G_{D2} Oligosaccharide: Target for Cytotoxic T Lymphocytes

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Summary

Carbohydrate antigens rarely provide target epitopes for cytotoxic T lymphocytes (CTL). Disialoganglioside G_{D2} is a glycolipid expressed at high levels in human tumors and a small group of murine lymphomas (EL4, RBL5, RMA, RMA-S, A13, and BALBRVE). Immunization of C57Bl/6 mice with irradiated EL4 cells stimulated a specific CTL response and protected these animals from engraftment of EL4 lymphoma. The CTL activity resided in the $CD4^-CD8^+$ population, was dependent on T cell receptor α/β, and was not removed by anti–natural killer cell immunoabsorption, but was restricted to G_{D2} and $H\text{-}2^b$ bearing targets. CTL activity could be completely inhibited by G_{D2}-oligosaccharide–specific monoclonal antibodies and their $F(ab')_2$ fragments, but not by immunoglobulin G_3 myelomas or antibodies against G_{D3} or G_{M2}. Soluble G_{D2} did not inhibit specific tumor lysis. RMA-S lymphoma cells ($G_{D2}{}^+H\text{-}2b^-TAP_2$ deficient) were resistant to G_{D2}-specific CTL. Sialic acid–containing peptides eluted from EL4 lymphoma cells could (*a*) stabilize H-2 molecules on RMA-S cells and (*b*) sensitize them for G_{D2}-specific CTL. Control peptides (derived from vesicular stomatitis virus nucleoprotein peptide and G_{D2}-negative lymphomas) could also stabilize H-2 on RMA-S, but were resistant to G_{D2}-specific CTL. These H-2–binding peptides could be purified by anti-G_{D2} affinity chromatography. We postulate a new class of naturally occurring epitopes for T cells where branched-chain oligosaccharides are linked to peptides with anchoring motifs for the major histocompatibility complex class I pocket. While analogous to the haptens trinitrophenyl and O-β–linked acetyl-glucosamine, the potential implications of natural carbohydrates as antigenic epitopes for CTL in biology are considerable.

Binding of peptide antigens to MHC class I proteins on APC is necessary for the activation of CTL precursors through recognition by TCR, interaction with adhesion molecules, and coactivation of other accessory proteins (1–3). Detailed structural analyses have identified unique octa- and nanopeptides that lodge specifically into the groove of class I molecules. Once activated, CTL will lyse targets carrying the specific peptide in association with the appropriate MHC class I molecules. Antigen systems involved in CTL activation (1) have been primarily peptide antigens. In model systems, haptenic determinants (e.g., TNP groups) have been shown clearly to require a peptide backbone that interacts specifically with class I MHC antigen grooves (4). However, no naturally occurring haptenic system has been described to date. Carbohydrate determinants have traditionally not been thought to function as target molecules for CTL, although they activate B cells in humoral immune responses. Glycolipids are poor immunogens for antibody response (5). Rare reports of T cell response to glycolipid (e.g., G_{M3}) have implicated the role of associated peptides (6). Recently, Haurum et al. (7) presented the first evidence of immunogenicity and specificity of synthetic O-glycosylated MHC-binding peptides. In this study, we describe evidence implicating a naturally occurring G_{D2} oligosaccharide as target antigen for CTL. CTL primed to $G_{D2}{}^+$ tumors killed G_{D2}-bearing tumors that expressed the appropriate MHC class I molecules. These CTL were $Thy1^+CD8^+CD4^-TCR\text{-}\alpha/\beta^+$. Since G_{D2} oligosaccharide is a natural component of neuronal cells and human tumors, it offers a potential target for specific CTL in human diseases.

Materials and Methods

Mice. C57Bl/6, BALB/c, and CAF_1 mice were purchased from Jackson Laboratory (Bar Harbor, ME). BALB/c nu/nu mice were bred at Memorial Sloan Kettering Cancer Center (MSKCC)[1]. Tumor cells were planted ($0.5–1 \times 10^5$ cells in a volume of 100 μl) subcutaneously into C57Bl/6 mice. After implantation, tumor sizes (maximum height, width, and length) were measured over time.

Cell Lines. EL4-IL2 (TIB181), EL4, and P815 were purchased from American Type Culture Collection (Rockville, MD). T2 and A13 were obtained from Dr. B. Cheeseboro (National Institute of Allergy and Infectious Diseases, Rocky Mountain Lab, Hamilton, MO). HFL/b was provided by Dr. Frank Lilly (Albert Einstein

[1] *Abbreviations used in this paper:* MSKCC, Memorial Sloan Kettering Cancer Center; NP, nucleoprotein peptide; VSV, vesicular stomatitis virus.

