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Murine Leukemia. Proposed Role for Gangliosides in Immune Suppression*†

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Abstract—*Glycolipid-bound sialic acid levels were elevated 2 to 4-fold in the sera of two strains of mice bearing thymic lymphoma produced either spontaneously (AKR/J) or due to chemical carcinogenesis [Swiss mice injected with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboximide]. The serum glycolipid-bound sialic acid level reflected the tumor burden of the AKR/J mice during the early stages of leukemogenesis. Furthermore, the elevation was found to coincide with the ontogenesis of thymic lymphoma and not to be simply an age-dependent phenomenon. TLC analysis of Florisil column-purified gangliosides from the sera of AKR/J and Swiss mice suggested presence of gangliosides with mobilities very close to GM2 and GM3 standards respectively. On the premise that the elevated glycolipid levels in circulation might interfere with normal lymphocyte functions, the immunoinhibitory properties of exogenously added mixed gangliosides were examined on tests of* in vitro *correlates of the immune response. Gangliosides inhibited concanavalin A and lipopolysaccharide-induced* [^{3}H]*-thymidine,* [^{14}C]*-leucine and* [^{3}H]*-lysine uptake by normal AKR/J mouse thymocytes and spleen cells. Mixed gangliosides also suppressed the two-way mixed lymphocyte reaction of AKR/J × Swiss and AKR/J × DBA/2 spleen cells. These and other results strongly suggest a general immunologically relevant role for gangliosides in the ontogeny of thymic lymphoma of mice.*

INTRODUCTION

TUMOR-INDUCED non-specific immunosuppression is a common feature of cancer. However, the complex relationship between serum factors and the cells of the immune system does not appear to be the same in different malignant situations. Acidic glycosphingolipids (gangliosides) are known to be membrane components of normal lymphocytes [1, 2] as well as lymphoid cell lines [3, 4], and have been found to be elevated in the circulation of tumor-bearing mice and human patients [5–7]. This laboratory has recently reported a higher content of total gangliosides in thymic lymphoma as well as in plasma of tumor-bearing AKR/J mice [8]. In this communication we have examined whether the serum glycolipid-bound sialic acid levels reflect the tumor burden of the leukemic mice or follows the time course of tumor development. Also, the studies have been extended to another animal model of chemical carcinogenesis [9], i.e., Swiss mice which develop thymic lymphoma as a result of a single injection of 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboximide (DTIC). Attempts have been made to determine the predominant species of serum gangliosides in these two tumor models.

The level of immunocompetence in tumor-bearing animals and human patients is known to be low [10, 11]. In particular, subnormal proliferative response of leukemic splenocytes to mitogens have been reported by different laboratories [12–14]. Also, leukemic serum was reported to contain factor(s) capable of inhibiting mitogenic reactivity of normal lymphocytes [15, 16]. We have previously reported that bovine brain gangliosides inhibit concanavalin A (Con A)-induced thymocyte blastogenesis *in vitro*, and this property is shared by different types of gangliosides [17, 18]. Miller and Esselman noted that addition of gangliosides as liposomes resulted in suppression of the num-

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Abbreviations: Con A: concanavalin A; MLR: mixed lymphocyte reaction; LPS: lipopolysaccharide; HMEM-TBH: Hanks' Minimal Essential Medium containing Tes-Bes-HEPES buffers; GM1: Monosialoganglioside; DTIC: 5-(3,3-dimethyl-1-triazeno) imidazole-4 carboximide; NeuAc: *N*-acetylneuraminic acid.

ber of plaque-forming cells by mouse spleen cells *in vitro* [19].

Further proof of definitive immunoinhibitory properties of exogenous gangliosides required demonstrations using other mitogens and cell types, as well as an assessment of different parameters of immune response. The current studies extend to the ganglioside effect on mouse spleen cells activated by the B cell mitogen lipopolysaccharide (LPS) or T cell mitogen phytohemagglutinin (PHA). In addition, we have tested the ability of exogenous gangliosides to interfere with the mixed lymphocyte reaction (MLR), another parameter of cell-mediated immune response. The results obtained appear to substantiate the concept of immunomodulatory role of gangliosides in tumorigenesis.

