Lack of Specificity of Brain Gangliosides in the Modulation of Lymphocyte Activation

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Received July 17, 1985

A potential role for glycolipid gangliosides to act as immunomodulating agents has been suggested. Most studies have employed brain gangliosides. We have systematically investigated highly purified murine brain gangliosides for their ability to modulate lymphocyte activation. All sialic acid classes of ganglioside inhibited lipopolysaccharide (LPS)-induced antibody secretion and all polysialated gangliosides inhibited LPS-induced DNA synthesis. Monosialated gangliosides had no effect on DNA synthesis induced by LPS. 8-BrcGMP-induced DNA synthesis was also inhibited, suggesting that a negative signal was delivered to B lymphocytes by co-cultivation with exogenous gangliosides. The lack of specificity with respect to sialic acid class observed in these studies suggests that further investigation of an immunomodulatory role for gangliosides focus on endogenous lymphocyte gangliosides.

INTRODUCTION

Gangliosides are amphipathic glycosphingolipids containing sialic acid and an oligosaccharide chain which is bonded to ceramide. The hydrophobic ceramide anchors the molecule in the plasma membrane lipid bilayer, while the oligosaccharidesialic acid is exposed to the external environment. The physiologic role of these plasma membrane glycolipids has been the subject of intense study which has focused on their role in neuronal events, since gangliosides are present in high concentrations in neuronal tissue [1]. In peripheral tissues, gangliosides appear to act as receptors, or as a component of a receptor complex, for a variety of ligands, including bacterial toxins [2,3], polypeptide hormones [4], fibronectin [5], interferon [6], and lymphokines [7]. They also have an important role in cellular growth processes, including contact inhibition [8]. It has been postulated recently that gangliosides may mediate the transmembrane events which occur during lymphocyte activation [9]. The pattern of gangliosides in lymphocytes is characteristic for cellular types and subtypes and may be related to the functional capacity of cells [10].

In this investigation, the ability of murine brain gangliosides to modulate lymphocyte responsiveness to activating agents has been assessed. Our earlier studies showed that certain bovine brain gangliosides inhibited murine B lymphocyte stimulation by bacterial lipopolysaccharide (LPS) and had little effect on phytohemagglutinin (PHA) activation of T lymphocytes [11]. More recent studies have shown that leukemia cell gangliosides exert a similar inhibitory influence on human lymphocyte activation.
activation [12]. Other investigators have shown that specific gangliosides may exert profound effects on lymphocyte activation by lectins [13] and by antigens [14,15]. It has also been demonstrated that the expression of exogenous gangliosides and modified gangliosides on lymphocytes allows new immunologic reactivity to emerge [16,17]. In spite of these studies a clear understanding of the role of gangliosides in lymphocyte activation has not emerged. We now show that all sialic acid classes of exogenous brain gangliosides are able to modulate lymphocyte activation. Various possibilities for the inhibitory effects observed are discussed.

MATERIALS AND METHODS

Mice

Balb/c and C3H/HeN mice were obtained from the NIH breeding colony, Bethesda, MD. C3H/HeJ and C3H/HeB/FeJ mice were obtained from Jackson Laboratories, Bar Harbor, ME. All mice were female, 6–18 weeks old when used.

Cell Cultures

Splenic lymphocytes were obtained and cultured as previously described [18]. Eagles minimal essential medium (MEM) supplemented with 1 mM glutamine, 5 x 10^{-5}M 2-mercaptoethanol, 25 U/ml penicillin, 25 μg/ml streptomycin, 25 μg/ml gentamicin, 1 mM pyruvate, and 1 percent non-essential amino acids (NEAA) was used in the antibody secretion experiments. The same medium without pyruvate and NEAA was used for mitogenesis assays. Antibody secretion was measured using a modification of the Jerne plaque assay [19]. Data is presented as the number of plaque-forming cells (PFC) per 10^6 viable cells recovered on the day of assay. Lymphocytes were cultivated in one ml of medium (10^6 cells/ml) in Linbro plates.

Mitogenesis was measured by pulsing 0.2 ml cell cultures (5 x 10^5 cells/well) with 1 uCi of ^3HTdR for the final twenty-four hours of a seventy-two hour culture period [18].

Lymphocyte Activators

E. coli K235 lipopolysaccharide, prepared by phenol extraction [20] and butanol extraction [21], was kindly supplied by Dr. David C. Morrison, Emory University. Other LPS preparations and 8 BrcGMP were obtained from Sigma Chemical Co., St. Louis, MO.

