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# Gangliosides interact with interleukin-4 and inhibit interleukin-4-stimulated helper T-cell proliferation

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## SUMMARY

Gangliosides are potent immunosuppressive agents *in vitro*, and gangliosides shed from tumours *in vivo* may play an important role in the escape of tumours from immune destruction. We have investigated the effect of gangliosides on interleukin-4 (IL-4)-mediated processes in the murine helper T-cell line HT-2. Various gangliosides inhibited IL-4-stimulated DNA synthesis in HT-2 with $IC_{50}$ values in the range 26–60 $\mu$g/ml. However, the proliferation of four lymphokine-independent cell lines was unaffected by 500 $\mu$g/ml gangliosides, as was the IL-1-stimulated secretion of IL-2 by EL-4 NOB-1 cells. Gangliosides were highly effective inhibitors when added to $G_0$–$G_1$-synchronized HT-2 cells during the first 6 hr after IL-4 stimulation, indicating that they act early in the IL-4 signalling pathway. High levels of exogenous IL-4 completely reversed inhibition of proliferation by gangliosides, which suggests that gangliosides compete with cellular IL-4 receptors for available lymphokine. Receptor-binding experiments confirmed that gangliosides blocked binding of [$^{125}$I]IL-4 to receptors on intact HT-2 cells in a dose-dependent fashion. Gel-filtration fast protein liquid chromatography (FPLC) demonstrated that [$^{125}$I]IL-4 co-eluted with ganglioside micelles after co-incubation before chromatography, and an overlay technique showed that IL-4 bound efficiently to gangliosides on thin-layer chromatography plates. Taken together, these results indicate that gangliosides act as potent suppressors of IL-4-dependent processes in lymphocytes, and that their mechanism of action involves direct interaction with IL-4, thus preventing IL-4 binding to high-affinity IL-4 receptors. This information helps to explain the diverse immunosuppressive actions reported for gangliosides, both *in vitro* and *in vivo*.

## INTRODUCTION

Gangliosides are a structurally diverse class of sialoglycosphingolipids present in the outer leaflet of the plasma membrane of mammalian cells. Gangliosides are believed to be directly involved in activation of T lymphocytes by mitogens[1] and interleukin-2 (IL-2).[2–5] Over the past two decades, there have also been numerous reports on the ability of these glycolipids to negatively modulate cells of the immune system, both *in vitro* and *in vivo*. Several tumour types (neuroblastoma, melanoma, lymphoma, mammary carcinoma, hepatoma etc.) are known to shed high levels of tumour-specific gangliosides into the serum and ascites fluid *in vivo*, or the culture medium *in vitro*. Gangliosides shed by rapidly growing tumours of this type may thus be major contributors to the generalized immunosuppression often observed in cancer patients.[6–8] Recent work indicates that gangliosides are indeed immunosuppressive in the mouse *in vivo*,[9] and that tumour-derived species can enhance tumour growth.[10]

Gangliosides have been reported to inhibit the proliferation *in vitro* of many classes of immune cells, including lectin- and antigen-stimulated T lymphocytes and B lymphocytes, helper T-cells, monocytes, macrophages and natural killer (NK) cells.[6–8] The molecular mechanisms by which gangliosides exert such a wide range of immunosuppressive activities are not understood. Given the different characteristics and functions of the cell types they have been reported to inhibit, it seems likely that several different mechanisms could be involved.

Previous studies in our laboratory have investigated the immunoinhibitory effects of gangliosides on murine T lymphocytes. We initially showed that, in the two-step process of lectin-stimulated T-cell activation, gangliosides have no effect on the delivery of the first signal via the T-cell antigen receptor, which results in secretion of the lymphokine IL-2, and expression of

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Abbreviations: BSA, bovine serum albumin; FBS, foetal bovine serum; FPLC, fast protein liquid chromatography; HPTLC, high-performance thin-layer chromatography; IL-2, interleukin-2; IL-4, interleukin-4; IL-2R, interleukin-2 receptor; IL-4R, interleukin-4 receptor; rIL-2, recombinant interleukin-2; rIL-4, recombinant interleukin-4; TBS, Tris-buffered saline; [$^3$H]TdR, [methyl-$^3$H]thymidine.

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interleukin-2 receptor (IL-2R).[11] Instead, they appear to act exclusively at the second stage of the process, during which IL-2 binds to high-affinity IL-2R, thus transducing a signal for cellular proliferation. We have determined that the major mechanism by which gangliosides inhibit this stage of the activation process involves competition between gangliosides and IL-2R for binding of available IL-2.[12,13] The direct interaction of ganglioside micelles with IL-2[14] reduces the amount of lymphokine available for binding to both the p55 and p75 subunits of the IL-2R.[13]

The murine helper T-cell line HT-2 used in previous studies also expresses receptors for interleukin-4 (IL-4), and proliferates in response to this lymphokine.[15–17] It was thus of great interest to determine whether gangliosides were able to modulate IL-4-dependent processes. In this report, we demonstrate that gangliosides are more potent inhibitors of HT-2 cell proliferation mediated by IL-4 than that mediated by IL-2. Further investigation suggested that the molecular basis of this immunosuppressive effect involves the ability of gangliosides to bind and sequester IL-4, thus blocking binding of the lymphokine to cell surface IL-4R. The results help to explain the apparently broad specificity of gangliosides as immunosuppressive agents for both T and B lymphocytes.