MATERIALS AND METHODS

Mice

Three strains of mice were used. Male AKR/J mice (The Jackson Laboratory, Bar Harbor, ME) were used for most of the work. Approximately 80–90% of AKR mice develop thymic lymphomas in about 6–9 months of age. For the study of changes in glycolipid-bound sialic acid occurring during leukemogenesis, the mice were divided into 2 groups: (a) a group of normal young mice of 2–4 months of age with no evidence of leukemia; and (b) a leukemic group with thymic lymphomas frequently associated with splenomegaly. A complete description of the model is published elsewhere [18]. The second animal tumor model involves outbred male ARS HA/ICR Swiss Albino mice (Sprague–Dawley, Madison, WI), which develop thymic lymphoma following a single intraperitoneal injection of DTIC (0.32 mg/g body weight) at 4 weeks of age [9]. Starting at 70 days following injection, thymic lymphomas start to appear and more than 90% will have tumors within 125 days of injection. For MLR experiments, spleen cells were obtained from normal 2 to 4-month-old DBA/2 mice.

Chemicals

Mixed bovine brain gangliosides, and monosialoganglioside (GM1) were purchased from Supelco, Bellefonte, PA. TLC analysis of the mixed gangliosides showed the mono-, di- and trisialogangliosides (in decreasing order) as the major gangliosides. Ganglioside solutions were made in culture medium (HMEM-TBH described below) and sonicated for 30 sec with a Sonifier Cell Disruptor (Bronson Sonic Power Co., Plainview, NY), equipped with a microtip. DTIC and *N*-acetylneuraminic acid (NeuAc) were purchased from Clinical Branch, Collaborative Research, National Cancer Institute and Sigma Chemical Co. respectively. Florisil (100–200 mesh) was supplied by Fisher Scientific Co.

Cell preparation

Suspensions of thymocytes or spleen cells from 2 to 4-month-old AKR/J, Swiss or DBA/2 mice were prepared in Eagle's Minimal Essential Medium with Hanks' Salt Solution, containing Tris–BES–Hepes buffer, pH 7.4 (HMEM-TBH), as described previously [18]. Cell viability at the beginning and termination of cultures was determined by Trypan blue exclusion. Lymphocyte viability in presence of gangliosides was ±10% of control cells at the end of 2- or 3-day culture.

Serum

Individual mice of indicated strain (normal or tumor-bearing) were exsanguinated and sera obtained following centrifugation at 12,000 ***g*** for 1–2 min were stored frozen. Glycolipids were extracted by precipitation of the lipo- and glycoproteins by the citrate method described by Kloppel *et al.* [5], and lipid-bound sialic acid in the chloroform–methanol extract determined. A quantitative recovery (approximately 90%) of added [^{3}H]-GM1 (prepared as described in ref [20]) was noted under these conditions of extraction.

Extraction of serum gangliosides

The procedure of Saito and Hakomori [21], as modified slightly by S. Basu (Notre Dame University, Indiana), was followed. Two to three ml of clear, pooled sera were extracted overnight with 4 vol. of chloroform:methanol (1:1.5). After centrifugation, the upper and lower phases were removed and combined along with a chloroform:methanol (2:1) extract of the interphase precipitate. The combined extract was evaporated in a Rotovapor at 40–45°C. The residue was dissolved in 2 ml of chloroform:methanol (2:1), sonicated briefly and centrifuged (12,000 ***g***, 2 min). The sediment was extracted twice with the same solvent. The supernatant and washings were combined and evaporated under N_2 in a Teflon-capped centrifuge tube. The residue was suspended in 0.9 ml pyridine and 0.6 ml of acetic anhydride and kept airtight at 60°C. This acetylated lipid extract was extracted repeatedly with toluene (2 ml × 5 times), dissolved in 2 ml of 1,2-dichloroethane (2 ml × 5 times), dissolved in 2 ml of 1,2-dichloroethane

and applied to a Florisil column (0.5 g of 'Florisil' in 0.5 × 4 cm column equilibrated with dichloroethane). After washing the column with 2 ml of dichloroethane, acetylated gangliosides were eluted with 5 ml of dichloroethane:acetone mixture (1:1). The eluate was evaporated and subjected to deacetylation in 0.2 M NaOH in CH_3OH (0.75 ml) overnight at 37°C, tightly closed in the same container. The pH was then adjusted to 6–7 by adding small quantities of Dowex-50-H^+ suspended in methanol. Purified glycolipids in the supernatant were transferred to fresh tubes and evaporated under N_2 along with methanol washing of the Dowex-50-H^+. The final product was dissolved in chloroform:methanol (2:1) for use in the subsequent chromatographic separation.

