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ORIGINAL ARTICLE

**Saroj Basak · Brigitte Birebent · Enkhtsetseg Purev
Rajasekharan Somasundaram · Haruhiko Maruyama
Jan Zaloudik · Rolf Swoboda · Wolfgang Strittmatter
Weiping Li · Albrecht Luckenbach · Hong Song
Jian Li · Katrin Sproesser · Dupont Guerry
Sridar Nair · Koichi Furukawa · Dorothee Herlyn**

Induction of cellular immunity by anti-idiotypic antibodies mimicking GD2 ganglioside

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Abstract Gangliosides are potentially useful targets for tumor destruction by antibodies. However, the role of gangliosides in T cell-mediated immunity to tumors is unclear. We produced three murine monoclonal anti-idiotypic antibodies (Ab2) against a monoclonal antibody (Ab1) that binds strongly to melanoma-associated GD2 ganglioside and weakly to GD3 ganglioside. All three Ab2 induced anti-anti-idiotypic antibodies (Ab3) with Ab1-like binding specificity to tumor cells and antigen in rabbits. The Ab3 specifically bound to $GD2^+$ tumor cells and isolated GD2, and shared idiotopes with the Ab1. Two of the three Ab2 induced GD2-specific delayed-type hypersensitivity responses in BALB/c and C57BL/6 mice, but not in $C57BL/6/CD4^{-/-}$ mice. Peripheral blood mononuclear cells (PBMC) from a melanoma patient proliferated specifically in response to in vitro stimulation with Ab2. Proliferation was accompanied by Th1-type cytokine production. Our studies demonstrate the induction of ganglioside-specific T cell-dependent immunity by Ab2 in mice. These T cells showed specific reactivity to ganglioside expressed by tumor cells.

S. Basak · B. Birebent · E. Purev · R. Somasundaram
H. Maruyama · J. Zaloudik · R. Swoboda · W. Li
H. Song · J. Li · K. Sproesser · S. Nair · D. Herlyn (✉)
The Wistar Institute, 3601 Spruce Street,
Philadelphia, PA, USA
E-mail: dherlyn@wistar.upenn.edu
Tel.: +215-898-3962
Fax: +215-898-0980

W. Strittmatter · A. Luckenbach
Merck, Darmstadt, Germany

D. Guerry
Department of Medicine and Cancer Center,
University of Pennsylvania, Philadelphia, PA, USA

K. Furukawa
Department of Oncology, School of Medicine,
Nagasaki University, 1–12–4 Sakamoto, Nagasaki 852, Japan

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Abbreviations *Ab1* antibody directed to antigen · *Ab2* anti-idiotypic antibody directed to Ab1 · *Ab3* anti-anti-idiotypic antibody directed to Ab2 · *BSA* bovine serum albumin · *c.p.m.* counts per minute · *CRC* colorectal carcinoma · *DTH* delayed-type hypersensitivity · *FCS* fetal calf serum · *IL* interleukin · *i.v.* intravenous · *MHA* mixed hemadsorption assay · *MHC* major histocompatibility complex · *mAb* monoclonal antibody · *PBMC* peripheral blood mononuclear cells · *PBS* phosphate-buffered saline · *RIA* radioimmunoassay · *s.c.* subcutaneous · *SI* stimulation index

Introduction

The GD2 ganglioside is the target of a number of therapeutic monoclonal antibody (mAb) trials that have shown some in vivo anti-tumor effects in melanoma and neuroblastoma patients [4, 8, 10, 13, 18, 29, 44, 45]. In addition, GD2 is a potential target for treatment of gliomas, medulloblastomas, small cell lung carcinomas, retinoblastomas, and osteo- and soft tissue sarcomas, all of which express this antigen [6, 17, 21, 43]. mAb to GD2 have inhibited tumor metastasis formation in mice [28]. Induction of sustained immunity to GD2 by active immunization may be advantageous over passive administration of short-lived mAb [32, 59]. However, GD2 is weakly immunogenic when presented to the patient's immune system by the growing tumor [5, 15, 56]. Furthermore, GD2 is difficult to purify in amounts sufficient for immunization of cancer patients, and when administered in adjuvant, elicits a short-lived, low-titer IgM response of poor memory in animals and patients [26, 37, 41, 51]. To our knowledge, there are no reports on the induction of cellular immunity by isolated GD2 (or other gangliosides) in experimental animals or in patients.

Anti-idiotypic antibodies (Ab2), which bind to the antigen-combining site of other antibodies (Ab1), can mimic antigen and have advantages over antigen itself. Ab2 are easy to produce in large quantities, whereas this is often impossible for antigen. Moreover, Ab2 have been shown to break tolerance to bacterial polysaccharide in newborn mice, whereas immunization with the antigen itself was ineffective [48]. The latter aspect is important for the induction of immunity to tumor-associated antigens, such as GD2, which are also expressed by some normal tissues [54] and thus may induce immunological tolerance.

Based on the findings that: (1) Ab2 can mimic carbohydrates and induce carbohydrate-specific humoral immune responses in vivo [7, 9, 11, 32, 46, 58]; and (2) carbohydrates occasionally induce specific cellular immune responses in vivo [4, 20, 29, 31], we investigated whether Ab2 mimicking GD2 ganglioside could induce cellular immune responses in mice (delayed-type hypersensitivity; DTH) and a melanoma patient (proliferative lymphocytes). Ab2 were induced in mice against mAb ME361 (Ab1), which binds strongly to GD2 and weakly to GD3, lyses GD2-positive cultured melanoma cells in conjunction with complement or effector cells [54], and inhibits spontaneous metastasis of subcutaneously (s.c.) injected human melanoma xenografts to the lungs in nude mice [28]. In a dose escalation phase I clinical trial, mAb ME361 administered to 13 melanoma patients with metastatic disease was associated with a complete clinical response in one patient (our unpublished data).