Gangliosides

Mouse brain gangliosides were prepared using a modification of published methods [22]. Briefly, brain was homogenized and extracted with chloroform-methanol (1:2, v/v). The extract was filtered, applied to a DEAE Sephadex A-25 column, washed thoroughly to remove neutral lipids, and eluted with 0.1 M sodium acetate in methanol. The eluant was saponified with 0.1 M sodium hydroxide to destroy phospholipid; then it was dialyzed, lyophilized, and resuspended in chloroform-methanol (95:5, v/v) for further purification by chromatography on porous silica beads (Iatron Laboratories, Tokyo, Japan). The purified ganglioside fraction was obtained using a stepwise chloroform-methanol gradient. Sialic acid classes were separated by fractionation on DEAE Sephadex A-25, using a methanol-ammonium acetate gradient for elution [23]. Subclasses of sialic acid groups (i.e., individual gangliosides) were separated by Iatrobead chromatography using a gradient of chloroform-methanol-water for elution.
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Effect of brain gangliosides on LPS-induced DNA synthesis. Spleen cells from Balb/c mice were incubated in the presence of LPS (Salmonella minnesota R595, 10 µg/ml) and individual brain gangliosides (10 µg/ml). The open bars represent the mean cpm ± SD for triplicate cultures containing both LPS and ganglioside. The hatched bars show similar data for cultures without added LPS.

RESULTS

Inhibition of LPS-Induced Mitogenesis by Gangliosides

Isolated, purified murine brain gangliosides were investigated for their ability to modulate LPS-induced DNA synthesis in lymphocytes. The trisialoganglioside, G_{Tb} (IV³ NeuAc, II³ (Neu Ac)₂-GgOse₄Cer), was the most effective inhibitor of the four ganglioside preparations tested (Fig. 1). No inhibition was observed with the monosialoganglioside preparation. It appeared that more highly sialated gangliosides were more effective inhibitors. Considerable variation between experiments in the amount of inhibition observed was seen with a given concentration of ganglioside (50–95 percent for trisialogangliosides at 10 µg/ml) but the pattern of inhibition by individual gangliosides was consistent. The modest amount of inhibition of baseline thymidine uptake which was observed with G_{Db} (II³ (NeuAc)₂-GgOse₄Cer) and G_{Tb} (hatched bars) was felt to represent nonspecific toxicity of the gangliosides. This question of ganglioside-induced toxicity was investigated further by assessing lymphocyte viability after 72 hours of culture with LPS and gangliosides (Table 1). Minimal toxicity was evident at 10 µg/ml ganglioside, but a definite loss of cells (20–25 percent) was observed at 50 µg/ml.

Gangliosides (1.0 mg/ml) were stored in chloroform-methanol (1:1, v/v) at −20°C. Sialic acid was determined by the resorcinol method [24].

Asialoganglioside (ASG) was prepared by treating brain mixed trisialoganglioside (G₅ ganglioside) for one hour at 80°C with 0.1 N HCl. An oligomer of sialic acid (NANA)₅ was the kind gift of Stefan Svenson, Stockholm, Sweden.

Statistical Analysis

Students t test (unpaired, two-tailed) was used to test for statistical significance (p < 0.05).
TABLE 1
Viability in the Presence of Ganglioside and LPS

| Ganglioside | 10 µg/ml Ganglioside | 50 µg/ml Ganglioside |
|-------------|----------------------|----------------------|
|             | Control | LPS     | Control | LPS     |
| Control     | 43      | 48      | 43      | 48      |
| GM          | 37      | 39      | 39      | 28      |
| GD1a        | 39      | 43      | 34      | 30      |
| GT          | 38      | 37      | 24      | 27      |

*C3H/HeJ spleen cells (5 x 10^5 cells/well) were incubated in Eagles MEM without serum for 72 hours with gangliosides and LPS (butanol E. coli K235, 10 µg/ml) in flat-bottom microtiter plates. Each value represents the percentage of cells that were viable as assessed by trypan blue exclusion. This is a representative experiment from three separate experiments. At least 200 cells were counted to determine each point.

Role of Sialic Acid in Ganglioside Inhibition of Lymphocyte Activation

The importance of sialic acid groups in mediating the inhibition of lymphocytes by gangliosides was investigated by preparing asialoganglioside from GT and comparing its ability to inhibit mitogenesis with the intact ganglioside (Fig. 2). ASG had no effect on LPS-induced mitogenesis while the parent GT was a dose-dependent inhibitor. Free sialic acid also had no inhibitory effect at 100 µg/ml (10,316 ± 526 cpm vs. 13,336 ± 2,655 cpm). An oligomer of sialic acid, however, possessed inhibitory activity though it was not as potent as intact ganglioside (Table 2). These data suggest that the presence of bound sialic acid residues is a critical component for the inhibitory activity of gangliosides.