## MATERIALS AND METHODS

*Gangliosides, glycolipids and glycoproteins*

$GM_1$, $GM_2$, $GM_3$, $GD_{1a}$ and $GT_{1b}$ (>95% purity) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Mixed gangliosides were isolated from bovine brain using a modification of the procedure of Kanfer.[14,18] They were labelled with $^3H$ on sialic acid residues by mild periodate oxidation and subsequent reduction with $NaB[^3H_4]$ (230 mCi/mmol; Amersham International Canada, Oakville, Ontario, Canada).[14,19] Asialo-$GM_1$ and globoside were obtained from Matreya (Pleasant Gap, PA, USA). Bovine brain cerebrosides, sulfatides and calf serum fetuin were purchased from Sigma. Glycophorin A was isolated as described previously,[20,21] and desialylated by acid treatment.[22]

*Lymphocyte cell lines and cell cultures*

The murine IL-2-dependent helper T-cell line HT-2 was cultured in RPMI-1640 (ICN Pharmaceuticals, Montreal, Québec, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Burlington, Ontario, Canada), human recombinant interleukin-2 (rIL-2) (200 U/ml), 2 mM L-glutamine, 2 mM penicillin-streptomycin and 50 $\mu$M $\beta$-mercaptoethanol in humidified 5% $CO_2$ at 37°. The cell lines A20 [American Type Culture Collection (ATCC; Rockville, MD) No. TIB 208], Jurkat (ATCC No. TIB 152), K562 (ATCC No. CCL 243), and EL-4 NOB-1,[23] were maintained in RPMI-1640/10% FBS.

*Lymphokines*

Human rIL-2 was obtained from Boehringer–Mannheim (Dorval, Québec, Canada). One unit is defined as the amount required to support half-maximal [$^3$H]TdR incorporation into CTLL-2. One Boehringer–Mannheim unit approximates 1·8 NIBSC (National Institute for Biological Standards and Control) units, which has been established by the World Health Organization (WHO) as an interim standard. Murine recombinant interleukin-4 (rIL-4) (60 000 U/ml) was supplied by Genzyme (Boston, MA, USA), which defined one unit of bioactivity as the amount of IL-4 that induces half-maximal [$^3$H]TdR incorporation in 100 $\mu$l cultures containing $\sim 2 \times 10^3$ murine FDCP2 bone-marrow cells. rIL-4 was labelled with Na[$^{125}$I] (carrier free, 17 Ci/mg I, ICN Pharmaceuticals) using Enzymobeads (Bio-Rad Laboratories, Mississauga, Ontario, Canada) according to the manufacturer's instructions. The final specific activity of [$^{125}$I]IL-4 was $1{\cdot}7–3{\cdot}5 \times 10^5$ c.p.m./ng protein.

*Bioassay for DNA synthesis*

HT-2 ($2 \times 10^4$ cells/200 $\mu$l) were seeded in flat-bottomed 96-well microplates (Nunc) in RPMI-1640/10% FBS. After addition of rIL-2/rIL-4, and gangliosides as desired, cells were grown for 20 hr (IL-2) or 24 hr (IL-4) at 37°, pulse labelled with 1 $\mu$Ci/well of [$^3$H]TdR (6·7 Ci/mmol, Amersham) for an additional 6 hr (IL-2) or 16 hr (IL-4), and harvested onto glass-fibre filter strips using a Titertek cell harvester. Filters were dried and counted using anhydrous scintillant. Positive control wells contained HT-2 with rIL-2/rIL-4, whereas negative control wells contained no lymphokines.

HT-2 cells were synchronized in $G_0$–$G_1$ by IL-2 deprivation as previously described.[13,24] For determination of the time course of ganglioside-mediated inhibition, synchronized HT-2 ($2 \times 10^4$ cells/200 $\mu$l) were stimulated with 50 U/ml rIL-4 at time zero. Gangliosides (500 $\mu$g/ml) were added to the cultures at various times up to 16 hr following rIL-4 stimulation. After 10 hr, cells were pulse labelled with [$^3$H]TdR for a further 6 hr, harvested, and $^3$H incorporation into cellular DNA was determined as above.

A20, Jurkat, K562 and EL-4 NOB-1 cells were resuspended in RPMI-1640/10% FBS and seeded at $2 \times 10^4$ cells/200 $\mu$l in a 96-well microplate, with or without gangliosides. After a 20-hr incubation at 37°, cells were pulse labelled for another 6 hr with 1 $\mu$Ci/well [$^3$H]TdR prior to harvesting.