College of Medicine, New York, NY). HFL/d and HFL/k were obtained from Dr. Kenneth Blank (Hahneman Medical School, Philadelphia, PA); FBL3 from Dr. Lloyd Law (National Cancer Institute, Bethesda, MD); RBL5 and BALBRVE from Dr. Shuji Ikegami (Meiji Institute of Health Science, Odawara, Japan); and RMA and RMA-S from Dr. Hans-Gustag Ljunggren (8; Karolinska Institute, Stockholm, Sweden). EL4, $B6RV_1$, $B6RV_2$, and BALBRVE were provided by Dr. Elizabeth Stockert (MSKCC). All cell lines were cultured in 10% defined calf serum (Hyclone Laboratories Inc., Logan, UT) in RPMI with 2 mM L-glutamine, 100 U/ml of penicillin (Sigma Chemical Co., St. Louis, MO), 100 μg/ml of streptomycin (Sigma Chemical Co.), and 5% CO_2 in a 37°C humidified incubator. A G_{D2}-negative EL4 cell line was prepared by subcloning.

Antibodies. Anti-G_{D2} mAbs were produced in our laboratory as previously reported (9). They were manufactured by ascites induction in BALB/c or CAF_1 mice and purified by protein A affinity chromatography (IgG) or Superose 12 (Pharmacia Inc., Piscataway, NJ) chromatography under hypotonic conditions (IgM). 3F8 was an IgG_3, and 3G6, 3A7, and 5F11 were IgMs. $F(ab')_2$ fragments were prepared by pepsin digestion as previously reported (10). Anti-G_{D3} antibody (R24) was kindly provided by Dr. Alan Houghton and anti-G_{M2} (696) antibody by Dr. Philip Livingston (MSKCC). FLOPC21, an IgG_3 myeloma, was purchased from Sigma Chemical Co. TIB114 (N.S.7), a hybridoma secreting an IgG_3 control antibody, was obtained from American Type Culture Collection, and 6H12 (control IgM) was produced in our own laboratory. Rat antibodies against Thy1.2 (5.3-2.1), CD4 (RM 4-5), CD8 (53-5.8), NK, mouse mAb against H-$2K^bD^b$ (AF6-88.5) and H-$2K^dD^d$ (SF1-1.1), and hamster anti-TCR-α/β (H57-597) were purchased from PharMingen (San Diego, CA). HB11 (anti-H-$2K^bD^b$, clone 20-8-4S, an IgG_{2a} hybridoma) was purchased from American Type Culture Collection. Rat antiidiotypic mAbs were produced in nude mice as previously described (11).

Indirect Immunofluorescence. Surface antigen expression was studied by immunofluorescence flow cytometry. Anti-H-2 (PharMingen) and anti-G_{D2} antibodies were used at 2 μg per 10^6 cells. Antibodies against rat or mouse Ig (Tago, Inc., Burlingame, CA) were used according to the manufacturer's instructions. 10^6 cells were washed with PBS containing 0.02% sodium azide. After incubation with antibodies on ice for 30 min, the cells were washed and reacted with fluorescein-labeled affinity-purified goat anti-mouse/rat antibody on ice for 30 min. The cells were washed and resuspended in 100 μl of 1% formaldehyde after washing.

CTL Induction and ^{51}Cr Release Assay. C57Bl/6 mice were immunized i.p. with EL4 cells irradiated at 12,000 rads. Spleen cells were harvested ≥21 d after immunization and stimulated in vitro by irradiated EL4. Cytotoxic activity was measured 5–6 d after in vitro culture. In brief, 2×10^3 ^{51}Cr-labeled target cells were mixed with effector cells in a final volume of 0.2 ml of medium in 96-well flat-bottomed microtiter plates (Costar Corp., Cambridge, MA). The plates were incubated for 4 h at 37°C in 5% CO_2 and then centrifuged. 100 μl of assay supernatant was counted in a gamma counter. Target cell spontaneous chromium release ranged from 10 to 25%.

Immunoadsorption by Panning. Using goat anti-rat or anti-mouse Ig (PharMingen) at 3.3 μg/ml, plastic culture dishes were coated as follows: 15 ml per 100-mm plate and 7 ml per 60-mm plate at 4°C for at least 18 h. The antibodies were removed, and the plates were washed and blocked with PBS containing 10% BSA at 4°C for 2 h. Anti-CD4, CD8, Thy1.2, or NK were incubated with mouse spleen cells at a final concentration of 2 μg per 10^7 cells per ml on ice for 30–60 min. After washing the cells three times with cold PBS containing 2% BSA, the cells were added to goat antibody-coated plates and further incubated at 4°C for 1 h. Nonadherent cells were panned again using identical steps. All the adherent fractions were combined.

