associated galactosyltransferase isoenzyme II. Cancer Research; in press
2. Uemura, M., Winant, R.,C., and Brandt, A.,E., Immunoassay of serum galactosyltransferase isoenzyme II in cancer patients and control subjects. Cancer Research; in press
3. Sanai, M.,Yamasaki, Y., and Nagai, Y., Monoclonal antibody directed Hanganutziu-Deicher active ganglioside, GM_2 (NeuGc) Biochimica et Biophysica Acta; in press
4. Hakomori, S., Aberrent glycosylation in cancer cell membranes as focused on glycolipids; overview and perspectives. Cancer Research **45** 2405-2414 (1985)
5. Weiser, M., M., Podolsky, D., K., and Wilson, J., R., Cancer associated isoenzyme of serum galactosyl transferase. Proc. Natl. Acad. Sci. USA **73** 1319-1322 (1976)
6. Podolsky, D., K., and Isselbacher, K., J., Characterization of monoclonal antibodies to serum galactosyl-transferase. Proc. Natl. Acad. Sci. USA **81** 2529-2533 (1984)
7. Higashi, H., Hirabayashi, Y., et al. Characterization of N-glycolylneuramic acid containing gangliosides as tumor-associated Hanganutziu-Deicher antigen in human colon cancer. Cancer Research **45** 3796-3802 (1985)
8. Hirabayashi, Y., Higashi, H., et al. Occurrence of tumor-associated ganglioside antigens with Hanganutziu-Deicher antigenic activity on human melanomas. Jpn. J. Cancer Res. **78** 614-620 (1987)