We show here that Ab2 against Ab1 ME361 induces GD2-specific IgG responses in rabbits, and DTH responses in mice. Induction of DTH responses in mice was dependent on the participation of $CD4^+$ T lymphocytes. In addition, Ab2 induced proliferative responses in the lymphocytes of a melanoma patient in vitro. Thus, Ab2 are effective reagents to induce both IgG and cellular immunity to carbohydrate antigens, whereas the carbohydrate itself usually induces only IgM and no cellular immune responses.

Materials and methods

Patient and tumor cells

Human melanoma cell lines WM164 and WM115, human glioma cell lines U87 and 251 MG, and human colorectal carcinoma (CRC) cell line SW1116 have been described [24, 35, 36, 53]. Heparinized blood and melanoma spleen metastasis were obtained from non-vaccinated melanoma patient 3122 at the time of surgery. PBMC were obtained by Ficoll-Hypaque centrifugation of heparinized blood, and melanoma cell line WM3122 was established from the spleen metastases as described [24]. These cells express GD2 and both HLA class I and II (97.2, 93.2, and 94.8% of the cells positive for GD2, HLA class I and II, respectively). All human cell lines were maintained in L-15 medium supplemented with 10% fetal calf serum (FCS). Murine melanoma transfectant D142.34, which expresses human GD2 and GD3, and parental B78.96 cells, which are GD2- and GD3-negative, were derived from C57BL/6 mice as described [19]. Neither cell line expresses major histocompatibility complex (MHC) class I or class II antigens, even after interferon-γ (IFN-γ) stimulation (our unpublished results). Cells were maintained in RPMI medium supplemented with 10% FCS, 400 μg/ml geneticin (Gibco, Gaithersburg, Md.), and 50 μg/ml hygromycin B (Calbiochem, La Jolla, Calif.).

Antibodies

mAb ME361 (IgG2a) has been described [24, 54]. It binds strongly to GD2 and weakly to GD3, but not to GM: 1, 2, 3, 4; GD1a ± 1b; GT1a; GQ1b [24, 54]. $F(ab')_2$ of mAb ME361 was produced by pepsin digestion of intact IgG and the fragments were purified and tested for purity as described [52]. mAb ME313 (IgG1) to proteoglycan [4, 42] and mAb JC4 (IgG2a) binding to an unidentified antigen on human melanoma cells (our unpublished results) were used as controls. Normal mouse IgG was purchased from Cappel JCN Pharmaceuticals (Aurora, Oh.). mAb W6/32 (IgG2a) against HLA class I was obtained from Bice Perussia (Thomas Jefferson Medical School, Philadelphia, Pa.), and mAb SK37–7 (IgG2a) against HLA DR and DQ was produced in our laboratory [35]. Anti-IFN-γ mAb B133.1.1 and B133.5.1 (both IgG1) and anti-interleukin (IL)-4 mAb 4F2 and 5A4 (both IgG1) were obtained from Giorgio Trinchieri (Wistar Institute).

Antigens

GD2 was purified by β-galactosidase treatment of bovine brain GD1b ganglioside and purified by Sephadex-LH20 column chromatography. β-Galactosidase was isolated from bovine testes as described [12]. GD3 and GD1a + 1b were purchased from BioCarb Chemicals (S-22370 Lund, Sweden).

Immunizations of mice and rabbits

All studies in mice and rabbits were approved by the Wistar Institute's Institutional Animal Care and Use Committee (IACUC). For production of monoclonal Ab2 to Ab1 ME361, BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.), were immunized repeatedly s.c. with 20 μg of Ab1 ME361 $F(ab')_2$ coupled to keyhole limpet hemocyanin and emulsified in complete (first injection) or incomplete (subsequent injections) Freund's adjuvant. Mice that were seropositive for Ab2 (for assay, see below) were boosted with 20 μg of Ab1 ME361 $F(ab')_2$ intravenously (i.v.) 3 days before the fusion of splenocytes. For induction of Ab3, rabbits (New Zealand White; Hare-Marland, Hewitt, N.J.) received 4 s.c. injections of 300 μg (first immunization) and 100 μg (following immunizations) each of alum-precipitated monoclonal mouse Ab2 at bi-weekly intervals. Control animals received normal mouse IgG instead of Ab2. For induction of DTH reaction, mice [BALB/c, C57BL/6 and C57BL/6 $CD4^{-/-}$ knock-out mice (Jackson Laboratory, Bar Harbor, Me.)] were immunized s.c. with 100 μg of Ab2 (or normal mouse IgG) emulsified in complete Freund's adjuvant.

Hybridoma production

Splenocytes from mice that were seropositive for Ab2 (for assay, see below) were fused to SP2/O myeloma cells using polyethylene glycol-1500 (Boehringer Mannheim, Germany) as described [36].

Antibody binding assay

The presence of Ab2 in sera of mice immunized with Ab1 ME361 was determined by radioimmunoassay (RIA). Wells of microtiter plates were coated with ME361 $F(ab')_2$ and incubated with mouse Ab2-containing sera. Ab2 binding to the plates was detected by ^{125}I-labeled goat anti-mouse Fc (Cappel Organon Teknika, Durham, N.C.) preabsorbed 3 times to a mAb ME361 $F(ab')_2$-coupled immunoaffinity column to remove any residual antibody reactivity

to constant region determinants (CH_1 domain) present in the $F(ab')_2$ preparation.

All assays for the detection of Ab3 have been described [23, 38]. Briefly, binding of rabbit Ab3 to cultured tumor cells was determined in mixed hemadsorption assay (MHA) using, as indicator cells, sheep red blood cells (SRBC) sensitized with rabbit anti-SRBC antibody bound to goat anti-rabbit IgG. Binding of rabbit Ab3-containing sera to ganglioside was determined in RIA with antigen used as target (5 μg/well in ethanol) and ^{125}I-labeled goat anti-rabbit IgG as tracer.