Thin-layer chromatography

Samples or standards were applied to silica gel G-coated plates (Brinkman Instruments, Inc.) and developed ascending in solvent A ($CHCl_3$:CH_3OH:H_2O; 60:35:8 v/v containing 0.05% $CaCl_2$) or solvent B ($CHCl_3$:CH_3OH:2.5 N NH_4OH; 60:40:9 v/v) for 2.5 hr at room temperature. Air-dried plates were briefly exposed to iodine vapor, which produced yellow spots. After complete disappearance of the yellow spots, plates sprayed with resorcinol [22], developed at 120°C for 15–20 min and NeuAc positive areas noted. Standards used include *N*-glycolyl neuraminic acid containing GM3 ($GM3_{(G)}$, a kind gift from Dr. Hakomori), *N*-acetyl neuraminic acid containing GM3 ($GM3_{(A)}$, a kind gift from Dr. S. Basu), GM2, GM1, GD1a (bovine brain, Supelco, Inc.) and NeuAc.

Determination of N-*acetylneuraminic acid and ganglioside content*

Lipid-bound sialic acid from serum or from tissue extracts was hydrolyzed at 80°C in 0.05 M H_2SO_4 for 2 hr to release *N*-acetylneuraminic acid and taken for colorimetric determination by micro-modification of Warren's procedure (total volume of 1.4 ml instead of 4.3 ml, as originally described) and corrected for absorption due to interfering substances [23].

Mitogen induced lymphocyte transformation

Cells (1.25×10^6 viable count) were incubated in 0.25 ml of HMEM-TBH containing 5% (v/v) heat-inactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and glutamine (2 mM) in microtiter plates (Falcon plastics, Oxnard, CA) in a humidified atmosphere of 5% CO_2–95% air at 37°. Optimal doses of mitogens, Con A, from Miles Laboratory, Kankakee, IL (0.5 μg/well) or Lipopolysaccharide W of *S. typhose*, obtained from Difco Laboratories, Detroit, MI (10 μg/well), or phytohemagglutinin (PHA-P) from Gibco (10 μg/well), that induced maximal blastogenesis were added at the beginning of cultures. Twenty-four hr prior to cell harvest, 1 μCi of [methyl-^{3}H]-thymidine (5 Ci/mmol, New England Nuclear, Boston, MA) or 2 μCi of [U-^{14}C]-lysine (264 mCi/mmol, Amersham) were added per well and cells harvested on glass fiber filter and washed sequentially with saline-EDTA (1 mM), 5% TCA, water and finally with methanol, using a MASH harvester [18]. In some experiments, cultures were pulsed with 0.1 μCi of [U-^{14}C]-L-leucine (342 mCi/mmol, Amersham) 24 hr prior to harvest. At the end of indicated periods, cultures were transferred to fresh 12 × 75 mm Falcon tubes and kept in an ice bath. To remove residual cells from the culture wells, saline-EDTA was added to each well and the washings collected in Falcon tubes after 20 min. In order to achieve uniform and better recovery of cells and proteins in the medium during subsequent acid-precipitation and centrifugation steps, 5×10^6 carrier thymus cells suspended in 0.3 ml of 33% fetal calf serum in saline-EDTA were added to each of the above Falcon tubes containing recovered cells and medium wash, and centrifuged. The supernatant (mostly containing labeled secreted proteins) was separated and subjected to 5% trichloroacetic acid precipitation at 0–4°C twice. The cell pellets were further washed with saline-EDTA and subjected to 5% TCA precipitation at 0–4°C twice. The TCA pellets were then solubilized in hyamine hydroxide at 55°C and radioactivity determined.

Mixed lymphocyte reaction (MLR)

Spleen cells obtained from normal young AKR/J, DBA/2 or Swiss mice were cultured either alone or in the indicated combinations (1:1 ratio, 1.25×10^6 total viable cells/well) in HMEM-TBH containing 10% fetal calf serum. Cultures were pulsed with 1 μCi of [methyl-^{3}H]-thymidine during the last 24 hr of culture period (3–4 days, as indicated), harvested on glass fiber filter and washed sequentially with saline-EDTA (1 mM), 5% TCA, distilled water and finally with methanol, using a MASH harvester [18].

