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Tumor Gangliosides Inhibit the Tumor-Specific Immune Response

Robert McKallip, Ruixiang Li, and Stephan Ladisch

Tumor gangliosides are highly immunosuppressive membrane glycosphingolipids that are shed into the tumor cell microenvironment. We directly tested the impact of shed gangliosides on the in vivo antitumor immune response in a syngeneic fully autochthonous system (FBL-3 erythroleukemia cells, C57BL/6 mice, and highly purified FBL-3 cell gangliosides). The major FBL-3 ganglioside was identified as GM1b by mass spectrometry. Substantial ganglioside shedding (90 pmol/10^8 cells/h), a requisite for their inhibition of the immune function of tumor-infiltrating leukocytes, was detected. Immunosuppression by FBL-3 gangliosides was potent; 5–20 μM inhibited the tumor-specific secondary proliferative response (80–100%) and suppressed the generation of tumor-specific CTLs (97% reduction of FBL-3 cell lysis at an E:T ratio of 100:1). In vivo, coinjection of 10 nmol of FBL-3 gangliosides with a primary FBL-3 cell immunization led to a reduced response to a secondary challenge (the increase in the draining popliteal lymph node mass, cell number, and lymphocyte thymidine incorporation were lowered by 70, 69, and 72%, respectively). Coinjection of gangliosides with a secondary tumor challenge led to a 61, 74, and 42% reduction of the increase in lymph node mass, cell number, and thymidine uptake and a 63–74% inhibition of the increase of draining lymph node T cells (CD3^+), B cells (CD19^+), and dendritic cells/macrophages (Mac-3^+). Overall, the clear conclusion that tumor-derived gangliosides inhibit syngeneic antitumor immune responses implicates these molecules as a potent factor in promoting tumor formation and progression.


The ability of the immune system to recognize and eliminate spontaneously arising tumor cells is considered important in the prevention of tumor formation (1). The immune response to tumors is complex and in many cases is directed against tumor-specific Ags or tumor-specific transplantation Ags (TSTA) expressed by the tumor cells (2–4). However, many tumor cells expressing these immunogenic TSTA or tumor-specific Ags are able to escape host responses and survive to form tumors. These enigmatic observations suggest that tumor cells possess mechanisms that allow them to escape initial immune recognition and destruction.

One proposed mechanism of tumor escape is the release, by tumor cells, of soluble factors into their microenvironment, leading to the suppression of the immune response (5–7). We have hypothesized that gangliosides, a class of biologically active cell surface molecules, may function as soluble modulators of the immune response (8). Evidence that gangliosides may be active in the suppression of the antitumor immune response include studies demonstrating that tumor cells synthesize and shed gangliosides into their microenvironment (5, 8–12), coupled with studies showing that gangliosides are highly immunosuppressive in vitro. Gangliosides inhibit multiple steps in the cellular immune response, including, for example, Ag processing and presentation (13, 14), lymphocyte proliferation (13, 15–19), and the generation of a cytotoxic response (20–22). A gap in our knowledge, however, is the fact that modulation of the immune response by gangliosides in a syngeneic tumor system in vivo has not been shown, leaving us with only indirect proof of this concept. Here we have addressed this issue.

The immune response to tumors is distinct from the responses to other stimuli such as polyclonal mitogens, soluble bacterial Ags, and allogeneic stimuli (23). Thus, although the effects of gangliosides on the response to several non-tumor-associated Ags have been well documented (16–18), the effects of gangliosides on the antitumor immune response have not been completely elucidated. To overcome these pitfalls, we performed a comprehensive examination of the critical aspects of ganglioside involvement in tumor cell escape from the immune system. This includes evaluating ganglioside synthesis, shedding, and immunosuppressive activity, in a physiologically relevant in vivo tumor model.

This study has taken advantage of the well-documented and fully characterized antitumor immune response developed in the FBL-3 tumor model (23). FBL-3, an erythroleukemia of B6 origin, expresses unique TSTA that can be recognized by the immune system. Priming syngeneic mice with irradiated FBL-3 tumor cells leads to the subsequent rejection of future live tumor challenges (23). Although FBL-3 is immunogenic, injection of live FBL-3 tumor cells into naive syngeneic mice results in tumor growth, suggesting that the FBL-3 tumor cells possess mechanisms of escaping immune recognition and destruction.

Using this model, we have specifically and comprehensively examined the impact of tumor gangliosides on the antitumor immune response. We were able to directly assess, in a single system, all aspects of the hypothesis (8) that tumor cells actively shed high concentrations of gangliosides into their microenvironment and that these shed gangliosides serve as a protective barrier, binding to host lymphocytes and preventing the host from mounting an
efficient antitumor immune response. With an autochthonous system (C57BL/6 mice, syngeneic FBL-3 cells, and highly purified FBL-3 gangliosides), the metabolism (synthesis and shedding) and tumor-specific inhibition of the immune response in vitro and in vivo by the tumor gangliosides was demonstrated. This is the crux of the concept that gangliosides enhance tumor formation by an immunological mechanism. These results provide direct evidence that tumor gangliosides are highly effective inhibitors of the syngeneic antitumor immune response, thereby establishing ganglioside metabolism as an active factor in the process of tumor formation.

