Monosialoganglioside GM$_1$ Increases Survival of Thymocytes

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Gangliosides are normal constituents of the plasma membrane. Exogenous gangliosides can be incorporated into the membrane and extensive research in nervous tissue has demonstrated a beneficial effect of gangliosides on the functional recovery of lesioned neurons and protection against neurotoxins. This paper shows that the effect of gangliosides is not restricted to neurons. The monosialoganglioside GM$_1$ efficiently increases the survival of thymocytes and protects them against both the lytic effect of the glucocorticoid prednisolone and the effect of a thymocytotoxic serum. The protective effect of GM$_1$ was achieved both in vitro and in vivo.

KEY WORDS: ganglioside GM$_1$; thymocytes; survival; glucocorticoid.

INTRODUCTION

Gangliosides are glycosphingolipids containing sialic acid and are ubiquitous components of the outer leaflet of the plasma membrane. Exogenous gangliosides can be incorporated into the membrane (Orlando et al., 1979; Brown and Thompson, 1987) decreasing its fluidity (McDaniel et al., 1986) and modulating its functions (Hakamori, 1981). The monosialoganglioside GM$_1$ has been reported to increase the survival and stimulate the functional recovery of lesioned neurons (review see Gorio, 1986). This has raised the hope that gangliosides might be beneficial in peripheral neuropathies and stimulate the functional restoration after brain and spinal cord lesions (Gorio, 1986; Sabel and Stein, 1986). The mechanism by which gangliosides exert their protection is unknown. We investigate here whether the protective effect of gangliosides can be extended to cells other than neurons.

The young thymus contains intensely proliferating cortical thymocytes, which are dying spontaneously in large amounts both in situ and when cultured in vitro. Guinea pig thymocytes are sensitive to the cytotoxic effect of human serum (Sandberg and Stenvinkel, 1988) and mouse thymocytes to the lytic effect of...
glucocorticoids (Munck and Crabtree, 1981). Taking advantage of these properties we have studied whether the monosialoganglioside GM₁ has a protective effect on thymocytes. Our results clearly show that GM₁ increases the survival of thymocytes and protects them from spontaneous cell death in vitro, as well as death induced by a glucocorticoid or by a cytotoxic serum.

MATERIALS AND METHODS

Preparation of Cell Suspensions

Thymocyte suspensions were prepared from the thymus of 4- to 6-week-old, male guinea pigs of the Dunkin-Hartley strain weighing 250 to 300 g, from young male NMRI mice weighing 20 g and from human infant thymus obtained during open-heart surgery. Thymocytes were isolated as described previously (Ernström and Nordlind, 1974; Sandberg and Kölare, 1984). Guinea pig thymocytes were separated by density centrifugation on Percoll® (Pharmacia Fine Chemicals, Uppsala, Sweden) into three subpopulations (1a, 1b, 2) with increasing buoyant density (Sandberg et al., 1983; Sandberg et al., 1988). Unseparated and separated cells were suspended in serum free α-MEM (Flow Laboratories) and incubated in microwells (5 x 10⁵ cells in 100 μl) at 37°C in a humidified atmosphere with 5% CO₂.

Incubation with GM₁ and Human Serum

Thymocytes suspended in α-MEM were incubated in a concentration of 5 x 10⁶ cells per ml. After 1 h, GM₁ was added in a final concentration of 50–500 μM. Asialo-GM₁ was used as control in specified experiments. GM₁ and asialo-GM₁ were extracted and purified from bovine brain at Fidia Research Laboratories, Abano Terme, Italy. The concentrations of the glycosphingolipids are based on actual weight of the pure substances. After another hour of incubation, 20 μl human serum, known to be cytotoxic to guinea pig thymocytes (Sandberg and Stenvinkel, 1988), was added to cultures. Human serum was obtained from heparinized venous blood, centrifuged for 20 min at 4600 rpm. The serum was collected and further centrifuged twice before being diluted 1:3 with α-MEM. In one set of experiments the preincubation period with GM₁ was varied and thus human serum was added after either 1, 2 or 3 h. The incubation was interrupted 0.5 h after addition of serum and the viability of the thymocytes determined.