RESULTS

Glycolipid-bound sialic acid level in serum

The level of glycolipid-bound *N*-acetylneuraminic acid was determined in individual serum samples from normal and thymic lymphoma-bearing mice (Table 1). A statistically significant elevation of 2.6 times and 1.4 times above control values were noted in spontaneous leukemia of AKR and chemically (DTIC)-induced leukemia of Swiss mice respectively. Since the magnitude of elevation was higher in AKR leukemic mice, further experiments on the correlation between tumor mass and level of serum glycolipid-bound sialic acid were carried out with AKR mice. Figure 1 shows the relationship between the wet weight of thymic lymphoma and the corresponding serum *N*-acetylneuraminic acid level at the time of killing. As can be seen, a doubling of the weight of thymus resulted in doubling of serum glycolipid-bound sialic acid level. Maxiumum serum *N*-acetylneuraminic acid was reached when the tumor mass was about 400 mg and remained about that high level. Data presented in Fig. 2 clearly shows that increased appearance of serum glycolipid-bound sialic acid coincides with the time of appearance of leukemia.

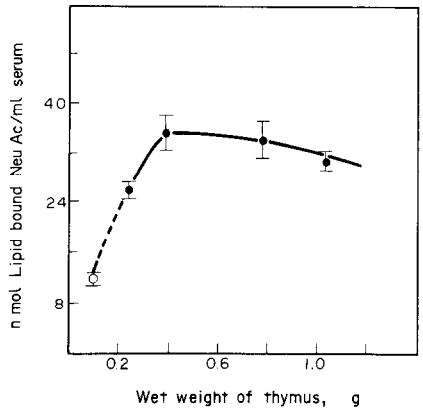

Fig. 1. Relationship between tumor mass and serum glycolipid-bound sialic acid level in leukemic AKR mice. Experimental conditions are as given in Table 1. Thymus wet weights were determined at the time of killing and serum collection. Tumor-bearing mice were divided into 4 groups based on tumor-weight as follows: (a) 0.2–0.25 g; (b) 0.35–0.45 g; (c) 0.75–0.85 g; and (d) 0.95–1.15 g. (○) Mean ± S.E. of normal AKR mice (72 mice); (●) mean ± S.E. of leukemic AKR mice (3–4 mice/group).

Analysis of serum gangliosides

To determine the predominant molecular species of ganglioside present in the sera of normal and leukemic mice, pooled sera were extracted by standard techniques [21]. As expected, the total lipid-bound sialic acid concentration of the serum gangliosides before TLC analysis was 2 to 4-fold higher in leukemic mice (see legends for Fig. 3). Upon thin-layer chromatography, the AKR/J and Swiss sera contained gangliosides with mobilities very close to GM2 and GM3 respectively (Fig. 3A). In solvent B, AKR/J gangliosides moved slightly below *N*-glycolyl NeuAc containing GM3, but close to or in line with the upper

Table 1. Glycolipid-bound sialic acid level in sera of normal and tumor-bearing AKR and Swiss mice

Strain of mouse	Serum lipid-bound sialic acid level (nmol NeuAc/ml) Normal	Thymic lymphoma bearing	Leukemia/normal
AKR/J	12.5 ± 0.9 (72)	33.0 ± 6.7* (37)	2.6†
Swiss	19.8 ± 0.7 (39)	27.9 ± 3.9 (22)	1.4

Glycolipids from individual serum samples were extracted following precipitation of lipo- and glycoproteins by the citrate procedure as described by Kloppel *et al.* [5], and lipid-bound sialic acid was estimated by the method of Warren [23].

Swiss mice were killed at about 4 months (range: 3–5 months) after DTIC injection and they were clearly leukemic as judged by the size of thymus.

*Values are the mean ± S.E.M. and the numbers in parentheses represent the number of animals.

†The differences between values for normal and tumor samples were highly significant at 99.9% (AKR) and 99% (Swiss) confidence levels. The statistical significance of differences in these data and those of subsequent tables were determined by the modified Student's *t* test.