Materials and Methods

Tumor cells

FBL-3, a Friend virus-induced erythroleukemia of B6 origin, was kindly provided by Dr. Philip Greenberg. B78H1 melanoma, a variant of B16 melanoma, was kindly provided by Dr. Hyam Levitsky. Both cell lines were maintained in vitro by serial passage in RPMI 1640 medium supplemented with 10% FCS, 1% nonessential amino acids, sodium pyruvate, t-glutamine, penicillin 50 U/ml, streptomycin 50 μg/ml, and 10 mM HEPES buffer (BioWhittaker, Walkersville, MD).

Mice

Female 5–7 wk old C57BL/6 mice (H-2b Thy-1.2), to which the FBL-3 and B78H1 tumor cell lines are syngeneic, were obtained from Harlan Sprague Dawley.

Ganglioside isolation

The cellular gangliosides were isolated from FBL-3 tumor cells which were pelleted, lyophilized, and extracted twice with chloroform-methanol (1:1). The total lipid extract was taken to dryness. Gangliosides were purified by partitioning the dried total lipids in diisopropylether/1-butanol/0.1% aqueous NaCl (24). The lyophilized final aqueous phase was dissolved in a small volume of water, after which Sephadex G-50 gel exclusion chromatography was used to remove salt and low m.w. impurities from the ganglioside fraction. Gangliosides were quantified as nanomols of lipid-bound sialic acid by a modified colorimetric resorcinol assay (25). Analysis of gangliosides was performed using 10–20 cm precotted Silica Gel 60 high performance TLC plates. The plates were developed in chloroform-methanol, 0.25% aqueous CacCl2H2O (60:40:9). Gangliosides were visualized as purple bands with resorcinol-HCl (26).

High performance liquid chromatographic purification of gangliosides

To obtain highly purified tumor cell gangliosides for the immunological studies, in a final step the gangliosides were purified by normal phase HPLC. Briefly, ~800 nmol lipid-bound sialic acid (LBSA) of FBL-3 cell gangliosides in 100 μl HPLC water were chromatographed with a Perkin-Elmer (Norwalk, CT) HPLC system, on a LiChrosorb-NH2 column (250 mm long, 10 mm inside diameter, Merck, Germany). The separation was conducted at ambient temperature with a solvent gradient program described by Gazzotti et al. (27). The eluting solvent system was composed of acetonitrile-5 mM Sorenson’s phosphate buffer (83:17), pH 5.6 (solvent A), and acetonitrile-20 mM Sorenson’s phosphate buffer (1:1), pH 5.6 (solvent B). The gradient elution program of 80 min, at a flow rate of 6.25 ml/min, was as follows: 100% of solvent A for 7 min, then a linear gradient from 100% solvent A to solvent A-solvent B (66:34) over 53 min, and then a linear gradient from solvent A-solvent B (66:34) to solvent A-solvent B (36:64) over 20 min. The elution profile was monitored by flow-through detection of UV absorbance at 215 nm. The isolated gangliosides were lyophilized, dissolved in a small volume of distilled water, desalted by Sephadex G-50 gel exclusion chromatography, and lyophilized.

Mass spectrometric analysis

The carbohydrate structure of gangliosides was characterized by negative ion fast atom bombardment mass spectrometry (FAB-MS). Ceramide structures were elucidated by negative ion FAB collisionally activated dissociation tandem MS (FAB CAD-MS/MS) without prior derivatization using linked scans. Approximately 1 μl of ganglioside-methanol solution was mixed with 2 μl of triethanolamine (matrix) on the FAB probe tip. Ions were formed by bombardment with a 6-kV beam of xenon atoms in a JEOL HX-110 double-focusing mass spectrometer. For analysis of the ceramide structure by FAB CAD-MS/MS, the [CerO]− fragment ion was selected as the precursor ion. Helium was used as the collision gas, and the helium pressure was adjusted to reduce the abundance of the precursor by 75%. A JEOL DA-500 data system generated the linked scans (28).

Ganglioside shedding

FBL-3 tumor cells were metabolically labeled by culture for 48 h in RPMI 1640 medium containing 10 μCi each of f-1-[14C]glucosamine hydrochloride and d-1-[14C]galactose, after which the cells were washed three times in RPMI medium and resuspended at 2.0 × 10⁶ cells/ml in 3 cm² tissue culture flasks. The supernatant and the cells from the same flask were harvested 24 h after the cells were resuspended. The gangliosides present in the cells and in the supernatant were then purified by previously described methods (24). The radiolabeled gangliosides were quantified by β-scintillation counting of the ganglioside-associated radioactivity, and equal aliquots of radiolabeled gangliosides were qualitatively analyzed by high performance TLC (HPTLC) autoradiography (29) with radiolabeled rat brain gangliosides as standards.

Proliferative responses

Mice were immunized by injecting 10⁷ γ-irradiated (10,000 rad) FBL-3 tumor cells in 100 μl PBS i.p. twice at 2-wk intervals (23). The spleens were removed from these mice for use in the immunological studies 2 wk after the second injection. Single-cell suspensions of splenocytes were prepared with a laboratory homogenizer (Stomacher, Tekmar, Cincinnati, OH). Contaminating erythrocytes were lysed by resuspending the splenocytes in 5 ml lysing buffer, ACK (BioWhittaker) for 1 min and then centrifuging three times in HB104 medium. After the third wash, the splenocytes were adjusted to 4 × 10⁶ cells/ml in HB104 medium containing 2% FCS and 1% HEPES buffer. This suspension was aliquoted (25 μl) into 96-well A/2 flat-bottom plates. FBL-3 cell gangliosides in 10 μl HB104 medium were added to each well to achieve a final concentration of 0–20 μM. Finally, the cultures were stimulated by addition of 10⁴ irradiated (10,000 rad) syngeneic FBL-3 tumor cells per well. The splenocyte proliferative response was determined 4 days later by adding 0.5 μCi [3H]thymidine (DuPont NEN, Wilmington, DE) to each well for 4 h. [3H]Thymidine uptake was determined by β-scintillation counting. The possibility that the gangliosides were directly toxic to the cells was addressed by determining the number and viability of the splenocytes present at the end of the culture period by trypan blue dye exclusion.