Antibody binding inhibition assays

All assays have been described in detail [23, 38]. Inhibition of mouse monoclonal Ab2 binding to Ab1 ME361 by rabbit Ab3 was determined by preincubating ^{125}I-labeled Ab2 TA17A (30,000 counts per minute; c.p.m. = 6 ng/well), TA412G (30,000 c.p.m. = 9 ng/well) or TB310B (30,000 c.p.m. = 7.66 ng/well) with various dilutions of rabbit Ab3 sera overnight at 4°C before adding the mixtures to Ab1 ME361 target (0.06 μg/well). Rabbit sera were preincubated with 400 μg/ml of normal mouse IgG (to block anti-mouse isotypic/allotypic antibodies present in the rabbit sera) before addition to the ^{125}I-labeled mouse Ab2. Inhibition of Ab1 ME361 binding to WM164 melanoma cells by rabbit Ab3 was determined in RIA. Melanoma cells were incubated with sera from rabbits immunized with either mouse Ab2 or normal mouse IgG (all sera were preincubated with 400 μg/ml normal mouse IgG). Ab1 ME361 (at 1.5 μg/ml, the concentration showing 50% maximum binding to WM164 cells) was added to the wells, and Ab1 binding inhibition to melanoma cells by the sera was detected by ^{125}I-labeled goat anti-mouse $F(ab')_2$ (30,000 c.p.m./well).

T cell proliferation assay

To determine whether Ab2 can stimulate cultured lymphocytes from a melanoma patient (no. 3122), Ficoll-purified PBMC were cultured in the presence of various stimulants. Adherent monocytes (5×10^4 cells/well of round-bottomed microtiter plates) were pulsed in triplicate for 8 h with Ab2, control mAb ME313 (both at 0.6 μg/well), GD2, GD3 or GD1a + 1b (each at 2 μg/well) or PHA in RPMI 1640 medium supplemented with 4 mM L-glutamine, 10% heat-inactivated human Ab serum, 10 mM Hepes and 5×10^{-5} M 2-mercaptoethanol (Sigma). Wells were washed once. PBMC (10^5 cells/well) were added in RPMI 1640 medium. In separate cultures, PBMC were incubated with irradiated (200 Gy) autologous WM3122 melanoma cells or allogeneic SW1116 CRC cells (10^4 cells/well). After 5 days of incubation, proliferative lymphocyte responses were determined in ^{3}H-thymidine incorporation assay, as described above for murine lymphocytes.

Replicate lymphocyte cultures were restimulated with the various preparations for an additional 5 days before ^{3}H-thymidine was added to the wells. Results are presented as c.p.m. incorporated into proliferating lymphocytes.

Cytokine determinations

Human IFN-γ and IL-4 levels produced by a patient's PBMC cultured with various stimulants were determined after 2 days of culture as described [30].

Lymphocyte phenotyping

Human PBMC restimulated with various stimulants for an additional 5 days were incubated with phycoerythrin-labeled anti-CD4 and anti-CD8 mAb (Pharmingen, San Diego, Calif.) in phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA; Sigma) for 30 min at 4°C. mAb binding was detected by flow cytometry (Coulter, Hialeah, Fla.).

DTH reaction in mice

Immunized mice were challenged with 5×10^5 irradiated (400 Gy from a cesium source) $GD2^+$ human WM115 or murine D142.34 melanoma cells in the right ear and with 5×10^5 irradiated $GD2^-$ human SW1116 CRC cells or murine B78.96 cells in the left ear [38]. A third group of mice was challenged with 1 μg of purified GD2 antigen in the right ear and 1 μg of purified GD1a + 1b antigen (negative control) in the left ear. Ear thickness was measured with a caliper (Poco Test, Mitutoyo, Japan) before and 24, 48, and 72 h after challenge. Increase in thickness was calculated for each ear at various time points after challenge relative to thickness before challenge.

Statistical analyses

Differences between mean experimental and control values were analyzed by Student's *t*-test using the statistical program Stat View.

Results

In vitro characterization of monoclonal mouse Ab2 TA17A clone (Cl)2, TA412G Cl1, TB310B Cl1, TA41G Cl1, TB39C Cl4, and TB14A Cl1, Monoclonal mouse Ab2 TA17A Cl2 (IgG2a), TA412G Cl1 (IgG2b), TB310B Cl1 (IgG2b), TA41G Cl1 (IgG2b), TB39C Cl4 (IgG2a), and TB14A Cl1 (IgG2a) against Ab1 ME361 (IgG2a) bound to this antibody and not to unrelated mAb ME313 (not shown). Maximal inhibition of Ab1 ME361 binding to tumor cells by the Ab2 ranged between 50% and 90% (not shown). In contrast, mAb JC4 binding to melanoma cells was not significantly inhibited (< 10% inhibition) by any of the six Ab2.

Humoral immune responses in Ab2-immunized rabbits

Induction of antigen-specific Ab3 across species barriers, i.e. in a species different from the species of origin of Ab1, after immunization with Ab2 demonstrates the internal image nature of Ab2 [23]. Sera of the two rabbits immunized with Ab2 TA17A Cl2 (nos. 859, 061), Ab2 TA412G Cl1 (nos. 807, 572), or Ab2 TB310B Cl1, (nos. 885 and 551) showed significant binding ($P < 0.01$ as compared to control sera) to WM115 (Fig. 1A–D) and WM164 (not shown) melanoma cells, and to 251MG (Fig. 1E-H) and U87 (not shown) human glioma cells, but not to antigen-negative SW1116 CRC cells (< 10% of the cells maximally bound to Ab3-containing sera derived from both rabbits; not shown). Sera obtained from the same rabbits before immunization and sera from the rabbit (no. 637) immunized with normal mouse IgG showed no significant binding to melanoma or glioma cells (< 10% of the cells maximally bound; Fig. 1D and H). Ab2 TA41G Cl1, TB39C Cl4 and TB14A Cl1 did not induce anti-melanoma antibodies (not shown) and were not studied further.

