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Establishment of a Human Monoclonal Antibody to Hanganutziu-Deicher Antigen as a Tumor-associated Carbohydrate Antigen

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We have established a human-human hybridoma producing a monoclonal antibody against a tumor-associated carbohydrate-specific antigen, Hanganutziu-Deicher (HD) antigen. Human spleen lymphocytes from a patient with esophageal varices complicated with liver cirrhosis were cultured in serum-free medium and co-stimulated with both anti-human μ-chain antibodies and supernatants of concanavalin A-stimulated human spleen cell culture (ConA sup). The activated lymphocytes were subsequently primed *in vitro* with particulate HD3 antigen and fused with a parent hybrid myeloma cell line, KR-12. A hybridoma, 1F43E31G7 produced anti-HD human monoclonal antibody (IgM λ). This monoclonal antibody reacted strongly with N-glycolylneuraminyl α2-3 lactosylceramide (HD3) and slightly with N-glycolylneuraminyl α2-3 lactoneotetraosylceramide (HD5), but did not react with N-glycolylneuraminyl α2-3 lactoneohexaosylceramide (HD7), N-acetylneuraminyl α2-3 lactosylceramide (GM3) and other derivatives of HD3 prepared by chemical modification of the sialic acid residue of HD3, which indicates that the monoclonal antibody is directed precisely toward the terminal sialic acid and whole structure of HD3.

Key words: HD antigen — Monoclonal antibody — Human hybridoma — Tumor-associated antigen

Hanganutziu-Deicher (HD)[*7] antigen is one of the heterophile antigens.[1-6] HD antigens were extracted and purified as glycosphingolipids by Higashi *et al.*[7] and Merrick *et al.*[8] from equine and/or bovine erythrocytes. HD antigens with N-glycolylneuraminic acid at the terminal carbohydrate do not exist in normal tissues of human and chicken. However, HD antigen is expressed with a high frequency on the surface of their tumor cells,[9-11] and the antibodies are often found at a high level in their sera.[9] Several antigenic glycosphingolipid molecules were identified in human colon cancers,[9] melanomas[10] and retinoblastoma cell lines.[11] It seems difficult to analyze precisely the nature of HD antigens expressed in tumor tissues using antisera obtained from the chicken or human. A monoclonal antibody to HD antigen would be a more suitable tool to resolve these problems. However, it is difficult to develop anti HD monoclonal antibodies through immunization of mice with HD antigen because it is a self antigen in mice. Another difficulty is that we are not able to utilize fetal calf serum because of the presence of strong HD antigens. Therefore we tried to establish a human-human hybridoma producing a monoclonal antibody against HD antigen in serum-free medium. The present communication describes the process of development of a human anti-HD monoclonal antibody and its immunological specificity.

Materials and Methods

Glycosphingolipids Glycosphingolipids used are listed in Table I. HD3 was isolated and purified from equine erythrocytes.[7] HD5 and HD7 were obtained from bovine erythrocytes.[7, 12] Purification of GM3 ganglioside from human erythrocytes was

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[*7] Abbreviations used in this paper: HD antigen, Hanganutziu-Deicher antigen; ConA sup, supernatant from concanavalin A-stimulated human lymphocytes culture; anti-μ antibody, anti human immunoglobulin μ-chain specific antibody; Igs, immunoglobulins; RIA, radioimmunoassay; PBL, peripheral blood lymphocytes; MoAb, monoclonal antibody.

Table I. Structures of Gangliosides Used

Abbreviation	Chemical structure
HD3	NeuGc(α2-3)Gal(β1-4)Glc-ceramide
HD5	NeuGc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glu-ceramide
HD7	NeuGc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc-(β1-3)Gal(β1-4)Glc-ceramide
GM3	NeuAc(α2-3)Gal(β1-4)Glc-ceramide
Oxidized and reduced HD3[a]	C_7 and C_8-NeuGc(α2-3)Gal(β1-4)Glc-ceramide
Reduced HD3[b]	1-reduced-NeuGc(α2-3)Gal(β1-4)Glc-ceramide
Methyl ester HD3[c]	1-methyl-NeuGc(α2-3)Gal(β1-4)Glc-ceramide

a) A mixture of C_7-analogue (60%) and C_8-analogue (40%) of HD3 was obtained by oxidation with $NaIO_4$ followed by $NaBH_4$ reduction.
b) The carboxyl group of HD3 was esterified by treatment with methyl iodine.
c) The carboxyl group of HD3 was esterified and then reduced to a primary alcohol by $NaBH_4$.