CTL response

Splenocytes (4 × 10⁶ cells/well) from mice preimmunized against FBL-3 tumor cells as described above and then cultured in the presence or absence of 20 μM FBL-3 gangliosides in 24-well plates in a final volume of 2 ml for 4 h before restimulation with 4 × 10⁶ γ-irradiated (10,000 rad) FBL-3 tumor cells. The splenocytes were harvested 4 days later and washed three times in HB104 medium to remove unbound gangliosides. The number of splenocytes added was adjusted to achieve the desired E:T ratios and aliquoted (100 μl) to 96 round-bottom plates. Tumor targets were labeled by exposing to 1 × 10⁵ cpm 35Cr-chromate (DuPont NEN) for 1 h in RPMI medium containing 10% FCS. The tumor target cells were then washed three times in HB104 medium, adjusted to 10⁵ cells/ml, and added (5 × 10⁵ cells/50 μl/well) to 96-well round-bottom plates. Lysis of the 35Cr-labeled tumor cells was assessed in a standard 4-h chromium release assay (22). 35Cr-labeled EL-4 tumor cells were used as nonspecific control targets. Supernatants were harvested via the Skatron supernatant collection system (Skatron, Sterling, VA). Spontaneous release of label was measured in parallel wells containing a 100 μl HB104 medium and resuspended at 2.0 × 10⁶ cells/ml in 3 cm² tissue culture flasks. The supernatant and the cells from the same flask were harvested 24 h after the cells were resuspended. The gangliosides present in the cells and in the supernatant were then purified by previously described methods (24). The radiolabeled gangliosides were quantified by β-scintillation counting of the ganglioside-associated radioactivity, and equal aliquots of radiolabeled gangliosides were qualitatively analyzed by high performance TLC (HPTLC) autoradiography (29) with radiolabeled rat brain gangliosides as standards.

In vivo primary immune response

To assess the effects of gangliosides on the primary antitumor immune response, naïve C57BL/6 mice were immunized by injecting 10⁴ irradiated (10,000 rad) FBL-3 tumor cells in the presence or absence of 10 nmol FBL-3 gangliosides s.c. into their left rear footpads. Two weeks later, the effect of gangliosides on the primary immunization was assessed by determining the response to a secondary tumor challenge, measured with a modified version of the popliteal lymph node (PLN) assay (30, 31). Here, mice were rechallenged by injecting them in their left rear footpad with 10⁴ irradiated (10,000 rad) FBL-3 tumor cells in 50 μl PBS. The immune
response was measured 5 days later by determining the changes in PLN mass, cell number, and [3H]thymidine incorporation, and comparing them with those of unstimulated lymph nodes isolated from the right rear footpad. The cell number was determined after preparing a single-cell suspension of the lymph nodes with a laboratory homogenizer. The lymph node cell suspensions were adjusted to 10^6/ml in RPMI medium containing 10% FCS, and 100 μl were added to triplicate wells in a 96-well flat-bottom plate, pulsed for 18 h with 0.5 μCi [3H]thymidine, and harvested, and [3H]thymidine uptake was determined by β-scintillation counting.

In vivo secondary immune response

Mice were immunized by injecting 10^7 γ-irradiated (10,000 rad) FBL-3 tumor cells in 100 μl PBS i.p. twice at 2-wk intervals (23) and were used for the immunological studies 2 wk after the second injection. The immunized mice received a secondary challenge by injecting them in their left rear footpad with 2.5 × 10^6 irradiated (10,000 rad) FBL-3 tumor cells in 50 μl PBS, admixed with or without 10 nmol of purified FBL-3 tumor cell gangliosides. As a positive control of inhibition, immunized mice were rear-footpad injected with irradiated FBL-3 tumor cells and concomitantly received cyclosporin, 24 mg/kg i.p. daily) (32). As a control for inhibition of the antitumor immune response 2 wk after the second injection. The immunized mice received 2.5 × 10^6 irradiated (10,000 rad) FBL-3 tumor cells in 50 μl PBS, admixed with or without 10 nmol of purified FBL-3 tumor cell gangliosides. As a positive control of inhibition, immunized mice were rear-footpad injected with irradiated FBL-3 tumor cells and concomitantly received cyclosporin, 24 mg/kg i.p. daily) (32). As a control for inhibition of the antitumor immune response 2 wk after the second injection. The immunized mice received 2.5 × 10^6 irradiated unre-irradiated (876H1) tumor cells. Five days after the secondary challenge, the immune response was determined by aseptically removing the draining lymph nodes and quantifying the increase in lymph node mass, cell number, and lymphocyte DNA synthesis compared with those of unstimulated lymph nodes isolated from the right rear footpad, as described above.