![FIG. 2. Lack of effect of asialoganglioside in inhibiting LPS-induced DNA synthesis. Spleen cells from C3Heb/FeJ mice were incubated for three days with LPS (E. coli 0111:B4, Westphal, 10 µg/ml): either alone, black bar; with asialoganglioside, open bars; or with GT, hatched bars. Each value represents the mean cpm ± SD for thymidine uptake of triplicate culture wells.](image-url)
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TABLE 2
Effect of Sialic Acid Oligomer on the Inhibition of LPS-Induced Mitogenesis

| LPS (µg/ml)b | Control | GDis b | (NANA)3 |
|-------------|---------|--------|---------|
| 0           | 644 ± 167 | 556 ± 11 | 317 ± 30 |
| 10          | 23,807 ± 1,370 | 2,353 ± 1,227 | 7,843 ± 1,041 |
| 25          | 23,364 ± 2,065 | 1,667 ± 1,035 | 8,286 ± 1,169 |

*aEach value represents the cpm ± SD from triplicate culture wells of C3Heb/FeJ spleen cells (5 x 10⁵ cells/well). Experiments were repeated at least twice, and a representative experiment is shown.

bLPS from E. coli K235 (phenol-extracted) was employed. GDis was used at 50 µg/ml. The oligomer of NANA was used at 38 µg/ml, which represents an equimolar amount.

Ganglioside Modulation of Antibody Secretion

In order to assess whether the inhibitory effect of gangliosides on LPS activation extended to other lymphocyte functions, we investigated the ability of each sialic acid class of ganglioside to modulate polyclonal antibody synthesis induced by LPS. In these experiments the number of PFC reported is per 10⁶ viable cells recovered at the time of assay. This was done to eliminate all nonspecific toxic effects of the gangliosides during the four-day culture period. All sialic acid classes of ganglioside inhibited the generation of PFC by LPS (Table 3). As with mitogenesis, this inhibition was not observed with asialoganglioside.

Potential Mechanism for Ganglioside Inhibition

One attractive hypothesis is that the interaction of gangliosides with the B lymphocyte renders it unresponsive to LPS. To investigate this directly, spleen cells were preincubated with GThb, washed, and stimulated with LPS. No inhibition of LPS-induced DNA synthesis was observed. After a one-hour exposure to GThb, spleen

TABLE 3
Ganglioside Modulation of the Polyclonal Antibody Response to LPS*

| Ganglioside | LPS | LPS/Ganglioside |
|-------------|-----|-----------------|
| GM1         | 413 ± 59 | 246 ± 50 |
| GDis        | 429 ± 90 | 93 ± 10 |
| GDb        | 274 ± 19 | 104 ± 58 |
| GThb       | 372 ± 110 | 190 ± 13 |
| ASG         | 321 ± 9 | 374 ± 82 |

*E. coli K235 LPS (phenol-extracted), 10 µg/ml was used in all experiments. The number of IgM PFC to sheep red blood cells (SRBC) was assessed on day 4 of culture of C3H/HeN spleen cells (10⁶ cells/well).

Experiments were repeated on different days so independent controls were always done for each ganglioside. Each value represents the mean ± 2 standard error of mean (SEM) for the PFC/10⁶ cells of duplicate cultures from representative experiments. Each experiment was repeated at least twice.

50 µg/ml of ganglioside was added to each culture on day 0. PFC were related to viable cells plated on day 4. Inhibition was observed (p < 0.05) for each ganglioside employed.
cells were fully stimulable by LPS (Table 4). The same result was obtained after a four-hour exposure of spleen cells to G_{Tb}. Thus binding of G_{Tb} does not appear to be a sufficient signal to inhibit the lymphocyte response to LPS. In a separate set of experiments, uptake of G_{Tb} into thymocytes was assessed. After exposure to G_{Tb} at 50 μg/ml, thymocyte gangliosides were extracted and displayed by thin layer chromatography. G_{Tb} was easily seen in these chromatograms even when too few cells (3 × 10^6) were used to visualize endogenous thymocyte gangliosides. Similar results were obtained when the cells were left in culture for 72 hours after exposure to the ganglioside. 1–2 μg of ganglioside sialic acid was bound per 8 × 10^7 cells after washing.

We also tested the ability of gangliosides to inhibit B lymphocyte activation induced by a structurally independent lymphocyte activator. 8 BrcGMP has been shown to be a potent mitogen for B lymphocytes [24]. G_{Dm} (IV^3 NeuAc, II^3NeuAc-GgOse4Cer) was found to be a potent inhibitor of 8 BrcGMP-induced lymphocyte activation (Fig. 3). Thus the inhibitory effects of ganglioside were observed both with LPS-induced activation and 8 BrcGMP-induced activation of lymphocytes.