reported in our previous publication.[13] The derivatives of HD3 were obtained as follows. The polyhydroxyl side chain of the sialic acid residue of HD3 was modified by mild oxidation with periodate followed by reduction with sodium borohydride according to the method of Vell *et al.*[14] C_7 and C_8 analogues produced by the treatment were previously isolated by gas-liquid chromatography.[15] The carboxylic group of the sialic acid residue was esterified with methyl iodine according to Handa and Nakamura.[16] The methyl ester of HD3 was successively reduced with sodium borohydride to produce the C_1 primary alcohol according to our previous paper.[17]

Cell Preparation Peripheral blood mononuclear cells were prepared from heparinized blood of normal volunteers by centrifugation on Ficoll-Hypaque gradients.[18] To remove adherent monocytes, peripheral blood mononuclear cells were incubated for 2 hr at 37° in a tissue culture dish (3003, Becton Dickinson and Co., Oxnard, CA). Nonadherent monocytes were used as a lymphocyte source. Human spleen lymphocytes were prepared from operatively resected spleens of patients according to Mishell and Shiigi.[19] Briefly, spleen lymphocytes were mechanically dissociated from sterile pieces of human spleen. Contaminating erythrocytes were depleted by hypotonic shock and subsequently adherent mononuclear cells were removed as mentioned above.

Culture Medium Cells were cultured in serum-free medium (HB104, Hana Media, Berkeley, CA), supplemented with human serum albumin, insulin and transferrin, ethanolamine, sodium pyruvate (1 m*M*) and L-glutamine (500 μg/ml) at suitable concentrations for cell growth. These protein supplements did not have HD antigenicity. As antibiotics, streptomycin (100 μg/ml) and penicillin (100 IU/ml) were added. Human spleen lymphocytes (5×10^6/ml) were cultured with ConA Sepharose 4B (50 μg protein/ml, Pharmacia Fine Chemicals, Uppsala) in the HB104 medium at 37° for 48 hr under 7% CO_2 and 93% air. After centrifugation, the culture supernatants were filtered through a Millex-HA membrane (0.45 μm, Millipore Corp., Bedford, MA) and kept frozen at −70° until used as ConA sup supplement.[20] $F(ab')_2$ fragment of goat anti-human Ig μ-chain specific antibody (Cappel, Cochranville, PA) was also used as another supplement. Insolubilized particulate HD3 antigen was prepared according to the method of Hirabayashi *et al.*[21] Briefly, HD3 antigen was conjugated to octyl-Sepharose CL-4B (Pharmacia) with 1.0*M* NaI (1:1, v/v) and then autoclaved before being added at a concentration of 1 μg/ml.

Cell Fusion As parent cells, KR-12[22] and UC729[23] were used. Cell fusions were performed by electrofusion using a somatic hybridizer (SSH-1, Shimadzu, Kyoto). Stimulated or non-stimulated lymphocytes (1×10^7) and parent cells (1×10^6) were suspended in 100 μl of fusion buffer containing glucose (0.25 *M*), $CaCl_2$ (0.1 m*M*), $MgCl_2$ (1.0m*M*) and Tris-HCl (0.2m*M*, pH 7.3). A 20 μl aliquot of cell suspension was fused in a fusion chamber, SSH-C12, at room temperature. Pearl formation of the mixed cells was completed by applying an alternating current (40 V/cm field strength) at a frequency of 1.0 MHz for 10 sec. Cell fusion were then induced with two direct current square pulses of 3.0 kV/cm field strength and 10 μsec duration at an interval of 1 sec. Dissociation of non-fused cells was performed by applying the same alternating current for 30 sec. Immediately after fusion, the cell suspension was transferred into 50 volumes of HB104 culture medium prewarmed at 37° and maintained for 5

min to complete membrane sealing. One hundred μl of the cell suspension (5×10^4 cells) was seeded into 96 half area wells and cultured for one day. The next day, a half of the medium was removed from each well and the same volume of fresh medium containing 0.1m*M* hypoxanthine (Sigma Chemical Co., St Louis, MO), 0.4n*M* aminopterin (Sigma) and 16 n*M* thymidine (Sigma) was supplemented according to Oi and Herzenberg.[24]