Glucocorticoid Treatment in vitro

Thymocytes from young male NMRI mice or from human infants were preincubated for 1 h before addition of 500 μM GM₁ or saline. After an additional hour of incubation the synthetic glucocorticoid prednisolone (Precortalone®, Organon, Netherlands) was added (0–100 μM), and the cells
were further incubated for different intervals up to 24 h before determination of viability.

**Glucocorticoid Treatment in vivo**

Young male NMRI mice, weighing 20 g, were injected with GM₁ (10 mg/kg i.p.) or saline and 2 h later with prednisolone (250 mg/kg i.p.) or saline and killed after 5 or 18 h (four experimental groups, n = 5). A suspension of thymocytes was prepared (see above), and without previous wash, cell viability was determined.

**Viability**

Fluorescein diacetate (2% in 50 µl PBS) and propidium iodide (0.5% in 15 µl PBS) were added to each microwell, staining the cytoplasm of living cells and the nucleus of dead cells, respectively (Jones and Senft, 1985). After 3 min at 20°C the multiwell plates were put on ice. Samples were examined in a fluorescence microscope, 100 cells counted per well, and the mean percentage of viable cells from duplicate cultures registered. Reproducible results are obtained with cells kept on ice for at least 40 min (Jones and Senft, 1985).

**RESULTS**

When fresh thymocytes are suspended in tissue culture medium and incubated at 37°C, a fraction of the cells die during the first hours. The addition of GM₁, resulting in a final concentration of 500 µM, increased the viability of the thymocytes from 80–90% to 95–98%. Figure 1 shows the increase in viability of mouse, guinea pig and human thymocytes after an incubation of 2.5 h, and we have also demonstrated a similar increase of viability after 16 and 24 h (data not shown). The effect of GM₁ on different subpopulations of thymocytes separated

![Graph showing increased survival of thymocytes](image)

*Fig. 1.* Increased survival of thymocytes by GM₁. Thymocytes from mouse, guinea pig and human were incubated at 37°C. After 1 h GM₁ was added in a final concentration of 500 µM and after another 1.5 h cell viability was determined.
Fig. 2. Protection of thymocytes by 500 μM GM₁ against spontaneous cell death and against the cytotoxic effect of human serum. GM₁ was added to guinea pig thymocytes after a preincubation for 1 h and serum after another 1 h. Viability was determined 30 min later.

according to their buoyant density was also investigated. Figure 2 shows that GM₁ increased the viability to a near 100% in all three subpopulations.

Human serum contains antibodies cytotoxic to guinea pig thymocytes. Within 30 min after addition of serum a marked cell death occurs (Stenvinkel and Sandberg, 1987). Addition of GM₁ to the thymocytes 1 h before exposure to serum dramatically reduced the cytotoxic effect. This protection by GM₁ was convincingly demonstrated both with unseparated cells and cells separated according to their buoyant density (Fig. 2). Figure 3 shows that asialo-GM₁ totally lacked the protective effect obtained with GM₁. Thus, the presence of sialic acid in the glycosphingolipid seems to be an absolute prerequisite for the effect on thymocyte survival.

Dose-response curves demonstrating the protective effect of GM₁ on guinea pig thymocytes exposed to cytotoxic serum show increased survival of cells with 50–500 μM GM₁ (Fig. 4). A prolongation of the preincubation with GM₁ from 1 to 2 or 3 h before addition of the cytotoxic serum did not enhance the protective effect.

