GD2 Oligosaccharide: Target for Cytotoxic T Lymphocytes

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Summary

Carbohydrate antigens rarely provide target epitopes for cytotoxic T lymphocytes (CTL). Disialoganglioside GD2 is a glycolipid expressed at high levels in human tumors and a small group of murine lymphomas (EL4, RBL5, RMA, RMA-S, A13, and BALBVE). 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 immunosorption, but was restricted to GD2 and H-2b bearing targets. CTL activity could be completely inhibited by GD2-oligosaccharide-specific monoclonal antibodies and their F(ab')2 fragments, but not by immunoglobulin G3 myelomas or antibodies against GD3 or GM2. Soluble GD2 did not inhibit specific tumor lysis. RMA-S lymphoma cells (GD2+ H-2b-TAP2 deficient) were resistant to GD2-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 GD2-specific CTL. Control peptides (derived from vesicular stomatitis virus nucleoprotein peptide and GD2-negative lymphomas) could also stabilize H-2 on RMA-S, but were resistant to GD2-specific CTL. These H-2-binding peptides could be purified by anti-GD2 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 hapten 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., Gm3) 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 GD2 oligosaccharide as target antigen for CTL. CTL primed to GD2+ tumors killed GD2-bearing tumors that expressed the appropriate MHC class I molecules. These CTL were Thy1+ CD8+ CD4− TCR-α/β+. Since GD2 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 CAF1 mice were purchased from Jackson Laboratory (Bar Harbor, ME). BALB/c nu/nu mice were bred at Memorial Sloan Kettering Cancer Center (MSKCC). Mice were on average 8 weeks of age at the time of tumor injection. C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). BALB/c nu/nu mice were bred at Memorial Sloan Kettering Cancer Center (MSKCC). Tumor cells were planted (0.5-1 × 105 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 College of Medicine).

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 BALB/VE from Dr. Shuji Ikemage (Meiji Institute of Health Science, Odawara, Japan); and RMA and RMA-S from Dr. Hans-Gustav Ljunngren (8; Karolinska Institute, Stockholm, Sweden). EL4, B6RV1, B6RV2, and BALB/VE 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% CO2 in a 37°C humidified incubator. A G02-negative EL4 cell line was prepared by subcloning.

Antibodies. Anti-G02 mAbs were produced in our laboratory as previously reported (9). They were manufactured by ascites induction in BALB/c or CAF mice and purified by protein A affinity chromatography (IgG) or Superose 12 (Pharmacia Inc., Piscataway, NJ) chromatography under hypotonic conditions (IgM). 3F8 was an IgG3, and 3G6, 3A7, and 5F11 were IgMs. F(ab’2) fragments were prepared by papain digestion as previously reported (10). Anti-G02 antibody (R24) was kindly provided by Dr. Alan Houghton and anti-G02 (696) antibody by Dr. Philip Livingston (MSKCC). FLOPC21, an IgG1 myeloma, was purchased from Sigma Chemical Co. TIB114 (N.S.7), a hybridoma secreting an IgG1 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&d (AF6-88.5) and H-2K’d (SFI-1.1), and hamster anti-TCR-α/β (H57-597) were purchased from PharMingen (San Diego, CA). HB11 (anti-H-2K&d, clone 20-8-4S, an IgG2a hybridoma) was purchased from American Type Culture Collection. Rat anti-idiotypic 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-G02 mAbs were used at 2 μg per 106 cells. Antibodies against rat or mouse Ig (Tago, Inc., Burlingame, CA) were used according to the manufacturer’s instructions. 106 cells were washed with PBS containing 0.02% sodium azide. After incubation with antibodies on ice for 30 min, the cells were washed and resuspended with 100 μl of 1% formaldehyde/0.1 M glycine. CTL Induction and 51Cr 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 × 103 51Cr-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% CO2 and then centrifuged. 100 μl of assay supernatant was counted in a gamma counter. Cell target 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 107 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 removed again using identical steps. All the adherent fractions were combined.

Class I Molecule Stabilizing by Peptide. RMA-S cells grown to a density of 106 cells per ml at 37°C were maintained at 25°C for 36 h in a container with 3% CO2. The cells were washed and resuspended to 106 cells per ml in 10% FCS-RPMI. 5–10 μg (in 300–500 μl) of vesicular stomatitis virus (VSV) nucleoprotein peptide (NP35-99, a kind gift from Dr. J. Nikolic-Zugic, MSKCC) or 500 μl of acid eluate from murine lymphoma cells was added per 106 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 51Cr 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, 106 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 kDa and then frozen at −80°C or used for RMA-S MHC class I stabilization experiments. For enzyme treatment, agarse-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.