Flow cytometric analysis

Cells (5 × 10^6) isolated from the PLNs of C57BL/6 mice were resuspended in cold PBS-0.5% BSA and incubated with fluorescently labeled mAb. The following mAb (PharMingen, San Diego, CA) were used: PE-conjugated hamster IgG isotype standard (anti-keyhole limpet hemocyanin); PE-conjugated anti-CD8; PE-conjugated anti-CD69; PE-conjugated anti-CD25; PE-conjugated anti-CD69; FITC-conjugated anti-CD3; FITC-conjugated anti-CD4; FITC-conjugated anti-CD19; and FITC-conjugated anti-Mac-3. The cells were stained with the mAbs for 30 min on ice, washed twice in PBS-0.5% BSA, and analyzed with a FACStarPlus flow cytometer (Becton Dickinson, Mountain View, CA).

Statistical analysis

Student’s unpaired t test (two-tailed) was used to determine the significance of differences between experimental and control data.

Results

Identification of the major FBL-3 ganglioside as G_{M1b}

FBL-3 gangliosides were isolated (24), quantified (25), and initially analyzed by HPTLC (27). FBL-3 tumor cells contained ~12 nmol gangliosides/10^6 cells. HPTLC analysis of the FBL-3 ganglioside migration pattern (Fig. 1) revealed the FBL-3 ganglioside fraction to contain one major ganglioside doublet. It migrated with HPTLC mobility between that of the human brain gangliosides standards G_m1 and G_D1a, similar to the migration of a ganglioside observed previously in several other mouse tumors and identified as G_{M1b} (28, 29).

To determine the molecular structure, the HPLC-purified FBL-3 gangliosides were characterized by negative ion FAB-MS analysis. The ganglioside preparation contained two species corresponding to the two bands of the doublet seen by HPTLC and represented by [M – H] ions at m/z 1516 and 1626 (Table I). They correspond to two molecular species of the ganglioside G_{M1b}. Fig. 2 shows the negative-ion mass spectrum of one of the molecular species of G_{M1b}. The peak at m/z 1626 represents the molecular ion, [M – H]^−. In addition, there are peaks in the mass spectrum that resulted from the loss of N-acetylneuraminic acid, galactose, N-acetylgalactosamine and a second galactose. Specifically, the molecular ion [M – H]^− at m/z 1626 yielded the peak at m/z 1335.6 by the loss of N-acetylneuraminic acid, and the peak at m/z 1173.6 by the loss of N-acetylneuraminic acid and galactose. The loss of N-acetylneuraminic acid, galactose, and N-acetylgalactosamine gave the peak at m/z 970.8, whereas the loss of N-acetylneuraminic acid, galactose, N-acetylgalactosamine, and a second galactose resulted in the peak at m/z 808.7. The corresponding [CerO]^− was also detected, at m/z 646.6.

The ceramide structures of the FBL-3 ganglioside were further examined by FAB CAD-MS/MS. In the case of the species of G_{M1b} which has a m/z 1626, the parent ion [CerO]^− at m/z 646.6 was used to produce secondary fragmentation S and T ions. The presence of an S ion at m/z 390 and a T ion at m/z 406 indicates that the long chain base is d18:1 and the fatty acid is C24:1. Therefore, the ceramide structure of this molecule was d18:1-C24:1 (Fig. 2). With this same approach, the ceramide structure of the second molecular species of G_{M1b} was identified. Thus, the analysis revealed that the G_{M1b} ganglioside had two ceramide structures, d18:1-C16:0 and d18:1-C24:1 (Table I) and led to the conclusion that two molecular species of G_{M1b}, differing in their ceramide structures, are the major gangliosides of FBL-3 tumor cells. This latter finding is consistent with the general concept, shown in a number of other studies, that tumor-derived gangliosides frequently express heterogeneous ceramide structures (28, 33–35).

FBL-3 cells shed gangliosides

Significant shedding of gangliosides by tumor cells into the cellular microenvironment is thought to be a critical element in satisfying the hypothesis that tumor gangliosides interfere with the antitumor immune response, because to interact with host leukocytes, these molecules should be released by the tumor cells. We quantified ganglioside shedding by the FBL-3 tumor cells and found that ~17% of cellular gangliosides were shed into the microenvironment in a 24-h period. This translates into a shedding rate of 90 pmol/10^6 cells/h, indicating that FBL-3 tumor cells shed gangliosides at a substantial rate. Qualitative analysis of the shed gangliosides by TLC/autoradiography revealed that G_{M1b} is both the major cellular and the major shed ganglioside (Fig. 3). This is consistent with previous observations and with the generally accepted concept that the major ganglioside species present on the
cell membrane are also the species most prominent as shed molecules in the cell culture supernatant (8, 12, 28).

**FBL-3 gangliosides suppress the in vitro secondary proliferative response to syngeneic tumor cell challenge**

As a first step in determining the effects of tumor-derived gangliosides on the antitumor immune response, the effect of FBL-3 gangliosides on the secondary proliferative response of splenocytes to syngeneic tumor cells was examined. In these experiments, splenocytes from preimmunized mice were cultured in the presence or absence of FBL-3 gangliosides and restimulated with irradiated FBL-3 tumor cells. Ganglioside concentrations as low as 5 μM caused an 80% decrease in the proliferative response, in comparison with the secondary proliferative response of control splenocytes cultured in the absence of added gangliosides. Splenocytes exposed to 20 μM gangliosides showed almost 100% inhibition of proliferation (Fig. 4A). These results demonstrate that tumor-derived gangliosides can inhibit the tumor-specific secondary proliferative response.