*Measurement of IL-2 secretion by EL-4 NOB-1*

In response to stimulation with IL-1, EL-4 NOB-1 secretes large amounts of IL-2, which was measured using an HT-2 cell bioassay as described previously.[25] For experiments in which stimulation was carried out in the presence of gangliosides, culture supernatants were diluted by at least 10-fold, so that the final ganglioside concentration added to the HT-2 cells was below the inhibitory range.[13,25]

*IL-4 receptor binding assay*

Binding of [$^{125}$I]IL-4 to IL-4 receptors on HT-2 was determined as previously outlined for [$^{125}$I]IL-2.[12,13] HT-2 ($1 \times 10^6$ cells/100 $\mu$l) were incubated with 100 pM [$^{125}$I]IL-4 in RPMI-1640/10% FBS, with or without gangliosides, for 20 min at 37°, or 30 min at 4°. Cell-bound [$^{125}$I]IL-4 was subsequently separated from free [$^{125}$I]IL-4 by centrifugation at 15 000 $g$ for 10 min through a 300 $\mu$l cushion of pre-chilled *n*-butylphthalate. After freezing in liquid nitrogen, tube tips containing the cell pellets were cut off, and cell bound [$^{125}$I]IL-4 was determined by gamma counting. Non-specific binding of [$^{125}$I]IL-4 was

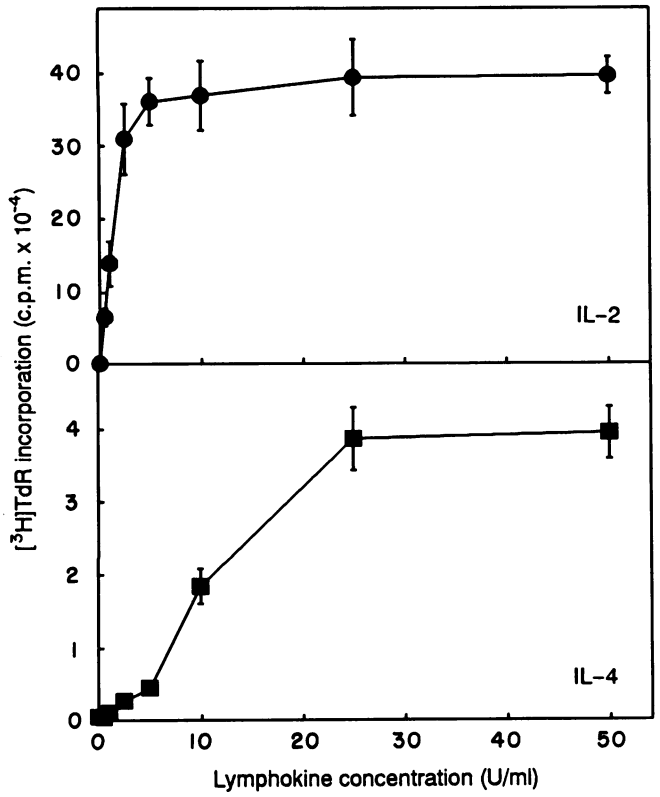


**Figure 1.** Cellular proliferation of HT-2 in response to IL-2 and IL-4. HT-2 ($2 \times 10^4$ cells/200 $\mu$l) were cultured in RPMI-1640/10% FBS with increasing concentrations of IL-2 or IL-4. After further incubation for 20 hr (IL-2) or 24 hr (IL-4) at 37°, cells were pulsed with [$^3$H]TdR (1 $\mu$Ci/well) for an additional 6 hr (IL-2) or 16 hr (IL-4), and harvested for determination of $^3$H uptake into cellular DNA. Values of individual data points represent means ± SD for triplicate determinations.

measured in the presence of a 200-fold excess of unlabelled IL-4, and was < 5% of the maximum amount bound.

*Fast protein liquid chromatography (FPLC) analysis of the interaction between gangliosides and IL-4*

Gel-filtration FPLC was carried out on a Superose 6 column (1 × 30 cm; Pharmacia) connected to a Gilson high-performance liquid chromatography (HPLC) system and a Gilson 111B ultraviolet flow detector. The column was calibrated using a series of standard proteins of known molecular mass as described previously.[14,26] A calibration curve of log $M_r$ versus elution volume was fitted by linear regression, and used to interpolate the apparent molecular mass of eluting peaks.

[$^3$H]-Gangliosides alone (100 $\mu$g, 0·16 $\mu$Ci), [$^{125}$I]IL-4 alone (40 fmol, 0·03 $\mu$Ci), or a mixture of both, were eluted through the column. Samples of 200 $\mu$l, in either TBS (50 mM Tris–HCl in 0·15 M NaCl, pH 7·4) or RPMI-1640/2·5% FBS, were incubated for 30 min at 37°, passed through a 0·45-$\mu$m filter, injected onto the column, and eluted at 0·5 ml/min. Fractions (0·5 ml) were collected and counted for $^{125}$I and $^3$H.