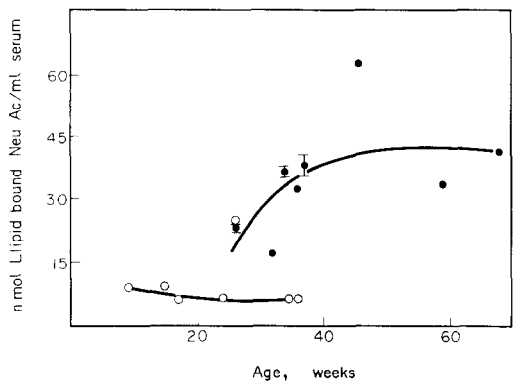

Fig. 2. Serum glycolipid-bound sialic acid level in non-leukemic and leukemic AKR/J mice as a function of age. Serum from individual mice were analyzed after being killed at indicated ages. O—O *Non-leukemic;* ●—● *leukemic.*

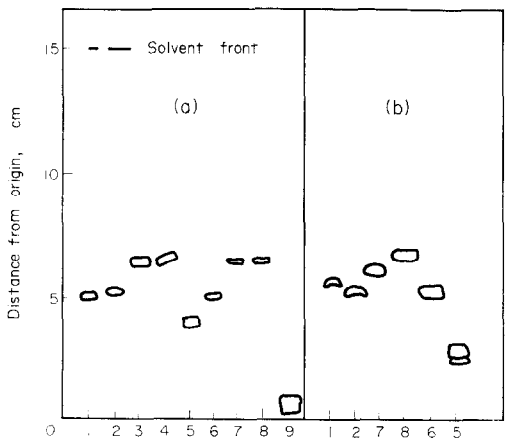

Fig. 3. Thin-layer chromatogram tracings of serum gangliosides. Gangliosides were extracted from sera as described in Materials and Methods, developed on silica gel G TLC plates and sprayed with resorcinol reagent. Spots shown are positive for both resorcinol and iodine (except NeuAc which was positive only for resorcinol). Solvent A = $CHCl_3:CH_3OH:H_2O$ *containing* 0.05% CaCl2 (60:35:8, v/v); *solvent B* = $CHCl_3:CH_3OH$:2.5 N NH_4OH (60:40:9, v/v). 1: *AKR/J Non-leukemic mice*; 2: *AKR/J leukemic*; 3: *Swiss normal*; 4: *Swiss leukemic*; 5: *GM*1; 6: *GM*2; 7: *GM*3 *containing N-glycolyl sialic acid*; 8: *GM*3 *containing NeuAc*; 9: *N-Acetylneuraminic acid. GM*2, *GM*1 *and GD*1*a were from bovine brain (Supelco, Inc). Total lipid-bound sialic acid concentration in serum of these mice (categories* 1–4) *before TLC expressed as* nmol/ml *serum (or* nmol/g *protein) were as follows*: 1 = 2.34 (22.8); 2 = 8.75 (64.8); 3 = 6.8 (62.3); 4 = 12.14 (131.3).

portion of GM2 (Fig. 3B). No other resorcinol-positive spot could be identified in normal or leukemic sera of either strain of mice.

Effect of mixed gangliosides on blast-transformation of splenocytes triggered by T and B cell mitogens

We have previously reported that addition of mixed gangliosides to cultures resulted in loss of ability of thymocytes to respond to the T mitogen, Con A [18]. This observation was extended to spleen cells which were separately exposed to T and B mitogens, Con A, PHA and LPS. The dose of mixed gangliosides used in these experiments was chosen from our previous experience with lymphocyte cultures [18]. As can be seen from Table 2, the ability of spleen cells to respond to either T or B mitogen as measured by [^{3}H]-thymidine incorporation into DNA or [^{14}C]-lysine incorporation into proteins *in vitro* was abolished or decreased by the gangliosides at the same dose (10 μg/well), thereby showing the indiscriminate action of gangliosides on both thymus and spleen cells.

Inhibition of mixed lymphocyte reaction of splenocytes by exogenous gangliosides

The ability of bovine brain mixed gangliosides to interfere with two-way mixed lymphocyte response (MLR) of spleen cells from three different strains of mice are shown in Table 3. These data show that the MLR of AKR × DBA/2 cultures as well as AKR × Swiss cultures was completely inhibited by the presence of mixed gangliosides in the medium, suggesting that the inhibitory effect of ganglioside is not restricted to a given set of genetic strains of mice. This observation provides a further proof of the suggested immunoinhibitory property of gangliosides, since the stimulus used in this assay was of a different nature, i.e., histoincompatibility.