The possibility that gangliosides could inhibit the immune response by merely altering the kinetics of the response was examined. [3 H]Thymidine incorporation of ganglioside-exposed and control splenocytes from preimmunized mice was assessed at times before and after the maximal control response was observed (days 3–7). In this experiment, the maximal control response was observed on day 4 (Fig. 4B); whereas in the presence of 20 μM FBL-3 gangliosides, the proliferative response never achieved a level greater than 15% of the control response on any day. Thus, the addition of tumor-derived gangliosides completely inhibited the proliferative response without altering the kinetics of the response.

We excluded the possibility that the immunosuppressive activity of the FBL-3 gangliosides was the result of a nonspecific direct cytotoxic effect on the splenocytes. We exposed splenocytes from preimmunized mice to FBL-3 gangliosides for 4 days and then determined the viable cell counts. The cell counts of splenocytes incubated in 20 μM FBL-3 gangliosides were nearly equal to those of control cells (2.28 and 2.40 × 10^6/ml, respectively, representing a cell recovery of 57 and 60% of the initial 4 × 10^6 cells/ml plated). Therefore, the inhibition of the proliferative response caused by exposure of splenocytes to FBL-3 gangliosides was not the result of a direct effect on cell viability. Further supporting this conclusion is the fact that mitogen-induced lymphoproliferative responses are relatively unaffected even at ganglioside concentrations which cause marked inhibition of Ag-induced responses (36).

**FBL-3 gangliosides inhibit the generation of CTL specific for syngeneic FBL-3 tumor cells**

The ability of the immune system to develop T cells capable of recognizing and lysing specific tumor targets is critical for tumor elimination in vivo (37). To examine the effects of FBL-3 gangliosides on the CTL response, splenocytes (4 × 10^6/well) from preimmunized mice were cultured in the presence or absence (control) of 20 μM FBL-3 gangliosides and restimulated with 4 × 10^5 irradiated FBL-3 tumor cells. After 4 days of culture, the nonadherent cells were harvested and redirected against 51Cr-labeled FBL-3 tumor targets at E:T ratios ranging from 100:1 to 3:1. A vigorous specific CTL response was observed; at an E:T ratio of 100:1, there was 72% specific lysis of FBL-3 targets and no significant lysis of the unrelated target, EL-4. FBL-3 gangliosides (20 μM) had a significant inhibitory effect on the CTL response, as shown in Fig. 5. At an E:T ratio of 100:1, for example, target cell
lysis was reduced by 97%. These results clearly show that FBL-3 gangliosides inhibit the generation of tumor-specific CTLs.

The effect of tumor gangliosides on the effector function of tumor-specific CTLs was also examined. In this case, splenocytes from preimmunized mice were stimulated with irradiated FBL-3 tumor cells for 4 days to allow generation and expansion of the tumor-specific CTL population. The CTLs were harvested and cytotoxicity directed against $^{51}$Cr-labeled FBL-3 tumor targets in the presence or absence of 20 $\mu$M FBL-3 gangliosides was assessed in a standard chromium release assay. Lytic activity of the ganglioside-exposed splenocytes at an E:T ratio of 100:1 (59% lysis of tumor targets) was only slightly lower than the 72% lysis by splenocytes not exposed to gangliosides (Fig. 5). Thus, the inhibitory effect of the tumor gangliosides on the antitumor CTL response reflects a significant inhibitory effect on the generation phase combined with only a minimal effect on the effector phase of the response.

C57BL/6 mice develop a measurable and specific antitumor immune response to FBL-3 cells

The first step in the establishment of an in vivo tumor system in which the antitumor immune response could be measured was to develop a mouse model that is capable of recognizing and lysing the specific tumor cell, FBL-3. To accomplish this, C57BL/6 mice were immunized with FBL-3 tumor cells as described by Kern et al. (23). Two weeks after the second immunization, we determined whether or not the immunization was successful by injecting immunized as well as naive mice (i.e., not immunized with FBL-3 cells) with a live tumor cell challenge ($5 \times 10^6$ FBL-3 cells) and observing them for survival. All three of three immunized mice rejected the tumor challenge, whereas all naive mice rapidly developed tumors.

FIGURE 4. Tumor-derived gangliosides inhibit the secondary proliferative response to syngeneic tumor challenge. A, Splenocytes ($4 \times 10^5$) from previously immunized mice were rechallenged with irradiated FBL-3 tumor ($4 \times 10^4$) cells in the presence (○) or absence (hatched area of 2.5–20 $\mu$M FBL-3 gangliosides. The cells were pulsed with 0.5 $\mu$Ci $[^3H]$thymidine for 4 h, 4 days after stimulation. B, The kinetics of the proliferative response was examined by stimulating splenocytes from preimmunized mice ($4 \times 10^5$) with irradiated FBL-3 tumor cells ($4 \times 10^4$) in the presence (○) or absence (□) of 20 $\mu$M FBL-3 cell gangliosides and determining the proliferative response on days 3–7 by $\beta$-scintillation counting. Data are means ± SE of triplicate determinations. The proliferative responses of splenocytes treated with 5–20 $\mu$M FBL-3 gangliosides were significantly different from that of control splenocytes, $p < 0.01$.