Ab3-containing sera from all 6 rabbits immunized with 3 different Ab2 significantly ($P < 0.01$ as compared to control sera) and specifically inhibited the binding of

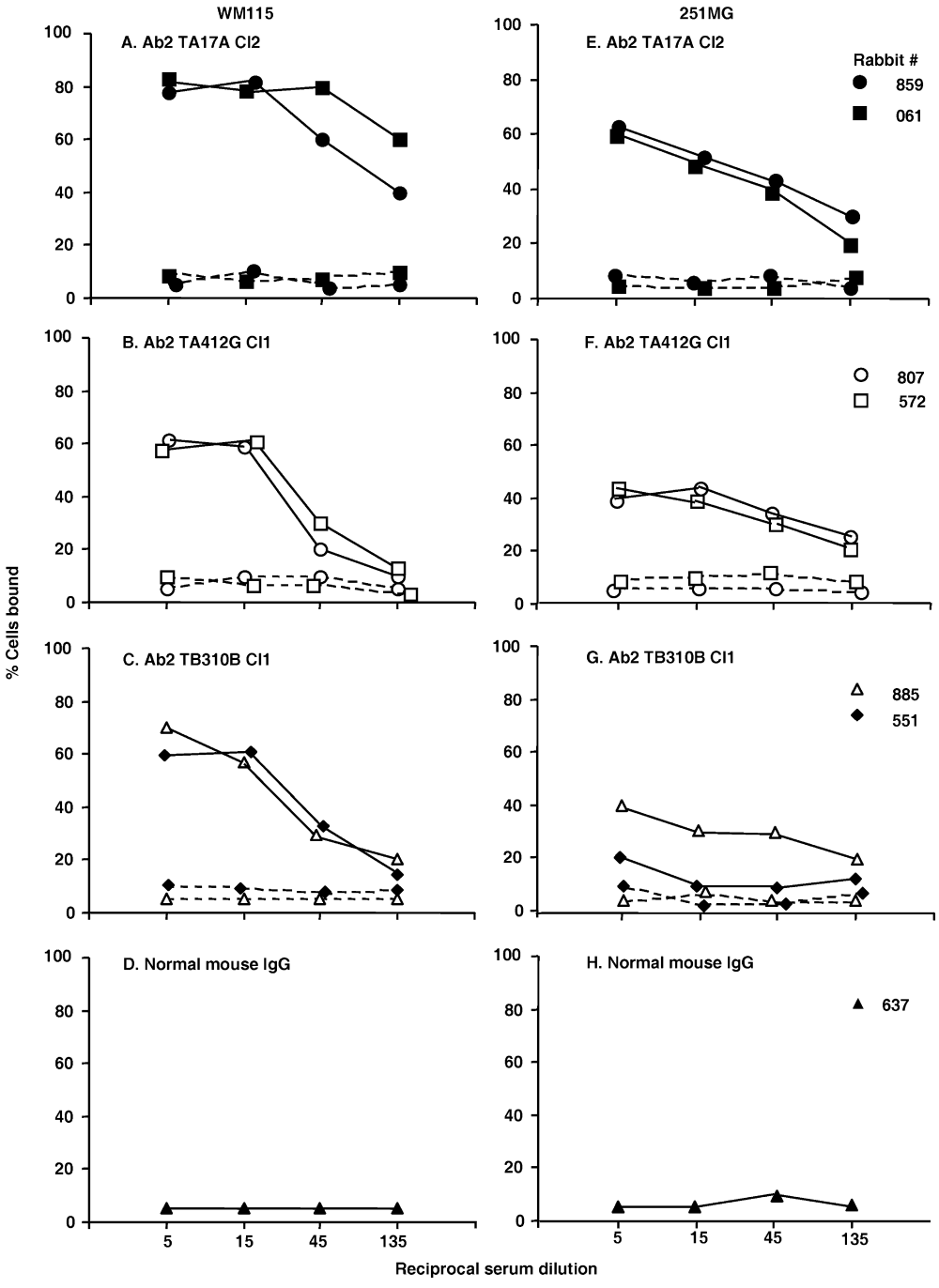

Fig. 1 Binding to WM115 melanoma cells (**A**–**D**) or 251MG glioma cells (**E**–**H**) of Ab3 elicited by alum-precipitated Ab2 TA17A Cl2 (A,E), Ab2 TA412G Cl1 (**B**, **F**), Ab2 TB310B Cl1 (**C**, **G**), or antibodies elicited by normal mouse IgG (**D**, **H**). Rabbits (*code numbers next to symbols*) were immunized with 300 μg of antibody in alum on day 0, followed by 100 μg of antibody in alum on days 15, 36, and 60. Sera were obtained from rabbits either before immunization or 10–19 days after the last immunization. Sera were tested for binding in MHA. Values obtained with post-immune sera (–) (dilution 1:5) of Ab2-immunized rabbits differed significantly ($P < 0.01$) from the corresponding values of pre-immune sera (–) and post-immune values of control rabbit no. 637

Ab1 ME361 to melanoma cells (Fig. 2). Thus, the rabbit Ab3 may bind to the same epitope as Ab1 ME361 does on melanoma cells. Binding of unrelated anti-melanoma mAb JC4 to melanoma cells was not inhibited by the Ab3 sera (not shown).

Sera from each of the 2 rabbits immunized with Ab2 TA17A Cl2 (nos. 859, 061), Ab2 TA412G Cl1 (nos. 807, 572), or Ab2 TB310B Cl1 (nos. 885 and 551) showed significant binding ($P < 0.01$ as compared to control sera) to isolated GD2 (Fig. 3) but not to GD1a + 1b (not shown). Immune sera showed no binding to GD3 (not shown). Thus, the Ab2 mimic GD2, but not GD3, although Ab1 ME361 binds to GD2 and, albeit weakly, to GD3. Ab3-containing sera from the two rabbits immunized with one of the three Ab2 significantly ($P < 0.01$) inhibited the binding of Ab1 ME361 to the Ab2 (Fig. 4). Thus, the rabbit Ab3 may bind to the same idiotope as Ab1 ME361 on Ab2.