Class I Molecule Stabilizing by Peptide. RMA-S cells grown to a density of 10^6 cells per ml at 37°C were maintained at 25°C for 36 h in a container with 3% CO_2. The cells were washed and resuspended to 10^6 cells per ml in 10% FCS-RPMI. 5–10 μg (in 300–500 μl) of vesicular stomatitis virus (VSV) nucleoprotein peptide (NP_{52-59}, a kind gift from Dr. J. Nikolic-Zugic, MSKCC) or 500 μl of acid eluate from murine lymphoma cells was added per 10^6 RMA-S cells. Cells were incubated at 25°C for 1 h and then at 37°C for 2 h before being separated into two aliquots: (*a*) for CTL assays, the treated cells were labeled with chromium at 37°C for 1 h, washed, and used as targets in a 4-h ^{51}Cr release assay; (*b*) a separate aliquot of cells was cultured for an additional 1 h at 37°C, and class I MHC molecules were measured by indirect immunofluorescence.

Acid Elution of Peptides from Tumor Cell Surface. EL4 cells were grown in F10 (RPMI 1640 containing 10% calf serum [Hyclone Laboratories, Inc.], 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin). For each preparative run, 10^9 viable murine lymphoma cells were pelleted and resuspended in 10 ml of 0.4% TFA (Sigma Chemical Co.) in PBS, pH 2, for 1 min at 4°C with mixing, and the supernatant was collected by microcentrifugation. After neutralization with 1 M Tris to a final pH of 7, the extract was millipore filtered (0.2 μm) and then ultrafiltered (Amicon Inc., Beverly, MA) with cutoff limits of 10 kD and then frozen at −80°C or used for RMA-S MHC class I stabilization experiments. For enzyme treatment, agarose-bound neuraminidase (Sigma Chemical Co.) and streptococcal protease (Sigma Chemical Co.) were washed in PBS and reacted with TFA tumor eluate at 0.1 U of neuraminidase or 1 U of protease per milliliter of extract. After incubation at 37°C for 2 h, the reaction was allowed to continue overnight with mixing. The enzyme beads were removed by centrifugation. For control experiments, tumor extract was treated at 37°C for 2 h and at room temperature overnight but without enzyme beads added.

Immunoaffinity Chromatography. Antibody 3F8 was dialyzed in 0.02 M sodium acetate, pH 5.0, containing 0.15 M sodium chloride, and was reacted with 50:1 vol/vol ratio of 0.5 M $NaIO_4$ for 60 min at room temperature. 1:20 vol/vol ratio of glycerol was added to neutralize the oxidizing agent for 10 min. Oxidized antibody was dialyzed in coupling buffer (0.1 M sodium acetate, 0.5 M Na_2SO_4, pH 4.5) at room temperature for 6 h and was reacted with washed Affi-Prep Hz Hydrazide Support (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions (1.5 mg of 3F8 for each milliliter of gel). Acid eluate was purified by anti-G_{D2} affinity chromatography using FPLC (Pharmacia Inc.). 0.5 M ammonium acetate was used to elute adsorbed peptides, which were dried by vacuum centrifugation and redissolved in PBS.

Vaccination Against EL4. C57Bl/6 mice were immunized with irradiated EL4 and then challenged with s.c. injection of tumorigenic EL4 tumor cells. Control mice were immunized with RMA-S and B6RV2 tumors. Engraftment was determined by periodic measurements using a fine caliper.

Results

Kinetics of Secondary CTL Response. C57Bl/6 mice were immunized i.p. (days 0 and 21) with irradiated H-2^b-bearing tumor cells EL4, EL4-IL2, RBL5, or RMA, and then challenged with s.c. injection of EL4 lymphoma 5–6 d afterwards.

These mice were protected from EL4 while the control mice immunized with saline, B6RV2, or RMA-S (both H-2^b) tumor cells showed earlier EL4 engraftment and higher mortality at 1 mo (Table 1). To study the protective immunity after i.p. injection of irradiated EL4 cells, spleen cells from C57Bl/6 mice were boosted in vitro for 5 or 6 d and assayed with EL4 targets (Fig. 1). Specific lysis was consistently found and was inhibitable by anti-G_{D2} mAb 3F8. The kinetics of the in vitro–boosted CTL response was investigated over 5 d (days 3–8). CTL activity first became detectable on day 4, peaked on days 5 to 6, and declined to half maximal killing by day 8 (Fig. 2). While most of the CTL activity was inhibitable by 3F8 during the early part of the secondary response, the degree of inhibition diminished substantially by day 8 of culture.