Effect of gangliosides on the distribution of [^{14}C]-*labeled proteins in Con A-stimulated thymocytes and culture medium*

Lymphocytes can synthesize different classes of proteins, viz. structural and other proteins integral to the lymphocytes and secretory proteins, which include certain soluble mediators of immunity such as lymphokines [24]. Since the gangliosides are known to bind to intact thymocytes [20], it was considered possible that the gangliosides, by some specific or non-specific cytotoxic mechanism, promoted the leaking of the labeled proteins or favored active release of exportable proteins. To exclude this possibility, thymocytes were cultured in the absence or presence of Con A and/or mixed gangliosides and pulsed with [^{14}C]-leucine during the last day of culture. The radioactivity in TCA-precipitable material in the cells and culture medium was then determined separately. There were 20- and 2½-fold increments in labeled proteins in cells and medium, respectively, from Con A-treated cultures (Table 4). The presence of gangliosides resulted in an almost total inhibition of cellular protein labeling, but did not result in a cor-

Table 2. Effect of mixed gangliosides on blast-transformation of splenocytes stimulated by T and B cell mitogens

Additions	cpm [^{3}H]-thymidine taken up/10^6 viable cells	cpm [^{14}C]-lysine incorporated into protein/10^6 viable cells
Control	3114 ± 826*	1161 ± 152*
+Con A	31,604 ± 7884	6020 ± 470
+ Con A + gangliosides	3119 ± 377	2262 ± 294
+LPS	14,341 ± 1964	2680 ± 207
+ LPS + gangliosides	1838 ± 618	1221 ± 276
+PHA	29,707 ± 9267	NT
+PHA + gangliosides	18,719 ± 1770	NT
+ Gangliosides	1570 ± 792	1127 ± 184

Spleen cells 1.25×10^6 from young (non-leukemic) AKR mouse were incubated for 72 hr. [^{3}H]-Thymide (0.1 μCi) or [^{14}C]-lysine (0.1 μCi) was added 24 hr prior to termination of cultures and cells processed by MASH harvester by successive washings with saline-EDTA, 5% TCA and methanol as described previously [18]. Con A, LPS, PHA or ganglioside were added at the initiation of cultures at their optimal concentrations of 0.5, 1.0, 2.0 and 10 μg/well respectively.
*Mean ± S.D. of 6 cultures.
NT: not tested.

Table 3. Effect of exogenous mixed gangliosides on the two-way mixed lymphocyte reaction

AKR × DBA/2		AKR × Swiss	
Source of cells	[^{3}H]-thymidine uptake (cpm/10^6 viable cells)	Source of cells	[^{3}H]-thymidine uptake (cpm/10^6 viable cells)
AKR	535 ± 27*	AKR	1786 ± 365
DBA/2	549 ± 40	Swiss	2782 ± 146
AKR × DBA/2	1224 ± 140	AKR × Swiss	5693 ± 794
AKR × DBA/2 +gangliosides	403 ± 121	AKR × Swiss +gangliosides	991 ± 10

Spleen cells (1.25×10^6) obtained from normal young AKR, DBA/2 or Swiss mice were cultured either alone or in the combinations mentioned above in absence or presence of mixed bovine brain gangliosides (20 μg/well). Cultures were pulsed with 1 μCi of [methyl-^{3}H]-thymidine during the last 24 hr of 3 day (AKR × DBA/2) or 4 day (AKR × Swiss) cultures and processed in MASH harvester, as described in the Materials and Methods section. In absence of allogenic stimulation, gangliosides did not significantly alter the [^{3}H]-thymidine uptake in control cultures.
*Mean ± S.E.M. of 3–4 cultures.

responding increase in the amount of labeled proteins in the culture supernatants, thereby ruling out the possibility of ganglioside-mediated specific release or leakage of proteins synthesized in response to Con A.

DISCUSSION

Although increased quantities of both neutral and acidic glycolipids have been reported to be synthesized by tumor tissues [4, 25], the latter are more likely to accumulate in the plasma of such tumor-bearers in view of the demonstration that the half-lives of gangliosides were $2.3–12.6 \times 10^2$ times higher than the neutral glycolipids [26]. Presence of sialic acid in gangliosides probably results in decreased hepatic clearance rate. Beginning at 24 weeks of age, the majority of AKR/J mice develop thymic lymphoma. Interestingly, the serum glycolipid-bound sialic acid level starts reflecting the presence of tumors at the same time. This increase is not an aging-related phenomenon, since non-leukemic mice as old as 37 weeks have serum sialic acid levels far below the concentration in mice of comparable age showing definitive evidence of leukemia (Fig. 2). The previous observation of elevated plasma lipid-bound sialic acid level in leukemic AKR mice reported from this laboratory [8] is further confirmed by testing the sera from individual mice of a larger sample number and using a different extraction procedure (Table