DTH responses in Ab2-immunized mice

BALB/c (Fig. 5A) and C57BL/6 (Fig. 5B) mice immunized with Ab2 TA412G Cl1 or Ab2 TB310B Cl1, but

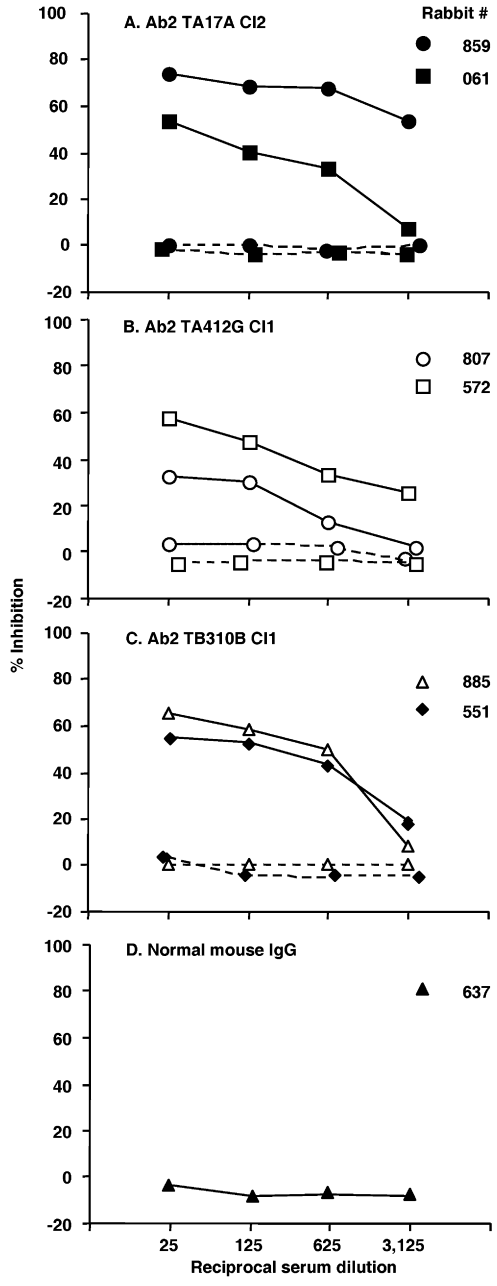

Fig. 2 Inhibition of binding of Ab1 ME361 to WM164 melanoma cells by Ab3 elicited by Ab2 TA17A Cl2 (**A**), TA412G Cl1 (**B**), or TB310B Cl1 (**C**), and absence of inhibition by antibodies elicited by normal mouse IgG (**D**). For immunization and serum collection, see Fig. 1 legend. Inhibition of binding of Ab1 ME361 to WM164 melanoma cells by the Ab3-containing sera was detected by ^{125}I-labeled goat anti-mouse F(ab')$_2$ in RIA. All sera were incubated with 400 μg/ml of normal mouse IgG prior to assay. Values obtained with post-immune sera (–) (dilution 1:25) of Ab2-immunized rabbits differed significantly ($P < 0.01$) from the corresponding values of pre-immune sera (—) and post-immune values of control rabbit no. 637

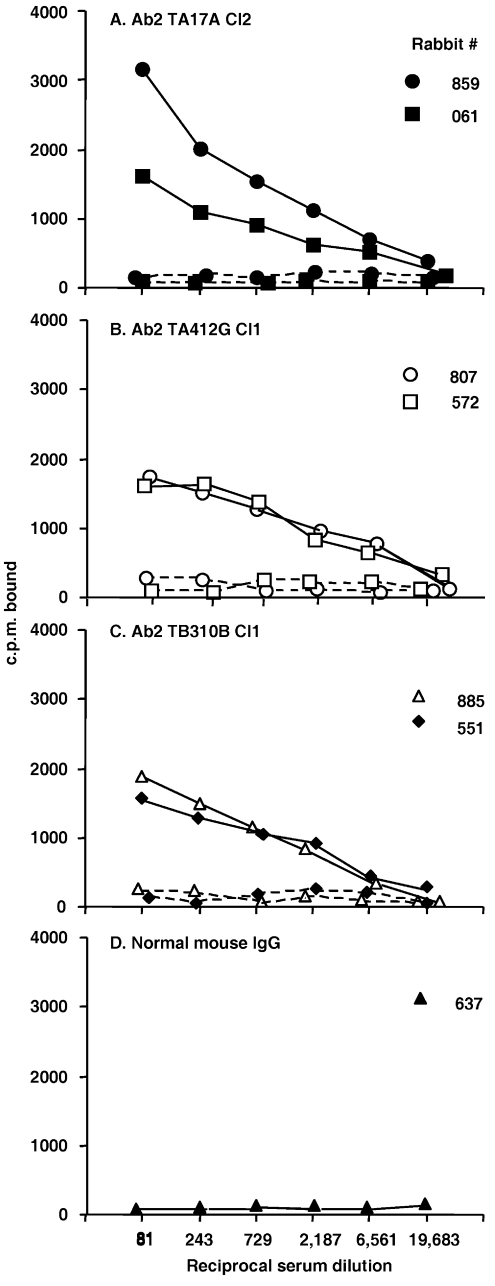

Fig. 3 Binding to GD2 antigen of Ab3 elicited by Ab2 TA17A Cl2 (**A**), TA412G Cl1 (**B**), or TB310B Cl1 (**C**), and absence of binding of antibodies elicited by normal mouse IgG (**D**). For immunization and serum collection, see Fig. 1 legend. Values obtained with post-immune sera (–) (diluted 1:81) of Ab2-immunized rabbits differed significantly ($P < 0.01$) from the corresponding values of pre-immune sera (—) and the post-immune values of control rabbit no. 637

not mice immunized with Ab2 TA17A Cl2 (not shown), showed significant ($P < 0.05$) DTH responses against a challenge with GD2-positive WM115 human melanoma cells as compared to GD2-negative human SW1116 CRC cells. DTH responses to challenge with the WM115 melanoma cells were significantly ($P < 0.01$) higher in mice immunized with Ab2 TA412G Cl1 or TB310B Cl1 as compared to normal mouse IgG-immunized mice (Figs. 5A and B). However, neither Ab2 TA412G Cl1 nor Ab2 TB310B Cl1 induced significant ($P > 0.05$) and specific DTH responses to challenge with the corresponding Ab2 or with GD2 in BALB/c and C57BL/6 mice (not shown). Ab2 TA412G Cl1 and