Effector. After 5 d of in vitro boost, effector cells were depleted of specific populations by immunoabsorption. Using cell panning on mAb-coated plastic dishes, CTL activity was found in the adherent populations for both anti-Thy1 and anti-CD8. In contrast, CTL activity resided in the nonadherent population after anti-CD4 immunoadsorption (Fig. 3). Anti-NK panning did not eliminate specific lysis. No differences were found in the phenotypic characteristics of CTL that were obtained by single immunization or hyperimmunization (data not shown).

Requirement of H-2 Molecules and TCR for Tumor Killing. Spleen CTL from EL4-primed mice were boosted in vitro for 5 d and assayed on various tumor cell lines (Table 2). Only target cells bearing G_{D2} and H-2^b molecules (EL4, RBL5, RMA, A13) were susceptible to lysis by the EL4-primed spleen CTL. RMA-S cells lacking surface class I antigens and G_{D2+} BALBRVE of a different H-2 background (H-2^d) resisted killing by these CTL. Although most of the EL4-specific CTL activity was inhibited by 3F8 when assayed against various $G_{D2}{}^+$ targets, the inhibition was close to complete only for EL4-IL2. Inhibition by anti–H-$2K^bD^b$ mAb was consistent (Table 3) and dose dependent (Fig. 4), in contrast to anti–H-$2K^dD^d$ mAb, which was ineffective. Anti–TCR-α/β mAb also strongly inhibited specific CTL activity.

Target Antigen. Anti-G_{D2} oligosaccharide antibodies during the killing phase (irrespective of isotype IgM vs IgG_3) inhibited CTL activity consistently (Table 2) and in a dose-dependent manner (Fig. 5). $F(ab')_2$ fragments were almost as effective as the intact 3F8 mAb (Table 3 and Fig. 5). Control mouse mAbs R24 (IgG_3 anti-G_{D3}), 696 (IgM anti-G_{M2}), FLOPC21 (IgG_3 myeloma), and NS.7 (IgG_3 myeloma) were

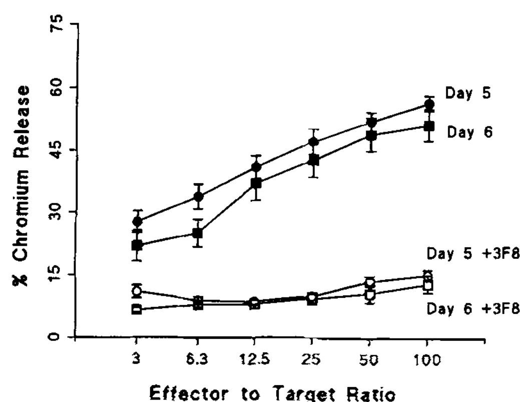

Figure 1. Specific lysis of EL4 targets measured by ^{51}Cr release. Spleen cells from EL4-immunized C57Bl/6 mice were cultured in the presence of irradiated EL4 cells for 5 or 6 d and then assayed with EL4 targets. *Solid circles,* CTL after 5 d of in vitro boost (n = 15); *solid squares,* CTL after 6 d of in vitro boost (n = 12); *open squares and circles,* CTL in the presence of 3F8 inhibitor.

Table 1. *Protection from Tumor Engraftment after Vaccination with EL4*

Immunogen	n	Size* of tumor at 2 wk	Size* of tumor at 3 wk	Alive 1 mo	NED 1 mo
EL4	7	0 (0/7)	333 (2/7)	3/7	2/7
RBL5	8	0 (0/8)	114 (3/8)	7/8	4/8
EL4-IL2	8	0 (0/8)	716 (2/8)	6/8	4/8
RMA	8	0 (0/8)	507 (7/8)	1/8	1/8
RMA-S	8	625 (4/8)	898 (7/8)	2/8	1/8
B6RV2	4	146 (3/4)	1,822 (4/4)	0/4	0/4
None	7	126 (5/7)	978 (6/6)	0/7	0/7

Mice were immunized i.p. with 5 × 10^6 irradiated (12,000 rads) tumor cells and boosted with the same number of tumor cells 21 d later. They were challenged s.c. with 10^5 EL4 cells 5–6 d afterwards. *NED,* no evidence of disease.

* Size of tumor equals the (product of maximum height, width, and length)/2, arithmetic means of tumors in tumor-bearing mice. Numbers in parentheses represent number of mice bearing tumors over total number of mice studied.