*Binding of [$^{125}$I]IL-4 to gangliosides by high-performance thin-layer chromatography (HPTLC) overlay*

HPLTC overlay experiments with [$^{125}$I]IL-4 were performed

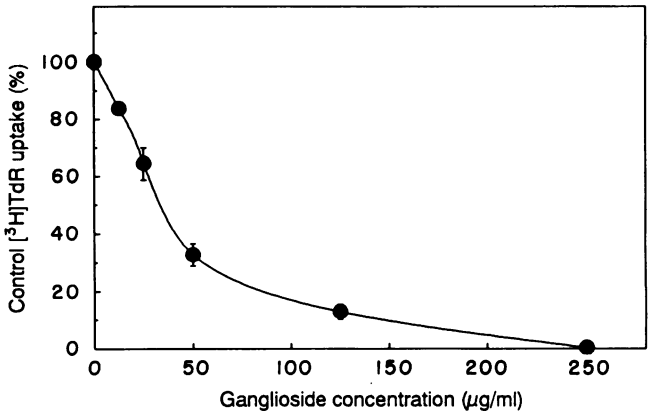


**Figure 2.** Effect of gangliosides on DNA synthesis in IL-4-stimulated HT-2 cells. HT-2 ($2 \times 10^4$ cells/200 $\mu$l) were cultured in RPMI-1640/10% FBS containing 50 U/ml of rIL-4. Increasing concentrations of mixed bovine brain gangliosides were added, and the uptake of [$^3$H]TdR into cellular DNA was determined as described in the Materials and Methods. Values of individual data points represent means ± SD for triplicate determinations.

essentially as described for [$^{125}$I]IL-2.[13] Developed plates were coated with poly(isobutylmethacrylate), blocked for 1 hr at 4° in TBS containing 1% BSA, and then incubated with [$^{125}$I]IL-4 (10 $\mu$Ci/$\mu$g) in TBS/1% BSA for 1 hr at 4°.

## RESULTS

### Gangliosides inhibit IL-4-stimulated proliferation of HT-2

The IL-2-dependent helper T-lymphocyte line HT-2 is also stimulated to proliferate by IL-4.[15–17] As shown in Fig. 1, HT-2 required 20–50 U/ml of IL-4 to elicit maximal DNA synthesis, compared to 5–10 U/ml for IL-2, and the upper limit of [$^3$H]TdR incorporation into cellular DNA after IL-4 stimulation was 10-fold lower than that seen for IL-2. HT-2 cells were routinely stimulated with 50 U/ml of rIL-4 in subsequent experiments.

Mixed gangliosides inhibited IL-4-stimulated proliferation of HT-2 in a dose-dependent fashion (Fig. 2). DNA synthesis was half-maximally inhibited at 35 $\mu$g/ml gangliosides, and completely suppressed at 250 $\mu$g/ml. Individual ganglioside species were tested in a similar fashion, and the concentration which resulted in 50% inhibition of DNA synthesis ($IC_{50}$) was determined. As indicated in Table 1, all ganglioside species tested were able to inhibit IL-4-induced HT-2 proliferation, with $IC_{50}$ values in the range 26–60 $\mu$g/ml. A complex trisialo-ganglioside was more highly inhibitory than simpler species. Related neutral glycolipids, such as asialo-$GM_1$ and globoside, were two- to five-fold less immunosuppressive, whereas other neutral or negatively charged species (cerebrosides and sulfatides) were essentially non-inhibitory (Table 1). As was observed previously with IL-2,[25] the human erythrocyte sialoglycoprotein glycophorin A, and its desialylated derivative, were also excellent inhibitors of IL-4-induced HT-2 proliferation, while the serum sialoglycoprotein fetuin was without effect. The similarity between this inhibition pattern and that seen for IL-2-mediated proliferation in the

**Table 1.** Effect of glycosphingolipids and glycoproteins on IL-2- and IL-4-stimulated proliferation of HT-2 cells

| Species added | $IC_{50}$ (μg/ml) IL-4-stimulated | $IC_{50}$ (μg/ml) IL-2-stimulated |
|---|---|---|
| Glycosphingolipids | | |
| $GM_1$ | 60 | 165 |
| $GD_{1a}$ | 40 | 130 |
| Mixed gangliosides | 35 | 140 |
| $GT_{1b}$ | 26 | 65 |
| Asialo-$GM_1$ | 125 | 175 |
| Globoside | 250 | 350 |
| Cerebrosides | > 1000 | > 1000 |
| Sulfatides | > 1000 | > 1000 |
| Glycoproteins | | |
| Glycophorin A | 180 | 200 |
| Asialoglycophorin A | 80 | 85 |
| Fetuin | > 1000 | > 1000 |

HT-2 ($2 \times 10^4$/200 μl) were cultured with 50 U/ml rIL-4 or 5 U/ml rIL-2, and various concentrations of glycosphingolipids and glycoproteins. After incubation for 20 hr (IL-2) or 24 hr (IL-4), cells were pulsed with 1 μCi/well [$^3$H]TdR for a further 6 hr (IL-2) or 16 hr (IL-4) before harvesting. The $IC_{50}$ value represents the concentration of each species which resulted in 50% inhibition of DNA synthesis.

same cell line suggests that a similar mechanism may be in effect.