FIGURE 5. Tumor-derived gangliosides inhibit the CTL response to FBL-3 tumor challenge. The effects of added FBL-3 gangliosides on the generation phase of the antitumor CTL response were determined by restimulating splenocytes from previously immunized mice with irradiated FBL-3 tumor cells for 5 days in the presence (○) or absence (□) of 20 $\mu$M gangliosides, washing them, and measuring cytotoxicity against $^{51}$Cr-labeled FBL-3 tumor targets in a standard chromium release assay. To determine the effect of tumor-derived gangliosides on the effector phase, CTLs were generated in the absence of gangliosides and then directed against $^{51}$Cr-labeled FBL-3 tumor cells in the presence of 20 $\mu$M FBL-3 gangliosides in a standard 4-h chromium release assay (■). The SE of the triplicates was routinely <10% of the total release.

C57BL/6 mice develop a measurable and specific antitumor immune response to FBL-3 cells

To determine whether an immune response to the FBL-3 tumor cells could be detected in vivo, we used a modified PLN assay (30, 31). Previously immunized mice were challenged in their left rear footpads with $2.5 \times 10^6$ irradiated FBL-3 tumor cells or B78H1 tumor cells. Five days after the injection, the immune response was quantified by harvesting the stimulated draining PLNs and comparing their mass and cell number to those of unstimulated PLNs. FBL-3-stimulated lymph nodes showed an increase in mass of $1.9 \text{ mg}$ and an increase in cell number of $1.7 \times 10^6$/node, compared with unstimulated lymph nodes, demonstrating that there was a
vigorouand quantifiable immune response to the syngeneic FBL-3 tumor cells. In contrast, mice preimmunized with irradiated FBL-3 and challenged with unrelated tumor cells (B78H1) did not produce a significant response (0.3 ± 0.2 mg increase in mass and no detectable increase in cell number), demonstrating that the immune response was specific for the FBL-3 tumor cells. These results confirm previous studies (38) showing that C57BL/6 mice immunized with FBL-3 cells can develop an effective and specific immune response to an FBL-3 tumor challenge and demonstrate that this model can be used to directly measure the effects of tumor gangliosides on the antitumor immune response to syngeneic tumor cells.

**FBL-3 tumor gangliosides inhibit the in vivo primary antitumor immune response to FBL-3 tumor cells**

The primary immune response to a tumor challenge most closely mirrors the likely initial interaction between the tumor cell and the immune system. Unsuccessful attempts to directly assess this response suggest that the FBL-3 tumor cells are not sufficiently immunogenic to produce a measurable primary immune response in the PLN assay. Therefore, to measure the effects of tumor gangliosides on the primary antitumor immune response in vivo, 10 nmol of FBL-3 gangliosides were coinjected with the primary FBL-3 cell immunization into naive mice. The modulatory effects of the tumor gangliosides on the primary immune response were assessed by determining how this influenced the response to a subsequent (secondary) challenge with 10⁵ irradiated FBL-3 cells alone. This challenge was administered 2 wk later, and the response was quantified 5 days after the challenge by determining the changes in PLN mass, cell number, and [³H]thymidine uptake, compared with unstimulated control nodes. Mice given a primary FBL-3 immunization in the absence of gangliosides had a significant secondary anti-FBL-3 immune response; the PLN mass increased by 2.8 ± 0.3 mg (Fig. 6A), the cell number increased by 2.7 × 10⁶ cells/node (Fig. 6B), and the thymidine uptake increased by 6.8 × 10⁵ dpm (Fig. 6C), compared with unstimulated control lymph nodes. However, when the immunizing FBL-3 tumor cells were admixed with 10 nmol FBL-3 gangliosides, there was a significant (p < 0.001) reduction in the secondary immune response (70, 69, and 72% reduction of the FBL-3 cell-induced increase in PLN mass, cell number, and thymidine incorporation, respectively), which directly reflects a reduction of the efficiency of the primary immune response. These results demonstrate that tumor gangliosides significantly inhibit the primary antitumor immune response in vivo.

**FBL-3 tumor gangliosides inhibit the increase in lymph node mass in response to secondary syngeneic tumor cell challenge in vivo**

In addition to examining the effects of tumor gangliosides on the primary antitumor immune response, using the same assay system, the modified PLN assay, we quantified the effects of FBL-3 gangliosides on the in vivo secondary antitumor immune response. The first parameter measured was the increase in PLN mass in response to syngeneic FBL-3 tumor cell challenge. When previously immunized mice were stimulated with FBL-3 tumor cells admixed with FBL-3 gangliosides (10 nmol), there was a marked reduction in the increase in PLN mass. Whereas the mean increase in lymph node mass in mice stimulated with FBL-3 tumor cells was 1.85 mg, the coinjection of purified FBL-3 gangliosides with FBL-3 tumor cells resulted in only a 0.72-mg increase in lymph node mass. This 61% inhibition of the response demonstrates that FBL-3 gangliosides significantly (p ≤ 0.02) suppress the increase in lymph node mass caused by syngeneic tumor challenge in vivo (Fig. 7A), and in fact to a degree similar to that caused by treatment of mice with cyclosporin (24 mg/kg i.p. daily for 4 days), a standard system for suppression of the cellular immune response (32).