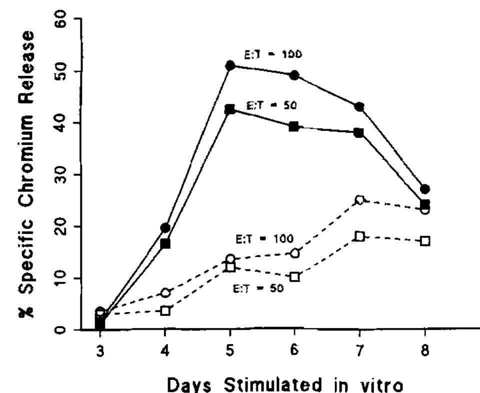

Figure 2. Kinetics of CTL response. Spleen cells from EL4-immunized C57Bl/6 mice were cultured in the presence of irradiated EL4 cells for 3–8 d and then assayed with EL4 targets. *Solid circles and squares,* specific lysis of EL4 targets; *open circles and squares,* specific lysis in the presence of anti-G_{D2} antibody (3F8, 10 μg/ml) as inhibitor.

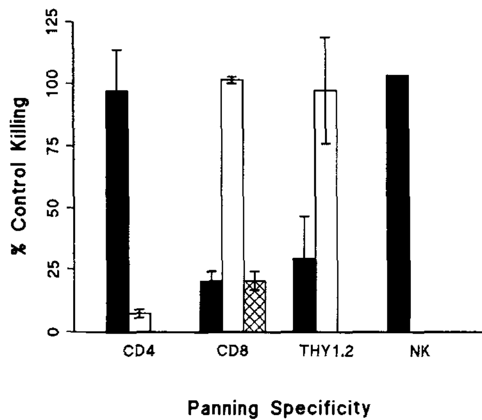

Figure 3. Distribution of CTL by panning. Spleen cells from EL4-immunized C57Bl/6 mice were cultured in the presence of irradiated EL4 cells for 5 d and then assayed with EL4 targets. *Solid bars*, nonadherent population; *open bars*, adherent population, *hatched bars*, adherent population in the presence of anti-G_{D2} (3F8, 10 μg/ml) inhibitor.

Table 2. *Percentage of Specific Lysis of Tumor Targets*

Target	GD2	N*	Percentage of lysis‡	Percentage of Inh
C57Bl/6, H-2^b				
EL4	+	4	45	80
EL4-IL2	+	4	29	98
RBL5	+	4	27	70
RMA	+	4	29	69
A13	+	4	38	89
RMA-S	+	3	7	nd
FBL3	–	2	<5	<5
B6RV$_2$	–	1	<5	nd
BALB.B, H-2^b				
HFL/b	–	4	<5	<5
BALB/c, H-2^d				
BALBRVE	+	1	<5	<5
T2	–	4	<5	<5
HFL/d	–	4	<5	<5
BALB.K, H-2^k				
HFL/k	–	4	<5	<5
DBA/2, H-2^q				
P815	–	1	<5	nd

* Number of experiments.
‡ Spleen cells from EL4-immunized mice were cultured in vitro in the presence of irradiated EL4 for 5 d and used as effectors against the targets at E/T ratio of 50:1.
%Inh, percentage of lysis inhibitable by 3F8; *nd*, not done.

Table 3. *Percentage of Inhibition of Specific Lysis by Antibodies*

mAb	Specificity	n*	Mean‡	(SE)
Mouse IgG				
20-8-4S, AF6-88.5	H-2^b	9	65	(2)
SF1-1.1	H-2^d	9	7	(2)
H57-597§	TCR-α/β	9	81	(2)
3F8	G_{D2}	9	68	(4)
3F8-F(ab')2	G_{D2}	6	47	(1)
R24	G_{D3}	6	3	(2)
FLOPC21	–	6	3	(2)
NS.7	SRBC	6	8	(2)
6H12	Breast CA	6	1	(1)
Mouse IgM				
3G6	G_{D2}	9	52	(2)
3A7	G_{D2}	6	58	(5)
5F11	G_{D2}	6	52	(13)
696	G_{M2}	6	1	(0)
Rat IgG				
A1G4	3F8 idiotype	3	7	(7)
Idio2	3F8 idiotype	3	0	(0)
2E6	Mouse IgG$_3$	3	3	(3)

Spleen cells from EL4-immunized mice were cultured in vitro in the presence of irradiated EL4 for 5 d and were used as effectors against EL4 targets at an E/T ratio of either 100:1, 50:1, or 25:1 in the presence of 10 μg/ml of inhibitor antibodies.
* Number of inhibition experiments; data at E/T ratios of 100:1, 50:1, and 25:1 were averaged.
‡ Arithmetic mean.
§ Hamster IgG.
CA, carcinoma.