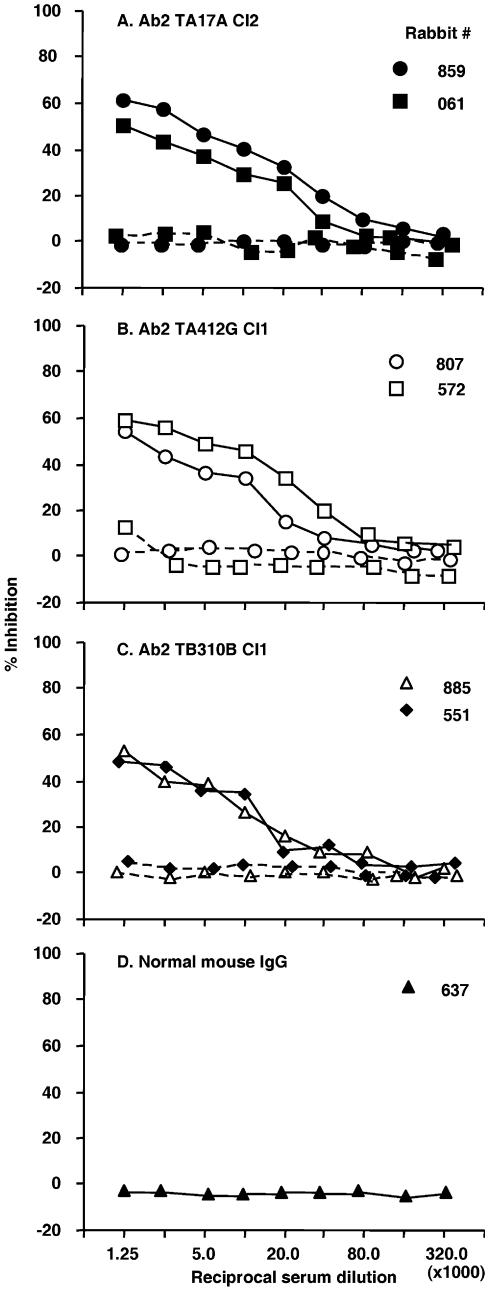

Fig. 4 Inhibition of binding of Ab1 to Ab2 by Ab3. Rabbits were immunized with Ab2 TA17A Cl2 (**A**), TA412G Cl1 (**B**), TB310B Cl1 (**C**), or normal mouse IgG (**D**). For immunization and serum collection, see Fig. 1 legend. Inhibition of binding of ^{125}I-labeled Ab2 to Ab1 ME361 by the sera was determined by RIA. All sera were incubated with 400 μg/ml of normal mouse IgG prior to assay. Values obtained with post-immune sera (–) (diluted 1:1,250) of Ab2-immunized rabbits differed significantly ($P < 0.01$) from the corresponding values of pre-immune sera (—) and the post-immune values of control rabbit no. 637

TB310B Cl1 were unable to elicit specific DTH responses to challenge with GD2-positive WM115 melanoma cells in C57BL/6 CD4$^{-/-}$ knock-out mice (Fig. 5C), suggesting the participation of CD4^{+} T cells in the DTH reaction.

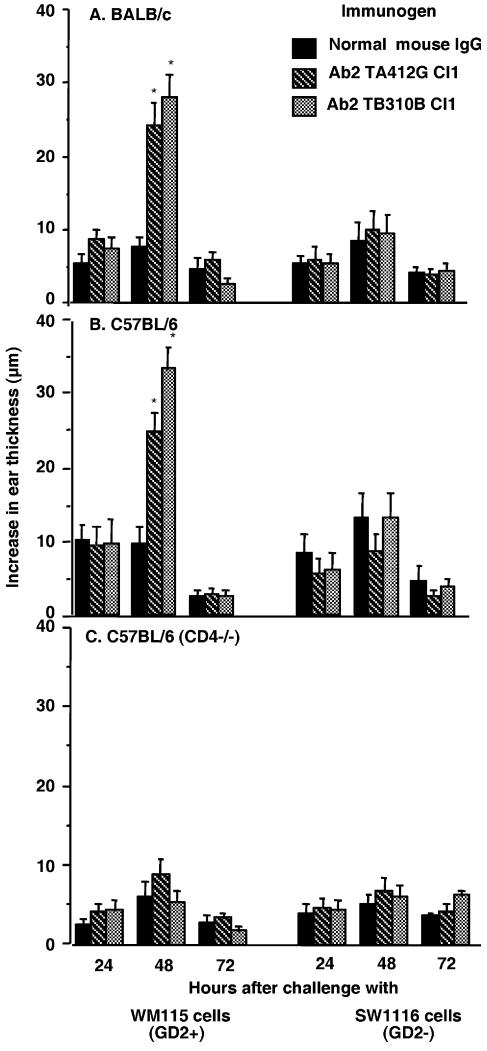

Fig. 5 DTH reactions in Ab2 (TA412G Cl1, TB310B Cl1)-immunized BALB/c (**A**), C57BL/6 (**B**) and C57BL/6/CD4$^{-/-}$ T cell knock-out (**C**) mice challenged with human GD2^{+} melanoma cells. Mice (6 per group) were immunized s.c. with 100 μg of Ab2 TA412G Cl1 or TB310B Cl1 or normal mouse IgG in complete Freund's adjuvant. Seven days later, mice were challenged with GD2-positive, irradiated human WM115 melanoma cells in the right ear and GD2-negative SW1116 CRC cells in the left ear; increase in ear thickness was calculated as described in Materials and methods. *Values (mean ± SD of different mice within a group) differed significantly from control values obtained in Ab2-immunized mice challenged with SW1116 cells ($P < 0.05$), or in normal IgG-immunized mice challenged with WM115 cells ($P < 0.01$)