Solid-phase Radioimmunoassay for HD Antibody Detection Solid-phase radioimmunoassay (RIA) was performed according to the method of Tsu and Herzenberg[25] with minor modifications.[21] Briefly, a Micro Test III 3911 flexible assay plate (Becton Dickinson and Co.) was coated by evaporation of 25 μl of glycosphingolipid antigen-containing ethanol solution (50 μl/ml). Nonspecific binding sites were blocked by incubation with 200 μl of 1% egg albumin-PBS at 37° for 2 hr. The plate was washed four times with 0.05% Tween-20 PBS, and 25 μl of culture supernatant of hybridoma cells was added to each well. The plate was incubated at 4° for 2 hr and then washed similarly. Twenty-five μl of unlabeled goat anti-human Igs (TAGO Inc., Burlingame, CA) at a concentration of 3.2 μg/ml, adjusted with 1% egg albumin-PBS, was added to each well. This reaction was performed at 4° for 2 hr. The plate was again washed four times, and approximately 1×10^5 cpm of [^{125}I]protein A (Amersham, Bucks, UK) in 25 μl of 1% egg albumin-PBS was added to each well. The plate was allowed to stand on ice for 1 hr, and after being washed as before, each well was cut out and counted with a gamma counter (RAC-501, Aloka, Tokyo).

Solid-phase Radioimmunoassay for Immunoglobulin Detection Twenty-five μl of culture supernatant was seeded in a Micro Test III 3911 and incubated at room temperature for 2 hr. After blocking with 1% egg albumin-PBS for 2 hr, approximately 1×10^5 cpm of [^{125}I]anti-human Igs (Amersham) in 25 μl of 1% egg albumin was added to each well. The plate was allowed to stand on ice for 1 hr, then washed. The radioactivity of each well was counted as described above.

Identification of Immunoglobulin Class of Monoclonal Antibody The double-immunodiffusion test was performed to determine the immunoglobulin class of MoAb in 0.8% agarose gel containing 0.9% NaCl.[26] Anti-human IgG_1, IgG_2, IgG_3, IgG_4 antibody preparation (ShAHu/$IgG_{1\text{-}4}$), anti-human IgM antibody preparation (GAHu/IgM(Fc)), anti-κ antibody preparation (RAHu/BJ-K(SD+HD)), and anti-λ antibody preparation (RAHu/BJ-L(SD+HD)) were purchased from Nordic Imm. Lab., Tilburg, The Netherlands. They were diffused at the original concentration against each culture fluid concentrated 10-fold by ultrafiltration with the use of a Minicon B15 membrane (Amicon Corp., Danvers, MA). Precipitin lines were observed each day for 1 week.

RESULTS

The Effect of Stimulation of Human Spleen Lymphocytes with ConA Sup and Anti-μ Antibody As a trial to induce proliferation and differentiation of immunoglobulin-producing B lymphocytes, the spleen cells from a patient were incubated and stimulated *in vitro* with 10% ConA sup and/or 15 μg/ml anti-μ antibody. As shown in Table II, immunoglobulin production at the 5th and 6th culture days was enhanced by stimulation with ConA sup plus anti-μ, compared with the non-stimulated

Table II. Immunoglobulin Production of Human Spleen Lymphocytes Stimulated with ConA Sup and Anti-μ Antibody

Stimulation		Immunoglobulin production[a] (cpm)		
		day 4	day 5	day 6
Anti-μ (−),	ConA sup (−)	273±23[b,c]	220±8[c]	288±16[c]
Anti-μ (−),	ConA sup 10%	270±11	301±29	245±25
Anti-μ 15 μg/ml,	ConA sup (−)	350±37	293±45	390±44
Anti-μ 15 μg/ml,	ConA sup 10%	423±16[c]	563±33[c]	703±43[c]

a) Human spleen lymphocytes (10^5) were cultured in each well of 96 half wells (3696, Costar, Cambridge, MA) containing 100 μl of medium supplemented with or without 15 μg/ml anti-μ antibody and 10% ConA sup. Twenty-five μl of each culture supernatant taken on the 4th, 5th and 6th days was transferred to another plate, and the immunoglobulin content was determined by the solid-phase RIA as described in "Materials and Methods."

b) Mean±SD of triplicate samples.

c) $P<0.01$.

group. On the other hand, anti-μ antibody alone or ConA sup alone did not enhance immunoglobulin production. An optimal concentration of anti-μ antibody titrated in another experiment was 15 μg/ml (data not shown).