### Gangliosides do not affect lymphokine-independent cell lines, or IL-1-dependent functions

To investigate whether gangliosides were generally inhibitory to proliferation of lymphocytes, we tested their effect on DNA synthesis in several lymphokine-independent lymphocyte cell lines, such as Jurkat (a human lymphoblastic leukemic T-cell line), A20 (a murine acute B-cell lymphoma), EL-4 NOB-1 (a murine thymocyte line) and K562 (a human chronic myelogenous leukemia line). Mixed gangliosides had very little effect on DNA synthesis; the $IC_{50}$ for inhibition of proliferation of all four cell lines was determined to be > 500 μg/ml. This indicates that gangliosides are not non-specifically inhibitory to growth of lymphokine-independent lymphocyte cell lines.

As an additional specificity control, we also investigated the effect of gangliosides on a process mediated by IL-1. The EL-4 NOB-1 cell line responds to stimulation with IL-1 by secreting IL-2, which can be measured in the culture supernatant with a bioassay using the HT-2 cell line. IL-2-containing supernatants were diluted at least 10-fold, so that the final ganglioside concentration was below the range known to inhibit HT-2 cells.[13] In the absence of gangliosides, IL-2 secreted by EL-4 NOB-1 cells in response to IL-1 resulted in $1{\cdot}32 \pm 0{\cdot}02 \times 10^5$ c.p.m. in the standard bioassay. In the presence of 250 μg/ml and 500 μg/ml gangliosides, this value was reduced only very slightly to $1{\cdot}25 \pm 0{\cdot}03$ and $1{\cdot}21 \pm 0{\cdot}18 \times 10^5$ c.p.m., respectively. Thus, at ganglioside concentrations that cause complete inhibition of IL-4-mediated proliferation in HT-2, IL-1-stimulation of a T lymphocyte line remains largely unaffected.

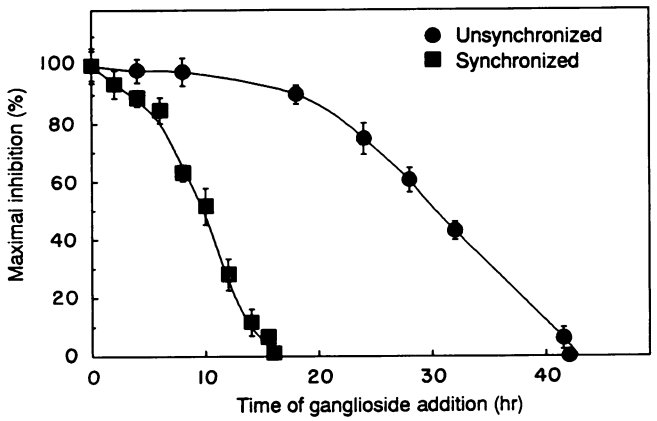


**Figure 3.** Time course of ganglioside-mediated inhibition of IL-4-stimulated proliferation of HT-2. Cells ($2 \times 10^4$ cells/200 μl) were either used directly as an unsynchronized population (●), or they were synchronized in $G_0$–$G_1$ (■) as described. At time zero, cells were stimulated with 50 U/ml rIL-4. Gangliosides (500 μg/ml) were added to the cultures at various times following rIL-4 stimulation, followed by measurement of the uptake of [$^3$H]TdR into cellular DNA. Values of individual data points represent means ± SD for triplicate determinations.

### Gangliosides act early in the IL-4 signalling pathway

Examination of the timeframe within which an agent acts to block DNA synthesis can give helpful information on its possible mode of action. As indicated in Fig. 3, gangliosides maximally inhibited proliferation of synchronized IL-4-stimulated HT-2 cells when added to the cultures at time zero, i.e. at the same time as IL-4 was added. Gangliosides were effective inhibitors when added as late as 6 hr after IL-4 stimulation, but became progressively less able to suppress DNA synthesis at later times, and were ineffective inhibitors at around 15 hr. When an unsynchronized HT-2 population stimulated with IL-4 at time zero was treated with gangliosides at different times (Fig. 3), results again showed that the sialoglycolipids had the maximum inhibitory effect when added at the same time as IL-4. In an unsynchronized population, cells completing one round of cell division and entering $G_1$ of the next cycle are not inhibited by gangliosides until some time after initiation. This leads to a time course of ganglioside inhibition that extends over a much longer period of time relative to that seen for synchronized cells. The data presented in Fig. 3 suggest that gangliosides modulate IL-4-induced proliferation by acting at a relatively early stage of the signalling process. This explanation is consistent with the proposal that ganglioside micelles bind to, and sequester IL-4 in the culture medium, thus lowering the levels of lymphokine available for binding to IL-4R.