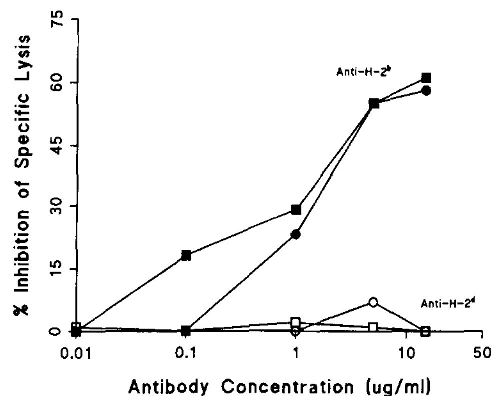

Figure 4. Inhibition of specific lysis by anti–H-2 antibodies. Spleen cells from EL4-immunized C57Bl/6 mice were cultured in the presence of irradiated EL4 cells for 5 d and then assayed with EL4 targets. *Solid and open squares*, E/T ratio of 25:1; *solid and open circles*, E/T ratio of 50:1.

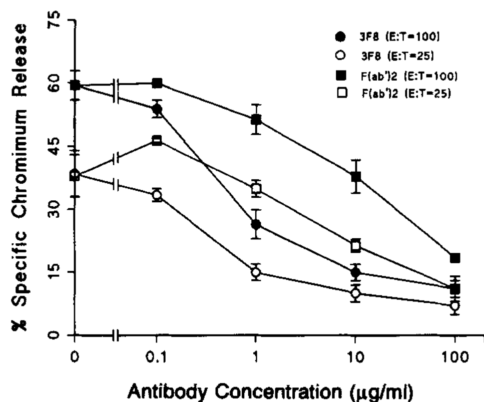

Figure 5. Inhibition of specific lysis by anti-G_{D2} antibody. Effector cells and targets as in Fig. 4. *Solid circles and squares*, E/T ratio of 100:1; *open circles and squares*, E/T ratio of 25:1; *circles*, whole 3F8 antibody; *squares*, 3F8 $F(ab')_2$ fragments.

ineffective (Table 3). Soluble G_{D2} or G_{D3} antigens (0.1–20 μg/ml) failed to inhibit CTL compared with ethanol controls; data not shown. Rat antiidiotypic mAb directed at 3F8 also failed to inhibit CTL activity. In other control experiments, anti-G_{D2} antibodies did not nonspecifically inhibit CTL activity from C57Bl/6 mice immunized with P815 or B6RV2 tumor cell lines (data not shown). In addition, CTL activity was not affected by panning with anti-G_{D2} mAb, eliminating the possibility that inhibition was mediated through G_{D2} on killer cells (data not shown). Neuraminidase treatment of EL4 targets completely eliminated anti-G_{D2} antibody–inhibitable CTL activity; however G_{D2}-independent killing was increased (data not shown).

Sialic Acid–containing Peptide Was the Target for CTL. RMA-S cells are deficient in *trans*-membrane transporters because of their defective transporter-associated with antigen processing (TAP_2) gene (12). Although both RMA-S ($H\text{-}2^{b-}$) and RMA ($H\text{-}2^{b+}$) expressed high levels of surface G_{D2}, only the latter was sensitive to G_{D2}-specific CTL induced by EL4. MHC class I antigens on RMA-S cells could be stabilized by exogenous VSV peptide, acid eluates from G_{D2}^+ or G_{D2}^- EL4 when measured by indirect immunofluorescence (Fig. 6 *A* and Table 4), but not if the acid eluate was treated by solid-phase protease (Fig. 6 *B*). Eluates from tumor cells stabilized H-2 to different degrees: those from G_{D2}^+/IL2-secreting and G_{D2}-negative EL4 cell lines were comparable to VSV, both being more potent that the eluate from G_{D2}^+ EL4 cell line. In addition, only acid eluate from G_{D2}^+ EL4 lines could sensitize RMA-S cells for killing by specific CTL, which was inhibitable by anti-G_{D2} antibodies. Neuraminidase treatment of the acid eluate did not affect its ability to stabilize H-2 on RMA-S (Fig. 6 *C* and Table 4) but eliminated its ability to sensitive RMA-S cells for killing by specific CTL. G_{D2}^+ EL4 acid eluate was purified by anti-G_{D2} affinity chromatography (Fig. 6 *C* and Table 4). While affinity-purified fractions from G_{D2}^+ EL4 cells could stabilize H-2 on RMA-S cells, those from control acid eluate could not. In addition, fractions from G_{D2}^+ EL4 cells could sensitize RMA-S targets for killing by specific CTL.