Fig. 3. Absence of protection of thymocytes by asialo-GM₁. Guinea pig thymocytes were incubated as in Fig. 2 but with 500 μM asialo-GM₁.
Fig. 4. Protective effect of preincubation of thymocytes with GM₄ for 1, 2 or 3 h and at different concentrations. A prolonged preincubation of guinea pig thymocytes with GM₄ did not enhance the protective effect. On the contrary, the prolonged incubation increased the vulnerability of the cells to the cytotoxic serum. An increased survival was found in the concentration interval 50–500 μM GM₄.
Fig. 5. GM₁ protection of thymocytes against the lethal effect of a glucocorticoid. A suspension of thymocytes was prepared from young male NMRI mice and incubated with 500 μM GM₁ and the synthetic glucocorticoid prednisolone (Precortalone®, Organon, Netherlands) for 16 h or 24 h. A 50% lethal effect was obtained with 10⁻⁷ M prednisolone. An increased survival of both normal and prednisolone-treated thymocytes was found with GM₁, except at high doses of the glucocorticoid.

In our experiments a 50% lethal effect on mouse and human thymocytes was obtained with 10⁻⁷ M and 10⁻⁵ M prednisolone, respectively. Figure 5 shows that GM₁ efficiently increased the survival of both mouse and human thymocytes exposed to prednisolone in vitro.

The effect of GM₁ in vitro raised the important question, whether GM₁ injected in vivo might achieve any protection of thymocytes. GM₁ was injected i.p. into young mice, followed 2 h later by a thymocytolytic dose of prednisolone. Control animals received saline injections. Groups of mice were killed by decapitation after 5 h or 18 h and the viability of the thymocytes was analyzed. The prednisolone treatment reduced the viability of the thymocytes from 88% to 81% after 5 h (p < 0.05) and from 87% to 74% after 18 h (p < 0.001). A single
Table 1. GM₁ protection of thymocytes against the lethal effect of glucocorticoids *in vivo*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viability (%)</th>
<th>Diff ± SE (n = 5)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Saline GM₁</td>
<td>86.7 ± 0.7</td>
<td>2.5 ± 1.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>2: Saline Saline</td>
<td>89.3 ± 1.3</td>
<td>6.6 ± 1.5</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>73.7 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisolone</td>
<td>80.2 ± 1.2</td>
<td></td>
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Young male NMRI mice, weighing 20 g, were injected (1) with GM₁ (10 mg/kg i.p.) or saline and (2) 2 h later with prednisolone (250 mg/kg i.p.) or saline and killed after 18 h. A suspension of thymocytes was prepared, and without previous wash the cell viability was determined. A protective effect of GM₁ against steroid-induced death of thymocytes was demonstrated. The statistical analysis was performed with Student's t-test (paired samples).

Injection of GM₁ resulted in an increased survival of the thymocytes (Table 1). The protective effect of GM₁ was most conspicuous in the animals treated with prednisolone and investigated after 18 h, when the treatment with GM₁ had resulted in an increased survival of the thymocytes, which was statistically significant (p < 0.05).

**DISCUSSION**

Gangliosides are constitutive elements of the plasma membrane, and research during the last decade implies that they may modulate membrane functions by interacting with enzymes, receptors and transducers. Different gangliosides are proposed to influence cell differentiation, cell growth and to interact with bacterial toxins, virus and hormones (Hakamori, 1981).

The finding that gangliosides can accelerate the outgrowth of neurites *in vitro* and produce axonal sprouting also *in vivo* (Gorio *et al.*, 1980) was followed by extensive research on the effects of gangliosides and particularly of GM₁ on regeneration and restoration in both the peripheral and central nervous system. A great number of clinical trials have raised the hope of a restorative medical treatment of both peripheral neuropathies and brain lesions (see Gorio, 1986; Sabel and Stein, 1986).