Trials for Establishment of Anti-HD Human Monoclonal Antibody under Various Conditions A parent cell line, UC729-6[22] or KR-12[23] was fused with *in vitro*-stimulated or non-stimulated human lymphocytes from either normal peripheral blood or the spleen of a patient with liver cirrhosis. The result of each trial for establishment of anti HD monoclonal antibody is shown in Table III. We succeeded in establishment of six anti HD monoclonal antibody-producing hybridomas when human spleen lymphocytes from a patient (j) stimulated with ConA sup, anti-μ and HD3 antigen were fused with KR-12 hybrid myeloma cells. In this fusion experiment, hybridoma growth was observed in 66 out of 104 wells. Immunoglobulin production was detected in 26 of the 66 wells, and 6 wells produced an antibody that reacts with HD3 antigen. The HD antibody titer of the patient (j) was quite low as determined by the equine

Table IV. Estimated Amounts of Anti HD3 Antibodies Produced by Several Hybridomas Recloned from a Parent Hybridoma, 1F4[a)]

Clones	HD antibody activity[b)] (cpm)
1F42E31G7	3,552
1F41D5B7	2,664
1F42E21E8	1,835
1F41C5D4	1,776
1F41D5E2	1,421
1F43C8C3	1,066

a) Recloning was performed 3 times successively by the limiting dilution method. At first, 1F4 parent cells (5 cells on average) were seeded in each well of 96-well, half area tissue culture clusters (Coster) together with X-ray-irradiated (2,000 R) human peripheral lymphocytes (2×10^5) as feeder cells. 1C5, 1D5, 2E2, 2E3 and 3C8 and other clones isolated were detected to be producing HD antibody. The 2nd cloning was performed similarly for each clone. Some clone produced no effective clone but 1C5 produced B7 and E2 clones. From the other clones, one clone producing the highest titer of the antibody was chosen. The final cloning was done by seeding 2 (average number) cells in each well and selecting the highest antibody titer-producing clone. Six clones finally obtained were producing the same immunoglobulin class of IgM(λ).

b) Antibody activity of each culture medium was estimated by solid-phase RIA. Cpm of KR-12 culture medium (that is, background) was 156.

Table III. Establishment of Human-Human Hybridoma Producing Anti HD Monoclonal Antibody

Parent cells	Human B cells	Stimulation *in vitro*[a)] ConA sup	anti-μ	HD Ag	No. hybrids[b)]/ No. total wells	No. Ig producing[c)]/No. hybrids	No. HD Ab producing[d)]/No. Ig producing
UC729-6[e)]	normal[f)] PBL	(−)	(−)	(−)	6/89	4/6	0/4
UC729-6	normal[g)] PBL	(+)	(−)	(−)	66/89	4/66	0/4
UC729-6	patient[h)] spleen cells	(+)	(−)	(−)	266/288	2/266	0/2
KR-12[i)]	patient[j)] spleen cells	(+)	(+)	(−)	27/33	4/27	0/4
KR-12	patient[j)] spleen cells	(+)	(+)	(+)	66/104	26/66	6/26

a) Lymphocytes were cultured with 10% ConA sup and/or 15 μg/ml anti-μ antibody for 5 days, and then on the final day immobilized HD3 was added at a concentration of 1 μg/ml before cell fusion.
b) Numbers of hybrids grown during 6 weeks of observation.
c) Numbers of immunoglobulin-producing hybrids screened by solid-phase RIA using [^{125}I]anti-human Igs.
d) Numbers of anti HD3 antibody-producing hybrids screened by solid-phase RIA using HD3-coated microplate.
e) A 6-thioguanine-resistant lymphoblastoid cell line established by Glassy *et al.*[22]
f) Twenty-nine-year-old healthy volunteer (male).
g) Thirty-one-year-old healthy volunteer (male).
h) Fifty-one-year-old female patient with liver cirrhosis and esophageal varices.
i) A 6-thioguanine- and ouabain-resistant human-human hybrid myeloma cell line established by Kozbor *et al.*[23]
j) Thirty-six-year-old male patient with liver cirrhosis and esophageal varices.