Discussion

We have shown that CTL derived from mice immunized against murine lymphoma EL4 reside in a $CD8^+CD4^-NK^-$ T cell population and will lyse tumor targets carrying cell surface ganglioside G_{D2}. Specific lysis is restricted to targets that share at least two antigens with EL4: the target antigen G_{D2} oligosaccharide and MHC class I antigen $H\text{-}2^b$. TCR-α/β is required, and CTL interaction with antigens on tumor targets appears to be inhibitable by anti-G_{D2} antibodies but not by soluble G_{D2}. The target antigen is sensitive to protease and neuraminidase treatment and can be purified by anti-G_{D2} affinity chromatography. We postulate a new class of epitopes for T cells where branched-chain carbohydrate residues are linked to peptides that bind to the MHC class I pocket. While analogous to the hapten TNP, the potential implications of carbohydrates as antigenic epitopes for CTL in biology are considerable. G_{D2} is expressed on human neuronal tissues as well as a wide spectrum of human tumors; it provides a unique model for studying T cell recognition of carbohydrate antigens in both malignant and autoimmune diseases.

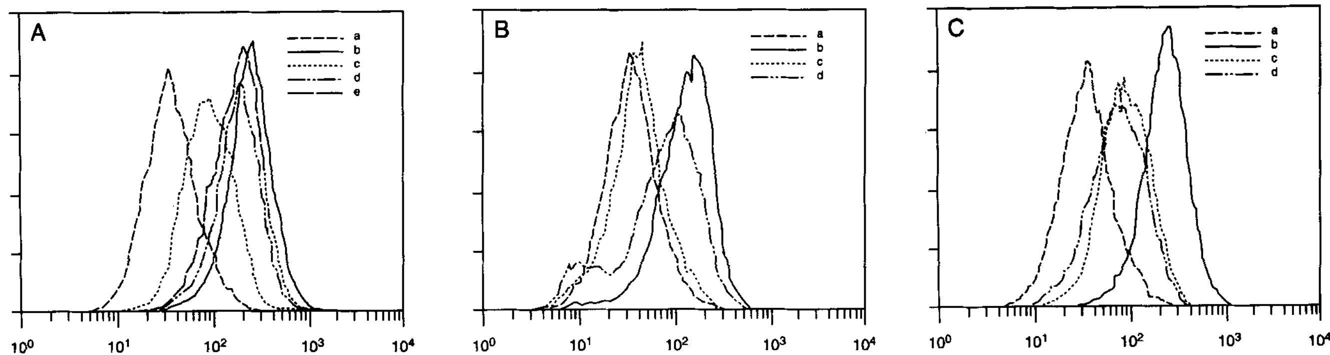

Figure 6. Histograms showing stabilization of $H\text{-}2^b$ on RMA-S cells. *y* axis = frequency; *x* axis = fluorescence channel. (*A*) *a*, control acid eluate; *b*, VSV NP peptide 5 μg/ml; *c–e* represent acid eluates from G_{D2}^+ EL4, G_{D2}^+/IL2-secreting EL4, and G_{D2}-negative EL4, respectively. (*B*) *a*, control acid eluate; *b–d* represent acid eluate from G_{D2}^+/IL2-secreting EL4 cell line before and after treatment with protease or neuraminidase, respectively. (*C*) *a*, control acid eluate; *b*, VSV NP peptide; *c* and *d* represent acid eluate from G_{D2}^+ EL4 cell line before and after affinity purification on anti-G_{D2} column.

Table 4. *Bioactivity of Eluted Peptides*

Cell line	Treatment*	H-2	Percentage of Lysis
RMA	None	+	29
RMA-S	None	−	7
RMA-S	VSV NP_{52-59}	+	2
RMA-S	Sham extract‡	−	6
RMA-S	G_{D2}^{+} EL4 extract§	+	30
	EL4-IL2 extract	+	31
	G_{D2}^{-} EL4 extract‖	+	1
RMA-S	EL4-IL2 extract		
	+ NANA bead¶	+	1
RMA-S	EL4-IL2 extract		
	+ protease bead	−	1
RMA-S	EL4 "peptides"**	+	18
RMA-S	Control‡‡	−	5

* RMA-S or RMA cells were cultured for 48 h at 25°C. The cells were washed and incubated with either medium alone or medium containing 5–10 μg/ml of VSV peptide for 1 h at 25°C, then 37°C for 3 h. H-2 expression was measured by immunofluorescence. Spleen cells from EL-4–primed mice were boosted in vitro with irradiated EL4 cells for 5 d and assayed with the cell lines as targets at E/T ratio of 50:1.
‡ TFA/PBS neutralized with Tris.
§ TFA/PBS extract from G_{D2}^{+} EL4 cell line.
‖ TFA/PBS extract from G_{D2}^{+} EL4 cell line.
¶ Acid eluate from G_{D2}^{+} EL4 cells was treated with solid phase neuraminidase (*NANA*).
** EL4 "peptides": TFA/PBS eluate from G_{D2}^{+} EL4 was purified by anti-G_{D2} affinity chromatography; n = 7; average lysis, 18% (range 10–30%); 39% inhibition by anti-G_{D2} 3F8.
‡‡ Control was sham extract purified by anti-G_{D2} affinity chromatography: n = 2; average lysis, 5%; 0% inhibition by anti-G_{D2} 3F8.