The availability of murine D142.34 melanoma cells, which express human GD2, enabled challenge of Ab2-immunized mice with syngeneic cells, thereby increasing the specificity of the DTH reaction as compared to challenge with the xenogeneic GD2-positive human cells used in the experiments described in Fig. 5. C57BL/6 mice immunized with Ab2 TA412G Cl1 developed significant ($P < 0.05$–$P < 0.001$) and specific DTH responses to challenge with syngeneic GD2-positive D142.34 cells as compared with GD2-negative B78.96 cells (Fig. 6). These two cell lines differ only in GD2 expression [19],

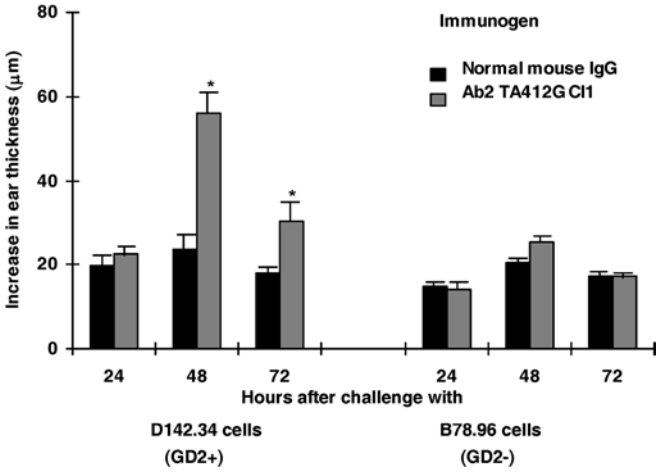

Fig. 6 DTH reactions in Ab2 TA412G Cl1-immunized C57BL/6 mice challenged with syngeneic $GD2^+$ melanoma cells. Mice (6 per group) were immunized s.c. with 100 μg of Ab2 TA412G Cl1 or normal mouse IgG in complete Freund's adjuvant. Seven days later, mice were challenged with $5x10^5$ syngeneic irradiated (400 Gy) $GD2^+$ D142.34 cells in the right ear and $GD2^-$ B78.96 cells in the left ear. *Values (mean ± SD of 6 mice per group) differed significantly from the corresponding control values obtained at the same time point: 48 h after challenge, $P < 0.001$ for D142.34 challenge in Ab2-immunized mice compared with normal mouse IgG-immunized mice, and $P < 0.001$ for D142.34 challenge compared to B78.96 challenge within the Ab2-immunized group; at 72 h after challenge, $P < 0.01$ for D142.34 challenge in Ab2-immunized mice compared with the control mice, and $P < 0.05$ for D142.34 challenge compared to B78.96 challenge within the Ab2-immunized group

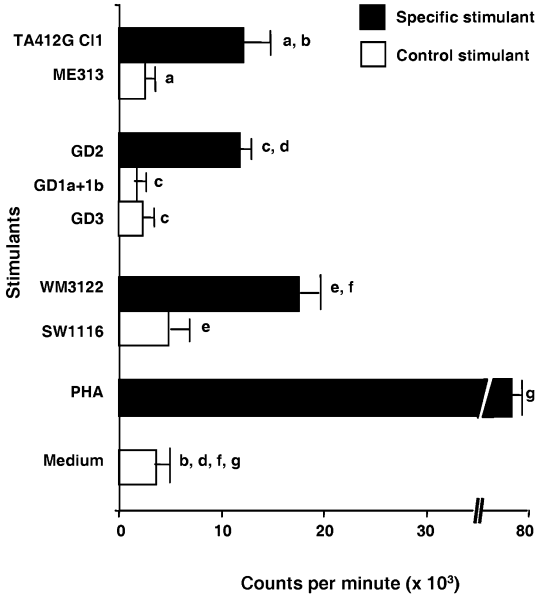

Fig. 7 Lymphoproliferative responses to stimulation with Ab2 or antigen in PBMC from patient no. 3122. Adherent monocytes ($5x10^4$/well) were pulsed with Ab2 TA412G Cl1, control mAb ME313 (both at 0.6 μg/well), GD2, GD3 or GD1a + 1b (each at 2 μg/well) and incubated with autologous PBMC (10^5/well). PBMC were also incubated with irradiated (200 Gy) autologous $GD2^+$ WM3122 melanoma cells or allogeneic $GD2^-$ SW1116 CRC control cells (10^4 cells/well). After 5 days, cultures were pulsed for 12 h with 1 μCi/well of ^{3}H-thymidine, cells were harvested and c.p.m. incorporation was determined. Results are given as mean c.p.m. incorporated (SD) of triplicate determinations. Values with identical letters differ significantly from each other (*a, b, e, g*: $P < 0.01$; *c, d, f*: $P < 0.05$)

and therefore the DTH response most likely is GD2-specific. DTH responses in the Ab2-immunized mice challenged with D142.34 cells were also significantly ($P < 0.01$–$P < 0.001$) higher compared to the responses of normal mouse IgG-immunized mice challenged with the same cells. Similar experiments using C57BL/6 $CD4^{-/-}$ knock-out mice immunized with Ab2 TA412G Cl1 and challenged with irradiated $GD2^+$ D142.34 cells or $GD2^-$ B78.96 cells revealed no DTH reaction to the $GD2^+$ cells versus the $GD2^-$ cells (not shown).