**FBL-3 gangliosides inhibit the secondary lymphoproliferative response in vivo**

The effect of FBL-3 gangliosides on the in vivo secondary lymphoproliferative response to syngeneic tumor challenge was directly examined in two ways: by determination of the increase in PLN cell number; and by quantification of the spontaneous proliferative response. Unstimulated lymph nodes contained 0.19 ± 10⁶ cells/node. Lymph nodes stimulated with γ-irradiated FBL-3 cells contained 1.83 ± 10⁶ cells/node, which represents an almost 10-fold increase in cell number. However, when the FBL-3 cell challenge was injected together with FBL-3 gangliosides, the draining lymph nodes harvested 5 days later contained only 0.73 ± 10⁶ cells, indicating a marked inhibition by gangliosides of the increase in cell number (Fig. 7B). FBL-3 gangliosides also significantly (p = 0.006) reduced the spontaneous proliferative response, as measured by [³H]thymidine uptake, by 41%, from
1.06 × 10^3 dpm/10^5 cells to 0.63 × 10^3 dpm/10^5 cells (Fig. 7C). Once again these results indicated a similar effect to that achieved by the repeated injection of cyclosporin (Fig. 7, B and C). These in vivo studies provide strong evidence that FBL-3 gangliosides significantly suppress the antitumor immune response in vivo.

**FBL-3 gangliosides inhibit the tumor-induced activation and expansion of lymph node cell populations in vivo**

Changes in the PLN populations induced by gangliosides were further delineated by determining the expression of cell surface markers specific for cell lineage and activation status. Lymph node cells from mice injected in the footpad with irradiated FBL-3 cells admixed with FBL-3 gangliosides (10 nmol) or injected with irradiated FBL-3 cells alone (control) were stained with fluorescence-conjugated mAbs for lineage-specific markers including CD3 (T cells), CD4 (Th cell), CD8 (CTL), CD19 (B cells), and Mac-3 (dendritic cells/macrophage) as well as activation markers CD69 (early activation marker), and CD25 (IL-2 receptor) and analyzed by FACS. This allowed us to determine changes in both the total number and activation status of cells from the various cell populations involved in the antitumor immune response in vivo. Comparing the cell populations of lymph nodes draining the site of FBL-3 cell injection with the cell populations of unstimulated lymph nodes, it was apparent that s.c. injection of the FBL-3 cells caused a significant increase in all cell populations examined (Fig. 8A). This increase was suppressed in the lymph nodes of mice also receiving the FBL-3 gangliosides. For example, the T cell population (CD3+) increased from 2.2 × 10^5 cells/node in the unstimulated lymph node to 11.1 × 10^5 cells/node in the stimulated, control lymph nodes, but to only 5.5 × 10^5 cells/node in the stimulated lymph nodes of ganglioside-treated mice (63% decrease). These ganglioside-induced changes were evident in the T cell subpopulations, in which there was 57 and 65% inhibition of the CTL and T helper cell populations, as well as in the B cell and dendritic cell/macrophage populations, which were decreased by 74 and 69%, respectively. The expression of activation markers was also affected; coinjection of FBL-3 tumor gangliosides with FBL-3 cells led to a significantly reduced number of lymph node cells expressing CD69 and CD25 (60 and 71%, respectively). Overall, ganglioside-induced inhibition of each lymph node cell subpopulation studied was statistically significant (p ≤ 0.04), whereas the distribution of the cell populations showed that the inhibition was not specific for any single population examined.

**FIGURE 7.** Tumor-derived gangliosides inhibit the draining lymph node response to syngeneic tumor cell challenge in vivo. In a modified PLN assay, preimmunized mice were injected s.c. (footpad) with 2.5 × 10^6 irradiated FBL-3 tumor cells together with (gan) or without (med) 10 nmol FBL-3 gangliosides. Mice treated with cyclosporin (CsA) served as positive controls for immunosuppression. The immune response was determined 5 days later as the net increase in the PLN mass (A), cell number (B), and [^3]H]thymidine uptake (C).

**FIGURE 8.** FACS analysis of the effect of in vivo tumor ganglioside administration upon draining lymph node cell subpopulations. Lymph node cells isolated from mice 5 days after receiving either a tumor cell challenge, alone (■) or together with 10 nmol FBL-3 gangliosides (□), were compared with control unstimulated lymph node cell populations (□). A, Absolute number of cells expressing the indicated markers. B, percent of the total lymph node cell population expressing the same markers. Data represent the mean ± SE of the results of three separate experiments.
(Fig. 8B). Thus, the in vivo administration of the tumor gangliosides caused a generalized suppression of the observed increase of cell populations associated with the antitumor immune response in vivo.

Discussion

The hypothesis that gangliosides shed by tumors bind to host leukocytes and suppress the antitumor immune response is supported by a number of observations. It has been shown that tumor cells synthesize and shed gangliosides (5, 8–12) and that these shed molecules directly bind to target cells in vitro (39, 40). Other work has demonstrated the immunosuppressive activity of gangliosides. Gangliosides inhibit the in vitro proliferative response of lymphocytes to both nonspecific mitogens (16–18) and soluble Ags (e.g., tetanus toxoid) (8, 41) and have been shown to be inhibitors of the cytotoxic response in vitro (20–22). Initial in vivo studies demonstrated that gangliosides are highly effective in suppressing the allogeneic immune response (31, 42), providing one possible explanation for the earlier observation that addition of gangliosides to poorly tumorogenic cells significantly increased their tumorigenic potential in vivo (29). Together, these findings add a possible role of gangliosides in modulating the ability of tumor cells to escape host immune destruction. However, before the present work, there has been no direct evidence, either in vitro or in vivo, of inhibition of the specific antitumor immune response by tumor-derived gangliosides.