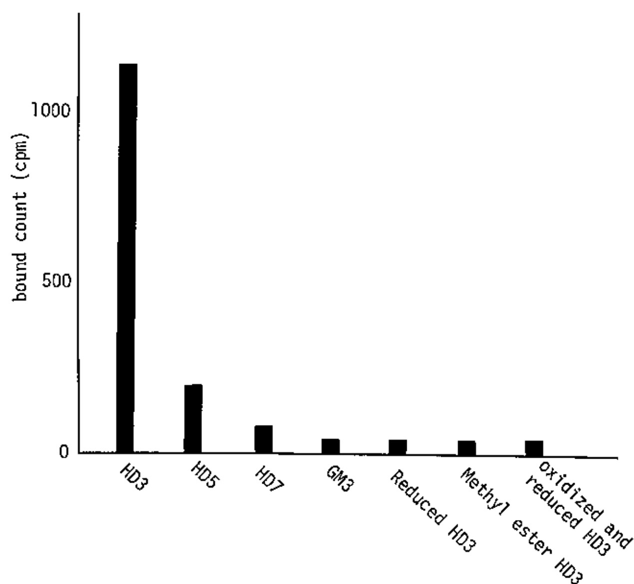

Fig. 1. Reactivities of monoclonal antibody 1F42E31G7 with HD3, HD5, HD7, GM3 and three chemically modified derivatives of HD3. Glycosphingolipid antigens coated on wells were reacted with 25 μl of culture medium containing the monoclonal antibody. The monoclonal antibody amounts reacted were measured by solid-phase RIA as described in "Materials and Methods."

erythrocyte hemagglutination test. We omitted to hybridize UC729 cells with spleen cells sensitized *in vitro* with HD antigen.

Characteristics and Immunological Specificity of Anti HD Human Monoclonal Antibody 1F4 clone among the 6 clones established was chosen as the clone producing the highest titer of anti HD antibody. Recloning of the clone was performed by limiting dilution. We obtained 6 clones that produce anti HD antibody after consequential limiting dilution. All clones produced IgM (λ-chain) class antibody. Antibody activities of these antibody-containing medium are listed in Table IV. The specificity of anti HD human monoclonal antibody, 1F42F31G7 was determined by solid-phase radioimmunoassay. As expected, horse and bovine red blood cells absorbed the antibody but human red blood cells did not (data not shown). This antibody reacted with HD3 in a dose-dependent manner (data not shown), but only a minute reaction was observed when HD5 was used as an antigen. No reaction with GM3 was seen. To examine the specificity of this monoclonal antibody more precisely, HD7 and three HD3 analogues in which the N-glycolylneuraminic acid moiety of HD3 was chemically modified were used in addition to the above compounds. Figure 1 showed that this monoclonal antibody strongly reacted only with HD3.

Discussion

We have established a human-human hybridoma producing anti HD monoclonal antibody. This is the first report of the establishment of monoclonal antibody against HD antigen. Human monoclonal antibody against malignant tumor cells should be a very effective tool for clinical cancer immunotherapy and the diagnostic radioimmunoimaging of cancer. Many human monoclonal antibodies against malignant tumor cells have been reported.[27-32] However, few papers have identified the epitope structure recognized by the monoclonal antibodies.[29] Furthermore, it is well known that human hybridomas are unstable in producing antibody. The same is true for Epstein-Barr virus-transformed B lymphoblast cell lines producing monoclonal antibody, although there are some exceptions.

We adopted the following strategy for establishment of anti HD human monoclonal antibody in the present study. 1) Stimulation of human lymphocytes with ConA sup. Many papers have reported that T cells stimulated