### Ganglioside inhibition of IL-4-stimulated proliferation can be reversed by IL-4

If the mechanism of ganglioside inhibition of proliferation involves binding to IL-4, it should be possible to overcome this

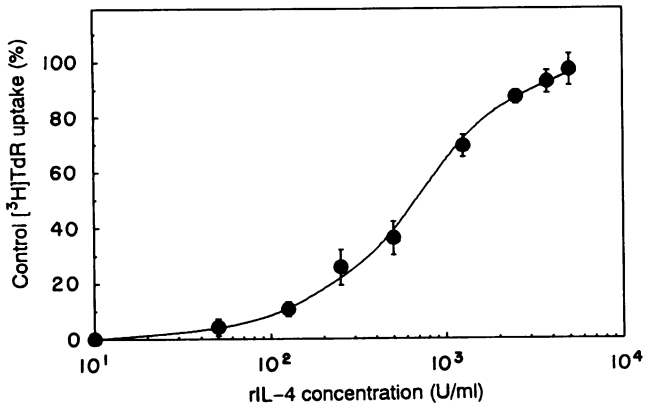


**Figure 4.** Reversibility of ganglioside inhibition by exogenous IL-4. HT-2 cells were stimulated with various concentrations of rIL-4 at time zero, in the presence of gangliosides (500 μg/ml). Measurement of the uptake of [$^3$H]TdR into cellular DNA was carried out as described. Values of individual data points represent means ± SD for triplicate determinations.

inhibition by addition of high concentrations of exogenous lymphokine. When increasing concentrations of IL-4 were added to HT-2 cells that were maximally inhibited by 500 μg/ml of mixed gangliosides, we observed a dose-dependent reversal of inhibition (Fig. 4). Cell proliferation was restored to > 90% of control levels at an IL-4 concentration of 3 500 U/ml. These results suggest that gangliosides can be 'titrated' with IL-4 in a stoichiometric fashion.

### Gangliosides block [$^{125}$I]IL-4 binding to high-affinity cell surface receptors

If gangliosides sequester IL-4 in the culture medium, then it is expected that much less IL-4 would be available for binding to cell surface IL-4R in the presence of the glycolipids. We therefore measured binding of [$^{125}$I]IL-4 to IL-4R on intact HT-2 cells, in the absence and presence of various concentrations of gangliosides. Measurements were carried out at two different temperatures, 37° and 4°. As shown in Table 2, gangliosides were highly effective at blocking the interaction of IL-4 with IL-4R at both temperatures, with the level of binding at 37° reduced to < 20% of the control at 250 μg/ml gangliosides. The ganglioside concentrations required for half-maximal inhibition of IL-4 binding at 37° and 4° were 85 μg/ml and 300 μg/ml, respectively. These values are 30–40% lower than the ganglioside concentrations previously reported to half-maximally block binding of IL-2 to the cell line CTLL-2 at the same temperatures.[13] Thus, gangliosides are able to block efficiently the binding of IL-4 to IL-4R.

**Table 2.** Effect of gangliosides on binding of [$^{125}$I]IL-4 to IL-4R on HT-2 cells

| Ganglioside concentration (μg/ml) | % control [$^{125}$I]IL-4 bound | |
|---|---|---|
| | 37° | 4° |
| 0 | 100 | 100 |
| 25 | 86 | 92 |
| 50 | 68 | 84 |
| 125 | 40 | 69 |
| 250 | 20 | 53 |
| 500 | 15 | 38 |
| 1000 | 12 | 21 |

Cells ($2 \times 10^6$/200 μl) were incubated with [$^{125}$I]IL-4 (100 pM), in the presence of various concentrations of mixed gangliosides at 37° for 20 min, or 4° for 30 min. Cell-bound and free [$^{125}$I]IL-4 were separated by differential centrifugation through a cushion of n-butylphthalate, and pellets and supernatants were counted. Reported values represent means for duplicate determinations in a typical experiment.