Table 4. Inhibition by gangliosides of Con A stimulated incorporation of [U-^{14}C]-leucine into thymocyte proteins

Addition to medium	cpm in TCA pellet/10^6 viable cells Cells	Medium	Total
None	105 ± 15* (8)	184 ± 37 (8)	290 ± 43 (8)
+Con A†	2060 ± 122 (8)	471 ± 123 (8)	2531 ± 204 (8)
+Con A + gangliosides (20 μg)	252 ± 44 (3)	576 ± 90 (4)	828 ± 110 (3)
+Gangliosides (20 μg)	160 ± 10 (3)	396 ± 21 (3)	556 ± 11 (3)
+Con A + gangliosides (100 μg)	30 ± 0 (3)	92 ± 5 (3)	122 ± 4.4 (3)
+Gangliosides (100 μg)	29 ± 0.6 (4)	115 ± 8 (4)	144 ± 8.8 (4)

Thymocytes (1.25×10^6) from normal AKR mouse were cultured for four days in absence or presence of Con A (0.5 μg/well) or mixed gangliosides (20 μg or 100 μg/well). See Materials and Methods for other details.

*Results of two experiments, each value being the mean ± S.E. of 3–8 cultures.

†Simultaneous cultures with Con A showed mitogenic response and complete inhibition by 20 μg of mixed gangliosides. [^{3}H]-Thymidine incorporation: control, 24; plus Con A, 1602; plus gangliosides, 64; and plus Con A + gangliosides, 72 cpm/10^6 viable cells.

1). A tumor burden of less than 0.25 g of thymic lymphoma in AKR mice appeared to provide lipid-bound sialic acid in serum sufficiently high to be detected (Fig. 1). Qualitatively similar results, but less dramatic in magnitude, were obtained with Swiss mice undergoing chemical carcinogenesis (Table 1). Our observations agree with previous reports on mice bearing transplantable mammary carcinoma [5], human carcinoma patients [5], rats bearing Morris hepatoma [7] and melanoma-bearing human patients [6]. Thus, an increase in serum glycolipid-bound sialic acid levels appears to be a common feature of significance in tumorigenesis, though the microheterogeneity of the molecular species of gangliosides altered remains to be established.

Based on the proposition that the biological significance of this *in vivo* accumulation of circulating gangliosides may be a factor in the generalized immunosuppression usually seen in tumor-bearing patients and experimental animals, we have previously reported that Con A responsiveness of thymocytes was inhibited by exogenously added mixed gangliosides as well as by four sub-classes of gangliosides, GM1, GM2, GD1a and GT1 [18]. As can be seen from Tables 2–4 of the present communication, the gangliosides proved to be immunosuppressive based on two *in vitro* correlates of cellular immunity: (i) blast transformation of thymocytes and splenocytes by T and B mitogens; and (ii) allogeneic MLR. Although extrapolation of our *in vitro* observation to *in vivo* situation may not be fully possible at this stage, it is interesting to note that the serum ganglioside concentrations of leukemic mice are not far different from the dose-responsive range of mixed commercial gangliosides used for immunosuppression. Assuming all the NeuAc were due to GM2 (in AKR/J) or GM3 (in Swiss mice), we arrive at approximately 40 and 50 μM serum ganglioside concentration for AKR/J and Swiss leukemic mice respectively (see legend for Fig. 3). We have previously shown that 50 μM GM2 could bring about 96% inhibition of lectin response [18]. In the experiments reported here a complete immunosuppression was noted with 10–20 μg of bovine gangliosides *in vitro* (Tables 2 and 4). On the basis of approximate composition of commercial mixed gangliosides (as checked by TLC), which is very close to the values reported by Tettamanti *et al.* [27], the above dose translated to 18–36 μM, a value in good agreement with leukemic serum ganglioside concentration.

Glycolipids are known to play a regulatory role in immune response. A soluble *N*-acetyl-

neuraminic acid-positive glycolipid bound to a protein component produced by dividing T cells was found to inhibit lymphocyte proliferation and immunoglobulin synthesis [28]. Fucose-containing glycolipids have been proposed to be the macrophage surface receptors for lymphokines, such as macrophage migration inhibitory factor [29].