The present study has taken advantage of the well-documented antitumor immune response developed in the FBL-3 tumor model, allowing us to comprehensively examine the impact of tumor gangliosides on the antitumor immune response. Here we directly tested the hypothesis that tumor cells actively shed biologically active gangliosides into their microenvironment, where they serve as a protective barrier, binding to host lymphocytes and preventing them from developing an efficient antitumor immune response. We found that FBL-3 tumor cells shed gangliosides at a high rate, creating a tumor microenvironment rich in tumor-derived gangliosides. Using the highly purified FBL-3 gangliosides, we showed in vitro inhibition of the tumor-specific secondary proliferative response and of the generation of tumor-specific CTLs. In vivo, we observed inhibition of both the primary and secondary antitumor immune responses. These studies provide the first direct evidence that tumor gangliosides are highly effective inhibitors of the syngeneic antitumor immune response and thereby establish tumor cell ganglioside metabolism as an active factor in the process of tumor formation.

Immunosuppressive activity is a general biological property of gangliosides that is not specifically limited by the source of the gangliosides but that is influenced by their molecular structure. In fact, gangliosides other than the disialoganglioside G_{M16} studied here, such as those isolated from LAN-5 neuroblastoma cells (which are mainly G_{D2} and G_{M2}) (43), also are effective inhibitors of the immune response to FBL-3 tumor cells (unpublished data). Variations in either the carbohydrate or the ceramide structure can lead to differing degrees of activity. For example, in comparing a series of highly purified normal human brain gangliosides, those with a terminal sialic acid linked to a compact neutral oligosaccharide had the greatest immunosuppressive activity (36). Other studies examining the relationship between structure and activity revealed that tumor gangliosides were frequently more immunosuppressive than corresponding normal human brain gangliosides of identical carbohydrate structure. These differences were inversely related to the length of the ceramide fatty acyl chain (34). Thus, immunosuppressive activity is quantitatively affected by ganglioside structure.

Another factor influencing activity is presence of these molecules in the tumor microenvironment. Because tumor cells shed gangliosides at a high rate, it was predicted that the concentration of gangliosides in the tumor microenvironment would be very high. This was supported by recent findings in an in vitro system in which the shedding rate and microenvironmental ganglioside concentrations could be estimated (39, 40). Therefore, in contrast to lower concentrations in the peripheral circulation, the tumor cell microenvironment may contain high concentrations of highly active gangliosides, leading to maximal inhibition of the antitumor immune response at the site of tumor formation and progression.

The mechanisms of ganglioside-induced immunosuppression are most likely multiple and remain to be fully elucidated. However, it is clear that both APC and T cells are affected by tumor gangliosides. Some of the known mechanisms are reversible inhibition of APC function (13, 14), inhibition of IL-2-induced T cell proliferation (44), and down-regulation of CD4 expression (45–47). Most recently, it has been shown that exposure of mouse splenocytes to gangliosides results in reduced gene transcription of the Th1-associated cytokines, IL-2 and IFN-\(\gamma\), while leaving gene transcription of the Th2-associated cytokines, IL-4 and IL-10, unaffected (48). Others have reported a ganglioside-induced increase in T cell IL-10 production (49). Together, these findings suggest that shed tumor gangliosides may shift the balance of the antitumor immune response from the normally predominant Th1 response toward the Th2 response, possibly leading to a reduction in the cellular antitumor immune response critical for tumor elimination. Because the earliest stage of the cellular immune response known to be affected by exposure to gangliosides is Ag presentation (13, 14), it is tempting to speculate that the initial step in tumor ganglioside-induced immunosuppression in vivo is interference with the functions of APC, which in the FBL-3 tumor model would predominantly be dendritic cells.

Notwithstanding gaps in knowledge regarding the mechanism of immunosuppression by tumor gangliosides, the conclusion supported by the present work, that gangliosides shed by tumor cells can suppress the ability of the immune system to recognize and eliminate tumor cells, may have direct relevance to human cancer. Corroborating evidence includes the observations that aggressive neuroblastomas shed significant quantities of gangliosides in vivo and that the level of shed gangliosides was directly related to the incidence of tumor recurrence and the rapidity of progression (50). It has been proposed that clinically it may be possible to boost the immune response, specifically or nonspecifically, to enhance tumor cell recognition and elimination (51–53). However, to date such attempts have had only limited success. This may stem in part from the ability of tumor cells to escape immune recognition by various mechanisms, one being the shedding of tumor gangliosides into the microenvironment. Thus, a potentially therapeutic enhancement of the immune response could be negated by the particular immunosuppressive activity of the tumor. Approaches designed to inhibit such tumor cell escape mechanisms would be desirable. Recently, it was shown that metabolic depletion of tumor gangliosides is possible by treating cells with inhibitors of the synthesis of glucosylceramide, a metabolic precursor of gangliosides (40, 43, 54, 55). With the recognition of a causative role of gangliosides in tumor-associated immunosuppression and a facilitating role in tumor progression, the use of such inhibitors of ganglioside synthesis might enhance the effectiveness of immunotherapeutic approaches to cancer.
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