with ConA sup secrete B cell growth factors, B cell differentiation factors and unknown other B cell growth factors,[33-37] and concanavalin A itself directly activated B cells.[38] For the above reasons, we used ConA sup as a possible factor which might induced proliferation and differentiation of B cells. 2) Stimulation with anti-μ antibody. The first step in B cell proliferation and differentiation is that antigen binds to antigen receptor on the surface of B cells. It has been reported that anti-μ antibody is able to replace this effect of the antigen.[39,40] Anti-μ stimulation enlarges B cells and causes them to enter the G_1 phase from the G_0 phase. However, anti-μ stimulation by itself does not induce DNA synthesis of B cells. It seemed possible that costimulation with ConA sup and anti-μ antibody might cause B cells to differentiate and proliferate. In fact, in our present experiments stimulation of lymphocytes with ConA sup and anti-μ antibody increased the immunoglobulin production of B cells. 3) *In vitro* immunization with HD antigen. The aim of the lymphocyte stimulation mentioned above was to increase the efficacy of *in vitro* immunization with antigens. Immunization with murine spleen cells *in vitro* had been established before Köhler and Milstein's hybridoma technique was reported.[41,42] *In vitro* immunization was then applied to the hybridoma technique. The efficiency of this method was proved in developing monoclonal antibodies against viruses, bacteria, tumor cells and soluble protein antigen.[43,44] In order to enhance the antigenicity of HD antigen *in vitro*, we utilized the insolubilized particulate antigen which we used for affinity chromatography to purify human polyclonal HD antibody. Namely, HD3 conjugated to octyl-Sepharose (Pharmacia) was used for *in vitro* immunization.

We established anti HD human monoclonal antibodies following the above-mentioned strategy. The established clones have been stable in immunoglobulin secretion and specificity for approximately one year since establishment. The strategy for establishment of human B cell hybridomas used in this study may be applicable to other antigen systems. However, we cannot rule out the possibility that our clones were established by chance irrespective of the method used. To generalize this method as a useful tool for developing human monoclonal antibody, we will need further investigations.

The monoclonal antibody 1F42E31G7 specifically reacted with N-glycolylneuraminic acid-containing HD3 ganglioside but did not react with N-acetyl-neuraminic acid-containing GM3 ganglioside which has the same chemical structure as HD3 glycosphingolipid except for the terminal sialic acid of the carbohydrate chain. Furthermore, the monoclonal antibody did not react with three derivatives of HD3 (oxidized and reduced HD3, reduced HD3 and methyl ester HD3) in which the sialic acid part was chemically modified. These results suggest that N-glycolylneuraminic acid is the dominant immunodeterminant recognized by the monoclonal antibody. This characteristic resembles that of avian anti HD3 immune sera rather than that of human polyclonal HD antibodies which were detected in some cancer patients.[15,17]

Four different patients' sera with HD antibodies reacted equally strongly with both HD3 and HD3 methyl ester, in which the carboxyl group of the sialic acid was esterified with methyl iodide.[17] HD3, HD5 and HD7 share a common disaccharide moiety, NeuGcα2-3Galβ1-4, at the terminal of the carbohydrate chain. The third carbohydrate moiety is glucose in HD3 but N-acetylglucosamine in HD5 or HD7. The monoclonal antibody developed here gave a strong reaction with HD3, but a minute reaction with HD5 and no reaction with HD7. These results indicate that the three-carbohydrate chain from the non-reducing terminal end of HD3 plays a key role in the monoclonal antibody recognition. Even in this respect, the monoclonal antibody resembles avian anti HD3 immune sera rather than human polyclonal HD sera.[15] Human HD sera mostly reacted with HD7 5 times as powerfully and HD5 2 times as powerfully as HD3.[17]

These results indicate that HD antibodies in human sera are heterogeneous. One out of multiple clones producing HD antibodies was chosen in this fusion experiment as the monoclonal antibody-producing clone. If we utilize another HD molecule than HD3, such as HD5, HD7 or 4-O-acetyl-N-glycolylneuraminic acid-containing GM2 ganglioside, in *in vitro* immunization, we could possibly obtain another monoclonal antibody specific for the

immunized molecule. A set of several monoclonal antibody preparations, each recognizing different N-glycolylneuraminic acid-containing glycoconjugates has to be established for more precise analyses of HD antigenic molecules expressed in human malignant tissues.

As to the reactivity of the monoclonal antibody to human tumor cells, we did not obtain a positive reaction in an immunohistochemical study with two malignant melanomas and four colon carcinomas examined. The following possibilities may be considered. 1) The antibody titer was not high enough to detect antigens in the immunohistochemical study. 2) HD3 might not be a major antigen on human tumor cells. 3) The tested tumors were antigen-negative by chance. To clarify these points and obtain positive reactions, further experiments are in progress.

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