Research on gangliosides has been concentrated on the protective and restorative effect on neurons, and almost no publications concern their effect on other cell types. In this paper we report that the monosialo-ganglioside GM₁ consistently protects thymocytes from different species from both spontaneous cell death and cell death induced by a thymocytotoxic serum or by a thymocytolytic dose of the glucocorticoid prednisolone. In contrast to GM₁, asialo-GM₁ completely lacked the protective effect. Thus, the presence of sialic acid in the glycosphingolipid seems to be a prerequisite for the effect on cell survival.

Glucocorticoids are lethal to thymocytes both *in vivo* and *in vitro* (Wyllie *et al.*, 1980). In this paper we have shown that GM₁ efficiently increases the resistance of both mouse and human thymocytes to prednisolone. To investigate whether it is possible to obtain a protective effect of GM₁ in intact animals also,
we performed experiments with mice treated with prednisolone \textit{in vivo}. The results showed that a single injection of GM\textsubscript{1} significantly protected the thymocytes against the thymocytolytic effect of prednisolone \textit{in vivo}.

Our results have shown that GM\textsubscript{1}, but not asialo-GM\textsubscript{x}, increases the survival of normal thymocytes as well as thymocytes exposed to cytotoxic antibodies or to a thymocytolytic glucocorticoid. Thus, irrespective of whether the cell death is spontaneous or induced in different ways, the exogenous GM\textsubscript{1} confers a conspicuous protection to the thymocytes. The experiment in the intact mouse also reveals that a single injection of GM\textsubscript{1} results in a significant protection of thymocytes \textit{in vivo}. The tissue concentration can be calculated to 50 \(\mu\text{M} \) GM\textsubscript{1}, if it is assumed that the injected dose is evenly distributed in the mouse. This roughly corresponds to the minimal effective concentration which increased survival of thymocytes \textit{in vitro}. It is probable that the mechanism behind the protective effect of GM\textsubscript{1} is a general one, increasing the resistance of different kinds of cells to different kinds of lesions.

Apoptosis (Wyllie and Morris, 1982), a physiological kind of cell death proposed to be fundamentally different from necrosis, is abundantly occurring in cortical thymocytes (Wyllie \textit{et al.}, 1980) and suggested to be connected with a Ca\textsuperscript{2+}-activated nuclear endonuclease cleaving DNA between nucleosomes into fragments (Wyllie \textit{et al.}, 1984). It has been shown that extracellular Ca\textsuperscript{2+} is required for toxic cell death (Schanne \textit{et al.}, 1979), and one possible mechanism behind the effect of GM\textsubscript{1} may be to stabilize the plasma membrane and prevent a lethal influx of Ca\textsuperscript{2+} ions. A reduced Ca\textsuperscript{2+} flux is in fact reported in synaptosomes of cats with GM\textsubscript{1} gangliosidosis (Koenig \textit{et al.}, 1987). Apoptotic cell death is also characterized by the extrusion of water (Wyllie \textit{et al.}, 1980), and it is also possible that GM\textsubscript{1} may stabilize the membrane and counteract such an extrusion. The \textit{in vitro} system with thymocytes presented here may allow a further exploration of the mechanism behind lethal cell injury including apoptotic cell death, and how the increased survival obtained with exogenous GM\textsubscript{1} is achieved. Screening of different pure gangliosides for their protective effect and potential usefulness in medical therapy, is much easier performed with our thymocyte viability assay than with a neuronal test system. In clinical studies improvement of peripheral neuropathies has been obtained with daily injections of a mixture of gangliosides (Gorio, 1986), and in mice a reduction of the neurotoxicity of vincristine without affecting its antitumor activity has been reported (Hellum \textit{et al.}, 1987).

Our results imply that GM\textsubscript{1} might be of clinical value in the protection of cells other than neurons against cytotoxic drugs or an autoimmune attack, but the possible effect of gangliosides on the survival of tumor cells must be taken into account.

\textbf{ACKNOWLEDGEMENTS}

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Thymocyte Survival

account of part of this work was presented at the International Leucocyte Culture Conference in Banff, Canada, May 1988.

REFERENCES