**DISCUSSION**

Exogenous gangliosides have been known to induce changes in the functional properties of lymphocytes for nearly a decade [10]. The mechanisms by which these alterations are produced remain obscure. One of the most attractive possibilities is that alterations in the ganglioside composition of the plasma membrane renders the cell incapable of receiving or responding to an activating signal [25]. This might occur because the ganglioside modifies the fluidity of the membrane [8] or because it induces a second signal within the cell such that the lymphocyte does not respond to LPS. There is evidence in other systems that gangliosides induce increases in cyclic AMP through an activation of adenylyl cyclase [26]. Such changes have not been demonstrated in lymphocytes. It has also been suggested that exogenous gangliosides may associate with endogenous membrane gangliosides which are critical components of a receptor complex [9]. This could inhibit the cross-linking of receptors and the transmembrane signal required for triggering the cell. Our data are totally consistent with this hypothesis.

**TABLE 4**

| LPS (μg) | Control | Preincubated |
|----------|---------|--------------|
| 0        | 696 ± 62 | 556 ± 50     |
| 1        | 2,054 ± 42 | 2,243 ± 242 |
| 10       | 7,118 ± 308 | 8,841 ± 425 |
| 50       | 9,956 ± 305 | 11,968 ± 255 |

*Balb/c spleen cells (10^6 cells/ml) were preincubated with 50 μg/ml of G_{Tb} for one hour, washed in BSS, and cultured with E. coli K235 LPS (phenol-extracted) for three days.

*Each value represents the mean ± SD for triplicate cultures which were preincubated with medium (control) or ganglioside (preincubated). There is no statistical difference between any of the paired values.
Another mechanism which might explain the inhibitory effects of ganglioside in these experiments is a direct interaction between the ganglioside and LPS. In other studies we have demonstrated that stable molecular complexes between ganglioside and LPS are produced by co-cultivation [27]. It is unlikely that this phenomenon can fully explain the observed data since there was equally potent inhibition of both LPS and 8 BrcGMP. In addition, others have found that ganglioside blocks lectin-mediated activation of cells without influencing the binding of the lectin [13]. The situation is more complex with LPS since binding of LPS to lymphocytes is nonspecific and not clearly associated with physiologic responses [28,29].

All sialic acid classes of ganglioside have an inhibitory effect on LPS activation of murine lymphocytes. It is clear that the presence of sialic acid is necessary for inhibitory activity and that more highly sialated gangliosides appear to have increased inhibitory activity. The lack of sialic acid specificity of the brain gangliosides used in inhibiting LPS activation of lymphocytes argues against a role for these brain gangliosides as receptors for endotoxins. Specific gangliosides have been shown to be receptors for other bacterial toxins, although the specificity is not absolute [30]. Binding of gangliosides to the lymphocyte surface does not appear to be a sufficient perturbation to alter LPS activation since these data show that G_{Th} binds to cells rapidly, but that it has no inhibitory effect even after a four-hour incubation. It appears that the continued presence of ganglioside is required to manifest inhibition.

Recent investigations have demonstrated that the composition of lymphoid gangliosides is profoundly different from brain gangliosides [31]. There are significant differences in the ganglioside composition of lymphoid cells between various species. Lymphoid gangliosides are often rich in the monosialated class, particularly G_{M3} [32,33,34,35]. Some disialated compounds are present in lower concentrations, but no higher order gangliosides have been demonstrated [32]. Additionally, the sialic acid moiety of non-neural gangliosides is different from brain gangliosides with N-glycoyl-neuraminic acid often predominating over N-acetyl neuraminic acid (NANA). Thus it remains possible that a lymphoid ganglioside which is distinct from

![Graph](image-url)
brain gangliosides may show specific binding with LPS. Further investigations of the endogeneous gangliosides of lymphoid populations will be needed to define their potential role as immunomodulators. Such studies are currently under way in our laboratory.

It has become evident that investigations on the role of gangliosides in immunomodulation will require the isolation and purification of specific gangliosides from lymphoid cells. Brain gangliosides represent a convenient source for highly purified gangliosides. They are structurally distinct, however, from the gangliosides found in lymphoid cells. The most prevalent gangliosides in murine brain are \( G_{M1} \), \( G_{Da} \), and \( G_{Tb} \). While it is clear that brain gangliosides may have profound effects on lymphocyte activation, the physiologic relevance of such observations is open to question. Further studies should concentrate on defining endogenous gangliosides from appropriate cellular sources. The inhibitory activities that we and others have observed using brain gangliosides are likely to be nonspecific and secondary to a modulation of the membrane of the cell or an interference with the ability of the ligand to trigger the cell. Further investigations of lymphoid gangliosides and their role in cellular communication is warranted.

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