Ganglioside G_{D2} has been used as a melanoma target for T lymphocytes armed with bispecific antibodies combining anti-G_{D2} and anti-CD3 fragments (13). Antibody-blocking experiments have also implicated G_{D2} as a target for LAK cells (14). However, CTL recognition of G_{D2} oligosaccharide through the classic model of TCR has never been demonstrated. Previously, tumor-associated antigens have been isolated from EL4 and RBL5 cells (15). However, they appeared to reside in glycoprotein fractions. More recently, the tumor antigens purified from RMA cells could sensitize RMA-S targets for specific lysis by RMA-immune CTL from C57Bl/6 mice (16). The nature of these tumor antigens is still being defined (16). Since the repertoire of CTL specificity against EL4 or RMA is likely to be heterogeneous, peptide-specific CTL might coexist with oligosaccharide-specific CTL. Alternatively, although only peptides have so far been identified in the MHC class I groove, their conjugation to haptens (e.g., TNP [4, 17, 18]) can expand the repertoire of antigen specificities. Oligosaccharides, analogous to TNP, may interact with or be directly conjugated to peptides. The interaction of ganglioside and peptides has previously been reported (19). G_{M3} was found to associate with gp18 and gp80 on B16 melanoma cells. CTLs raised against this melanoma did not have MHC restriction since they killed across species barriers. Contrary to what we observed with G_{D2} CTL, in these previous reports soluble G_{M3} was able to inhibit these melanoma-specific CTL, while specific anti-MHC and anti-G_{M3} antibodies were not inhibitory at microgram per milliliter concentrations. G_{D3} has also been proposed as a potential target for antimelanoma CTL (20). Definitive conclusions will require detailed biochemical and structural analyses of oligosaccharide-modified peptides derived from tumor cells. Haurum et al. (7) recently provided the first evidence that a class I MHC–binding peptide could be modified by O-glycosylation (O-β–linked acetyl-glucosamine) within the MHC binding region without affecting its binding to MHC class I. The resulting peptides were shown to be highly immunogenic and they elicited carbohydrate-specific MHC-restricted antiglycopeptide CTL. Proteins bearing the O-β–linked acetylglucosamine are frequently found in cytosolic and nuclear compartments, adding significance to the potential role of oligosaccharide residues in creating neoantigens, loss of tolerance, and development of autoimmunity (7). The relevance of T cell response to posttranslational modifications, such as glycosylation, depends on the identification of naturally occurring epitopes by direct elution from cells in their normal and diseased states.

Glycolipids are often thought to be poorly immunogenic in both humans and in rodents. The immunogenicity of G_{D2} oligosaccharide when presented by EL4 cells is intriguing. Previous studies have identified immunogenic and nonimmunogenic variants of EL4 (21). IL-2 release was an unlikely explanation since none of these variants were secretors. Although suppressor T cells were elicited, they were induced by both immunogenic as well as their nonimmunogenic counterparts. It is possible that other activation molecules expressed by EL4 cells may be necessary for the immunogenicity of G_{D2} (3). Alternatively, unique peptides that interact with G_{D2} and $H\text{-}2K^bD^b$ class I antigens that are present on EL4 or RBL5 cells may be necessary for successful induction of G_{D2}-specific CTLs. Lastly, EL4 may function as APC and effectively induce Th1 that facilitate specific CTL activation (22).

The identification of G_{D2} oligosaccharide–specific CTL may have implications for the understanding of human disease states. G_{D2} is found in the healthy human brain (23) as well as a wide spectrum of human tumors, including neuroblastomas (23, 24), osteosarcomas (25), and other soft tissue sarcomas (26), medulloblastomas (27), high grade astrocytomas (27, 28), melanomas (29), small cell lung cancer (30), and retinoblastomas (31). Gangliosides are potential targets for CTL because of the high surface antigen density, lack of modulation, and relative homogeneity in most tumors. MHC-restricted CTL have been used successfully to target immunotherapy (32). Although MHC antigens may be downregulated in many human tumors, IFN-γ has been very effective in inducing MHC expression (33). Given the potential of CTL in human disease states (34–36), the role of G_{D2} oligosaccharide as a target deserves further investigation.

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