A GM1-like substance was reported to be released by suppressor T cells and addition of exogenous gangliosides resulted in transient suppression of humoral [19, 30] and cellular immune responsiveness [31, 32]. The recent work of Sela and co-workers [33, 34] also implies an immunoregulatory role of gangliosides. Normal lymphocytes cultured with Con A have been shown to have a higher capacity to synthesize labeled glycolipids [35]. We have observed higher ganglioside synthetic capability in leukemic thymocytes [36], and we have shown here the immunosuppressive effect of added gangliosides. These results reveal the interrelationship between the ability of lymphocytes to respond to external stimuli and to the composition of lymphocyte glycolipids. Thus, it is speculated that translocation of normal or novel gangliosides among cellular elements of immune systems may be a mechanism of immunoregulation. It is conceivable that such a translocation could be achieved through the process of cell-surface turnover, by antigen-mediated specific release, or by excessive production and shedding during tumorigenesis.

The present data do not rule out the possibility that highly potent non-gangliosidic contaminant(s) in mixed ganglioside preparations may cause or contribute to the observed inhibitory effects. We consider this unlikely because (a) purified sub-classes of gangliosides still show dose-dependent inhibitory action [18]; (b) mixed and individual gangliosides purified over a silicic acid column were noted to suppress immune reactivity of human peripheral blood mononuclear cells [32] or murine thymocytes (results not shown); and (c) experiments with endogenous total ganglioside fractions isolated from pooled leukemic or normal thymuses showed comparable suppressive action [37] on cell-mediated immune response (Con A induced blastogenesis and MLR). More refined experiments and detailed data will be needed to specifically imply quantitative/qualitative alterations as the major factor in leukemogenesis. Nonetheless, this observation strongly argues against the species differences in composition and impurity of exogenous ganglioside preparations as being the cause of immunosuppression.

Non-availability of Con A to thymocytes due to a possible entrapment of the mitogen by exogenous gangliosides was ruled out as an artifact resulting in decreased mitogenesis. It was found that the amount of TCA non-precipitable labeled GM1 in the medium was the same in absence or presence of Con A under the usual culture conditions, i.e., GM1 did not co-precipitate with Con A (unpublished observation). This suggested that the gangliosides must be acting on the lymphocytes. Indeed, we obtained evidence for direct binding of [^{3}H]-GM1 to AKR/J thymocytes [20]. Results suggesting non-overlapping of GM1 binding site and Con A binding site have already been presented [20]. With regard to suppression of MLR, it remains to be shown if the gangliosides mask or interact with MLR-surface determinants.

The possibility of a non-specific cytotoxic effect of gangliosides was eliminated based on (i) the ability of treated cells to exclude trypan blue [18], and (ii) ability of cells to catabolize glucose at a normal rate [18]. Gangliosides are water-soluble lipids and in our cultures aqueous solutions of gangliosides are added in media containing 5–10% fetal calf serum. The possibility that the immunoinhibitory action of exogenous gangliosides were actually due to an aggregate or complex formed with lipoproteins, phospholipids and sterols present in the fetal calf serum cannot be ruled out at present. It may be pointed out that [^{3}H]-GM1 can bind murine thymocytes *in vitro* in the absence of the above compounds [20], thereby excluding any absolute requirement for these lipophilic molecules as far as cells surface interactions are concerned. We suspect that the gangliosodes owe their suppressive action to the ability to bind or insert into membranes [38] and influence the cell-surface components. Preliminary experiments have shown that intrathymocytic cyclic 3′, 5′ AMP levels are elevated within 2 min of exposure to GD1a or GT1 (from Supelco Inc.). Such a rise in cyclic AMP level could then antagonize the mitogenesis triggered by mitogens or mixed lymphocyte culture.

Apart from the immunobiological role of gangliosides being considered by us in tumor-bearing animals [18] and by others in non-pathologic situations [30], our results may have other implications in clinical research. Recently, attempts have been made to treat peripheral neuropathies with extracts of cerebral gan-

gliosides both in experimental models and human patients [39], wherein milligram quantities of gangliosides were injected for a period of up to 4 weeks. Such a chronic therapeutic schedule, while partially being effective in the symptomatic treatment of neuropathies, may have an undesirable immunosuppressive effect.

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