Proliferative lymphocyte responses of a melanoma patient

PBMC from patient no. 3122 showed significant proliferation in response to stimulation with Ab2 TA412G Cl1 ($P < 0.05$), GD2 ($P < 0.05$), or $GD2^+$ autologous WM3122 cells ($P < 0.01$) as compared to stimulation with the corresponding control preparations (Fig. 7). There was no lymphocyte reactivity to GD3. This experiment was repeated twice with similar results. Only the proliferating lymphocyte cultures produced significant amounts of IFN-γ (10.3, 38.0, and 36.0 U/ml of IFN-γ after stimulation with Ab2 TA412G Cl1, GD2, and WM3122 cells, respectively; $P < 0.05$–$P < 0.01$ versus the values obtained after PBMC stimulation with the corresponding control preparations). Ratios of $CD4^+$ to $CD8^+$ T cells ranged from 1.1 to 3.5 in the proliferating cultures and from 0.8 to 5.8 in the non-proliferating cultures.

Discussion

The Ab2 described here are highly specific stimulators of immune responses to the tumor-associated ganglioside GD2. Three of the six Ab2 produced against anti-GD2 mAb ME361 (Ab1) and binding to different idiotopes on the Ab1 (not shown) induced antigen-specific humoral immune responses against species barriers and, therefore, the Ab2 may bear the internal image of GD2 [23]. Two of the three Ab2 also induced DTH responses in mice.

Induction of humoral immunity by Ab2 mimicking carbohydrates, including GD2, has been described [7, 9, 11, 32, 46, 49, 57, 58]. In some of those studies, Ab3 were shown to bind specifically to native carbohydrate expressed on cells [32, 46, 57, 58], and these antibodies were of IgG isotype in one study [32]. In our study, the Ab3 were also reactive with the native GD2 antigen and were of IgG isotype (goat serum specific for rabbit IgG was used to detect rabbit Ab3 binding to tumor cells in MHA).

Although tumor-associated carbohydrates coupled to carrier and/or mixed with adjuvant have induced IgG responses to the carbohydrate in animals and in cancer patients, these antibodies, with the exception of antibodies induced by KLH-coupled GD2 [37], did not react to the native antigen expressed by tumor cells [2, 34]. Thus, Ab2 mimicking GD2 can induce GD2-specific IgG responses, whereas GD2 itself usually elicits short-lived, low-titer IgM response of poor memory [26, 37, 41, 51].

To our knowledge, our studies demonstrate for the first time induction of $CD4^+$ T cell-dependent cellular immune responses to carbohydrate by Ab2. The few studies that have addressed this issue failed to demonstrate carbohydrate specificity of the lymphocytes induced by Ab2 mimicking GD2 [31] or SSEA-1 [55]. In our study, DTH responses were induced in response to challenge with GD2-positive, but not GD2-negative, syngeneic mouse melanoma cells, indicating the GD2 specificity of the cellular immune responses of Ab2-immunized mice. Since the mouse melanoma cells do not express MHC class I or II, these cells most likely did not directly stimulate $CD4^+$ T cells, but may have stimulated the T cells after processing by antigen-presenting cells. DTH responses were only observed in $CD4^{+/+}$, not in $CD4^{-/-}$ mice, suggesting that it reflected reactivity of $CD4^+$ T cells to challenge with GD2 presented by syngeneic tumor cells, in agreement with the involvement of $CD4^+$ T cells in DTH responses to protein antigens [14, 50].

The demonstration of DTH responses in two different mouse strains suggests the absence of a genetic linkage of these responses. Thus, murine lymphocytes primed with Ab2 in vivo consistently showed specific DTH responses to challenge with GD2-positive syngeneic tumor cells. In contrast, in vivo challenge of Ab2-immunized mice with isolated GD2 did not induce DTH responses. GD2 most likely requires coupling to a carrier for stimulation of immunity in vivo [59]. Thus, GD2 presented by mouse tumor cells elicited GD2-specific cellular immunity in mice [60].

Absence of cellular immune responses to challenge with isolated GD2 in Ab2-immunized mice is in contrast with the demonstrated proliferative GD2 responses in the PBMC of a melanoma patient, suggesting that lymphocytes are able to respond to uncoupled GD2 stimulation, although this was demonstrated in one patient only. Patient's PBMC also responded to in vitro Ab2 stimulation with proliferation of predominantly $CD4^+$ lymphocytes of Th1 type. Our data suggest that the Ab2 induced an in vitro proliferative response in the PBMC of a melanoma patient, and this response was similar to the response induced by GD2 itself. Our studies aimed at determining whether the same lymphocytes responded to both Ab2 and GD2 stimulation were inconclusive (not shown).

Thus, Ab2 are highly specific inducers of cellular immunity to carbohydrates. Induction of cellular immunity by carbohydrates themselves is a controversial issue, although several studies have convincingly demonstrated carbohydrate reactivity of the induced T cells [1, 16, 31, 39, 47, 55]. Many of the structures recognized by the T cells, such as T or Tn antigen [31], TF antigen [22] or Le^y [47] are associated with tumor cells. However, in only one of those studies [25] were the T cells reactive with the native carbohydrate on tumor cells. Interestingly, GD2 presented by syngeneic mouse EL-4 lymphoma cells elicited MHC class I-restricted CTL in mice [60]. These CTL responses were blocked by anti-GD2 mAb. However, whole tumor cell vaccines thus far have not elicited cellular immunity to gangliosides in cancer patients. Therefore, Ab2 mimicking GD2 should be evaluated for their potential to modulate patients' cellular immunity to their tumors, although the recently experienced instability of the hybridomas TA412G Cl1 and TB310B Cl1 may not allow sufficient quantities of the Ab2 for clinical trials. Nevertheless, our studies provide proof of principle for cellular immunity induction to carbohydrate by Ab2.

Future studies will determine the structural basis for GD2 mimicry by Ab2 by identifying the peptide(s) derived from the hypervariable region of the Ab2 that can induce humoral and/or cellular immunity to GD2. The feasibility of such studies is emphasized by numerous reports on carbohydrate-specific antibody induction by carbohydrate peptide mimics [3, 27, 33, 40, 57].

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