Immunofluorescence. 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 NaClO for 60 min at room temperature. 1:20 vol/vol ratio of glyceral 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 Na2SO4, 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-G02 affinity chromatography using FPLC (Pharmacia Inc.). 0.5 M ammonium acetate was used to elute absorbed peptides, which were dried by vacuum centrifugation and resolubilized 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-2b-bearing tumor cell 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-2b) 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-GD2 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 immunoabsorption (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 GD2 and H-2b molecules (EL4, RBL5, RMA, A13) were susceptible to lysis by the EL4-primed spleen CTL. RMA-S cells lacking surface class I antigens and GD2, BALBVE of a different H-2 background (H-2k) resisted killing by these CTL. Although most of the EL4-specific CTL activity was inhibited by 3F8 when assayed against various Gna targets, the inhibition was close to complete only for EL4-IL2. Inhibition by anti–H-2KbDb mAb was consistent (Table 3) and dose dependent (Fig. 4), in contrast to anti–H-2KdDd mAb, which was ineffective. Anti-TCR-α/β mAb also strongly inhibited specific CTL activity.

**Target Antigen.** Anti-GD2 oligosaccharide antibodies during the killing phase (irrespective of isotype IgM vs IgG3) 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 (IgG3 anti-GD3), 696 (IgM anti-Gva), FLOPC21 (IgG3 myeloma), and NS.7 (IgG3 myeloma) were

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

| Immunogen | Size* of tumor at | Alive | NED |
|-----------|------------------|-------|-----|
|           | 2 wk 3 wk 1 mo 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 × 10⁶ irradiated (12,000 rads) tumor cells and boosted with the same number of tumor cells 21 d later. They were challenged s.c. with 10⁶ EL4 cells 5–6 d afterwards. NED, no evidence of disease.

* Size of tumor equals the (product of maximum height, width, and length)³/2, arithmetic means of tumors in tumor-bearing mice. Numbers in parentheses represent number of mice bearing tumors over total number of mice studied.

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![Figure 1](image1.png) **Figure 1.** Specific lysis of EL4 targets measured by ⁵¹Cr 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.

![Figure 2](image2.png) **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-GD2 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-GD2 (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-2b           |     |    |                    |                   |
| EL4                     | +   | 4  | 45                 | 80                |
| EL4-IL2                 | +   | 4  | 29                 | 98                |
| RBL.5                   | +   | 4  | 29                 | 70                |
| RMA                     | +   | 4  | 24                 | 69                |
| A13                     | +   | 4  | 38                 | 89                |
| RMA-S                   | +   | 3  | 7                  | nd                |
| FBL.3                   | -   | 2  | <5                 | <5                |
| B6RV.2                  | -   | 1  | <5                 | nd                |
| BALB.B, H-2b            |     |    |                    |                   |
| HFL/b                   | -   | 4  | <5                 | <5                |
| BALB/c, H-2d            |     |    |                    |                   |
| BALB.RVE                | +   | 1  | <5                 | <5                |
| T2                      | -   | 4  | <5                 | <5                |
| HFL/d                   | -   | 4  | <5                 | <5                |
| BALB.K, H-2d            |     |    |                    |                   |
| HFL/k                   | -   | 4  | <5                 | <5                |
| DBA/2, H-2d             |     |    |                    |                   |
| P815                    | -   | 1  | <5                 | nd                |

* Number of experiments.
† Percentage of lysis inhibitable by 3F8; nd, not done.

Table 3. Percentage of Inhibition of Specific Lysis by Antibodies

| mAb      | Specificity | n* | Mean² (SE) |
|----------|-------------|----|------------|
| Mouse IgG|             |    |            |
| 20-8-4S  | H-2b        | 9  | 65 (2)     |
| AF6-88.5 | H-2d        | 9  | 7 (2)      |
| SF1-1.1  | TCR-α/β     | 9  | 81 (2)     |
| H57-597s | GD2         | 9  | 68 (4)     |
| 3F8      | GD2         | 6  | 47 (1)     |
| R24      | GD2         | 6  | 3 (2)      |
| FLOPC21  |             | 6  | 3 (2)      |
| NS.7     | SRBC        | 6  | 8 (2)      |
| 6H12     | Breast CA   | 6  | 1 (1)      |
| Mouse IgM|             |    |            |
| 3G6      | GD2         | 9  | 52 (2)     |
| 3A7      | GD2         | 6  | 58 (5)     |
| 5F11     | GD2         | 6  | 52 (13)    |
| 696      | GM2         | 6  | 1 (0)      |
| Rat IgG  |             |    |            |
| A1G4     | 3F8 idiotype| 3  | 7 (7)      |
| Idio2    | 3F8 idiotype| 3  | 0 (0)      |
| 2E6      | Mouse IgG₁  | 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.
² 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.
circles and squares, 3F8 F(ab')2 fragments.

Figure 5. Inhibition of specific lysis by anti-GD2 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 GD2 or GD3 antigens (0.1-20 

Antibody Concentration (μg/ml)

Effector cells antibody-inhibitable CTL activity; however GD2-

Figure 5. Inhibition of specific lysis by anti-GD2 antibody. Effector cells

Figure 6. Histograms showing stabilization of H-2 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 GD2+ EL4, GD2+/IL2-secreting EL4, and GD2-negative EL4, respectively. (B) a, control acid eluate; b-d represent acid eluates from GD2+/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 eluates from GD2+ EL4 cell line before and after affinity purification on anti-GD2 column.