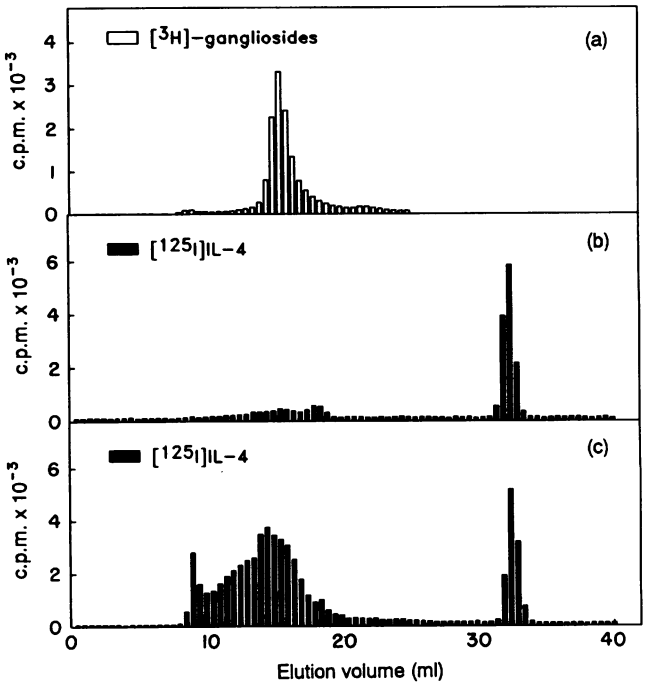


**Figure 5.** Association of [$^{125}$I]IL-4 with ganglioside micelles on Superose 6 gel-filtration FPLC. Samples (100 μl) of [$^3$H]-gangliosides alone (100 μg, 0·16 μCi), or [$^{125}$I]IL-4 alone (40 fmol, 0·03 μCi), were eluted through the column at a flow rate of 0·5 ml/min. Mixtures of gangliosides and IL-4 were incubated for 30 min at 37° prior to being injected onto the column. Fractions (0·5 ml) were collected and counted for $^{125}$I and $^3$H.

### IL-4 interacts directly with ganglioside micelles

To test whether gangliosides can interact directly with IL-4, gel-filtration FPLC experiments were performed. Ganglioside molecules aggregate to form large oblate ellipsoid micelles in aqueous solution, with a critical micelle concentration in the range $10^{-9}$ to $10^{-3}$ M, depending on the species.[27,28] $^3$H-labelled ganglioside micelles eluted as a broad peak, with $M_r$ centred around 440 000 MW (Fig. 5a), whereas [$^{125}$I]IL-4 eluted close to the included volume of the column (Fig. 5b). When gangliosides were co-incubated with IL-4 prior to chromatography, two distinct peaks of IL-4 radioactivity were observed.

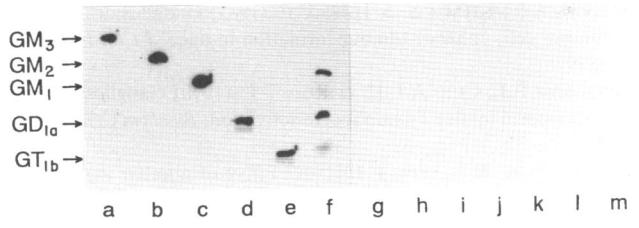


**Figure 6.** Binding of [$^{125}$I]IL-4 to gangliosides using the HPTLC overlay technique. Various ganglioside standards (5 $\mu$g), mixed bovine brain gangliosides (20 $\mu$g), glycosphingolipids and other lipids (5 $\mu$g) were spotted 1 cm from the bottom of the plate. Gangliosides and other lipids were separated in $CHCl_3$/MeOH/0·25% KCl (5:4:1 v/v) on HPTLC plates, which were dipped in 0·1% poly(isobutylmethacrylate). After blocking with 1% BSA in TBS (pH 7·4) for 1 hr at 4°, the plate was overlaid with [$^{125}$I]IL-4 for 1 hr at 4°, washed extensively in cold TBS, and autoradiographed. (a) $GM_3$, (b) $GM_2$, (c) $GM_1$, (d) $GD_{1a}$, (e) $GT_{1b}$, (f) mixed bovine brain gangliosides, (g) ceramide trihexose, (h) asialo-$GM_1$, (i) globoside, (j) lactosyl ceramide, (k) galactosyl ceramide, (l) phosphatidylserine, and (m) sulfatides.

A small amount of free [$^{125}$I]IL-4 eluted at an elution volume of 32–33 ml as expected, while the bulk of the lymphokine eluted in the high $M_r$ region corresponding to the ganglioside peak (Fig. 5c). The ganglioside peak was broadened, and its position was shifted to a higher molecular mass range, possibly indicating cross-linking or aggregation of the micelles in the presence of IL-4. Very similar elution profiles were obtained when samples were incubated and chromatographed in culture medium containing 2·5% FBS (data not shown). The co-elution of IL-4 with the micelles strongly suggests that IL-4 interacts directly with gangliosides.

To confirm this proposal, we used an overlay technique to demonstrate the direct binding of IL-4 to purified gangliosides on thin layer chromatograms. This highly sensitive method was previously used successfully to investigate binding of IL-2 to gangliosides and other lipid/glycolipid species.[13] Figure 6 shows that [$^{125}$I]IL-4 bound directly to several different ganglioside species on the HPTLC plate ($GM_3$, $GM_2$, $GM_1$, $GD_{1a}$ and $GT_{1b}$), but not to neutral glycolipids, or anionic lipids such as phosphatidylserine and sulfatides.

## DISCUSSION

IL-4 is produced by activated T lymphocytes and mast cells, and was originally identified as a stimulator of B lymphocyte proliferation *in vitro*. It has a broad spectrum of biological activity on both B and T lymphocytes, mast cells, macrophages, granulocytes, megakaryocytes and erythrocytes. IL-4 is recognized as a B-cell differentiation factor, which induces expression of class II antigen and Fcε receptors, and enhances secretion of IgG and IgE by B-cells. We previously established that gangliosides block IL-2-dependent proliferation of two IL-2-dependent lymphocyte cell lines by direct interaction with, and sequestration of, IL-2.[12–14] In this study, we have demonstrated that gangliosides are also very effective suppressors of proliferation mediated by the lymphokine IL-4. The pattern of inhibition of IL-4-mediated proliferation of HT-2 by glycolipids and glycoproteins is similar to that observed for suppression of IL-2-mediated proliferation in the same cell line.[12,13,25] It seems likely that an analogous mechanism of immunosuppression is in effect for IL-4-dependent processes.