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 GD2. Specific lysis is restricted to
targets that share at least two antigens with EL4: the target antigen GD2 oligosaccharide and MHC class I antigen H-2b.

TCR-α/β is required, and CTL interaction with antigens on tumor targets appears to be inhibitable by anti-GD2 antibi-

tod but not by soluble GD2. The target antigen is sen-
titive to protease and neuraminidase treatment and can be

purified by anti-GD2 affinity chromatography. We postulate

a new class of epitopes for T cells where branched-chain car-

bohydrate residues are linked to peptides that bind to the MHC

class I pocket. While analogous to the hapten TNP, the po-
tential implications of carbohydrates as antigenic epitopes for

CTL in biology are considerable. GD2 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.

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 sta-

bilized H-2 to different degrees: those from GD2+/IL2-

secreting and GD2-negative EL4 cell lines were comparable
to VSV, both being more potent that the eluate from GD2+

EL4 cell line. In addition, only acid eluate from GD2+ EL4

lines could sensitize RMA-S cells for killing by specific CTL, which was inhibitable by anti-GD2 antibodies. Neuramini-

dase treatment of the acid eluate did not affect its ability to

stabilize H-2 on RMA-S (Fig. 6 C and Table 4) but elimi-

nated its ability to sensitive RMA-S cells for killing by specific

CTL. GD2+ EL4 acid eluate was purified by anti-GD2 affinity

chromatography (Fig. 6 C and Table 4). While affinity-purified

fractions from GD2+ EL4 cells could stabilize H-2 on RMA-S

cells, those from control acid eluate could not. In addition,

fractions from GD2+ EL4 cells could sensitize RMA-S targets

for killing by specific CTL.

Stable 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 pro-

cessing (TAP2) gene (12). Although both RMA-S (H-2b-)
and RMA (H-2b+) expressed high levels of surface GD2, only

the latter was sensitive to GD2-specific CTL induced by EL4.
MHC class I antigens on RMA-S cells could be stabilized
by exogenous VSV peptide, acid eluates from GD2+ or GD2−
Table 4. Bioactivity of Eluted Peptides

| Cell line | Treatment* | H-2 Percentage of Lysis |
|-----------|------------|------------------------|
| RMA       | None       | +                      | 29                     |
| RMA-S     | None       | –                      | 7                      |
| RMA-S     | VSV NP52,59| +                      | 2                      |
| RMA-S     | Sham extract$^*$ | –                      | 6                      |
| RMA-S     | Gd2$^*$ EL4 extract$^*$ | +                      | 30                     |
| RMA-S     | EL4-IL2 extract$^*$ | +                      | 31                     |
| RMA-S     | Gd2$^*$ EL4 extract$^*$ | +                      | 1                      |
| RMA-S     | EL4-IL2 extract + NANA 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 Gd2$^*$ EL4 cell line.  
§ TFA/PBS extract from Gd2$^*$ EL4 cell line.  
∥ Acid eluate from Gd2$^*$ EL4 cells was treated with solid phase neuraminidase (NANA).  
** EL4 “peptides”: TFA/PBS eluate from Gd2$^*$ EL4 was purified by anti-Gd2 affinity chromatography; n = 7; average lysis, 18% (range 10-30%); 39% inhibition by anti-Gd2 3F8.  
*** Control was sham extract purified by anti-Gd2 affinity chromatography: n = 2; average lysis, 5%; 0% inhibition by anti-Gd2 3F8.

Ganglioside Gd2 has been used as a melanoma target for T lymphocytes armed with bispecific antibodies combining anti-Gd2 and anti-CD3 fragments (13). Antibody-blocking experiments have also implicate that Gd2 as a target for LAK cells (14). However, CTL recognition of Gd2 gangliosaccharide 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 T cell 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). GM3 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 Gd2 CTL, in these previous reports soluble GM3 was able to inhibit these melanoma-specific CTL, while specific anti-MHC and anti-GM3 antibodies were not inhibitory at microgram per milliliter concentrations. Gd2 has also been proposed as a potential target for antimealoma 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 acetyl-glucosamine 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 Gd2 gangliosaccharide 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 Gd2 (3). Alternatively, unique peptides that interact with Gd2 and H-2KbD$^*$ class I antigens that are present on EL4 or RBL5 cells may be necessary for successful induction of Gd2-specific CTLs. Lastly, EL4 may function as APC and effectively induce Th1 that facilitate specific CTL activation (22).

The identification of Gd2 gangliosaccharide–specific CTL may have implications for the understanding of human disease states. Gd2 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 down-regulated 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 Gd2 gangliosaccharide as a target deserves further investigation.
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