A detailed examination of the time course of ganglioside-mediated immunosuppression of IL-4-mediated proliferation, using HT-2 cell populations synchronized in $G_0$–$G_1$, indicated that the glycolipids act early in the signalling pathway, which is consistent with the proposal that they interfere with lymphokine binding, the first step necessary to initiate the proliferation process. It also seems likely that gangliosides are able to displace previously bound IL-4 from its receptor, as was shown earlier for IL-2.[13] Also, like other lymphokines, we would expect that IL-4 must remain bound to its receptor for several hours in order to commit the cells to entry into S-phase. This information helps to explain why ganglioside addition as late as 6 h after IL-4 stimulation still results in substantial inhibition of proliferation. Ganglioside inhibition was also fully reversible by exogenously added IL-4, which suggests that the glycolipids compete directly with cellular IL-4R for available lymphokine. However, unlike the situation with IL-2,[13] reversal of inhibition with high IL-4 could be achieved in unsynchronized cell populations. This is likely due to the fact that IL-4 is much more stable under standard culture conditions than IL-2, so that it persists long enough to 'rescue' cells from ganglioside inhibition up to 24 hr after initial stimulation (for more discussion of this issue, see ref. 13).

Experiments on binding of IL-4 to high-affinity receptors on HT-2 cells confirmed that gangliosides blocked binding of [$^{125}$I]IL-4 to receptors on intact HT-2 cells in a dose-dependent fashion. Thus it appears that the gangliosides can effectively compete with IL-4R for available IL-4 in the culture medium. Further experiments showed that IL-4 co-chromatographs with ganglioside micelles during gel-filtration FPLC, indicating the existence of a stable association between the two. HPTLC overlay experiments demonstrated that IL-4 binding was indeed to ganglioside species, rather than to minor components of the preparation. Thus, both FPLC and HPTLC overlay experiments showed that IL-4 interacts directly with ganglioside micelles, but not with other lipid and glycolipid species. Taken together, these results indicate that gangliosides act as potent suppressors of IL-4-dependent processes in lymphocytes, and that their mechanism of action involves direct interaction with IL-4, thus preventing IL-4 binding to high-affinity IL-4R. High-affinity IL-4R are also expressed by non-haematopoietic cell types, especially stromal epithelial cells, and cells of the brain, muscle, and liver.[16] It seems likely that the biological effects of IL-4 on all receptor-expressing cells will be inhibited by gangliosides.

The $IC_{50}$ values for ganglioside-mediated HT-2 immunosuppression observed using IL-4 are substantially (around three-fold) lower than those we reported earlier for IL-2[12] (see Table 1), which indicates that gangliosides are more powerful inhibitors of DNA synthesis stimulated by IL-4 compared to that mediated by IL-2. This suggests that IL-4 may have a higher 'affinity' for binding to ganglioside micelles than IL-2. Two other pieces of information obtained in this work tend to support this idea. The FPLC experiments indicated that a substantially higher fraction of [$^{125}$I]IL-4 co-eluted with ganglioside micelles (80%), compared to IL-2 chromatographed under the same conditions (53%[14]). In addition, a higher concentration of IL-4 (3 500 U/ml) was necessary to reverse ganglioside inhibition, compared to

IL-2 (1000 U/ml[13]). Given the low $IC_{50}$ values observed for gangliosides/glycoproteins in this study, suppression of lymphokine-driven responses may be highly significant clinically, not only in cancer, but also in other diseases where serum levels of gangliosides and/or acute phase sialoglycoproteins are elevated.

The IL-4R and the $\beta$-subunit of the IL-2R are structurally homologous; both are members of the haematopoietin growth factor receptor superfamily.[29,30] It has been suggested that these homologies extend to the lymphokine ligands as well.[29] Indeed, recent reports on the tertiary structure of IL-4 indicate that, like IL-2, it contains a four-helix bundle motif.[31,32] It has previously been suggested, by ourselves[13,14] and others,[33] that IL-2 contains one or more lectin-like sites for interaction with the carbohydrate moieties of gangliosides and specific glycoproteins. The IL-4 molecule may contain similar binding sites. It is possible that other interleukins or cytokines with receptors in the haematopoietin growth factor receptor superfamily (such as IL-3, IL-6, IL-7, and GM-CSF) have similar 3-dimensional structures, and may also interact with gangliosides. The information presented in this work helps to explain the diverse suppressive actions reported for gangliosides on a wide variety of immune cells, both *in vitro* and *in vivo*, and suggests that they may also have inhibitory effects on cytokine interactions with other cell types.

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