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Gangliosides inhibit T-lymphocyte proliferation by preventing the interaction of interleukin-2 with its cell surface receptors

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SUMMARY

Gangliosides are known to be actively shed from tumour cell membranes, and increased levels of circulating gangliosides may cause tumour-induced T-lymphocyte immunosuppression *in vivo* by interfering with the actions of interleukin-2 (IL-2). We have investigated the effect of gangliosides on the interaction of IL-2 with its cell surface receptors (IL-2R). Gangliosides inhibited IL-2-stimulated proliferation in synchronized populations of the IL-2-dependent cell lines CTLL-2 and HT-2. The immunosuppressive effect was most effective when gangliosides were added during the first 4 hr after IL-2 stimulation, indicating that they acted early in the IL-2 signalling pathway. Inhibition could be completely overcome by exogenous IL-2, suggesting that gangliosides inhibited growth solely by competing with IL-2R for available IL-2. In support of this proposal, gangliosides induced a concomitant dose-dependent decrease in binding of [^{125}I]IL-2 to high-, medium- and low-affinity IL-2R. Ganglioside-treated cells recovered their high-affinity [^{125}I]IL-2 binding after washing. The glycolipids also prevented chemical cross-linking of [^{125}I]IL-2 to the p55/p75 complex, as well as to both IL-2Rα (p55) and IL-2Rβ (p75) independently. A thin-layer chromatography overlay technique was used to demonstrate that IL-2 binds directly to gangliosides, but not to simple neutral glycolipids or acidic lipids. Taken together, these findings indicate that gangliosides directly block the interaction of IL-2 with IL-2R, and may explain, in part, the immunosuppressive activities of gangliosides *in vivo*.

INTRODUCTION

Gangliosides are a class of sialic acid-containing glycosphingolipids, located primarily in the outer leaflet of the plasma membrane of mammalian cells. In recent years, it has become widely recognized that these acidic glycolipids may be directly involved in both T-lymphocyte activation[1] and the negative modulation of the immune system.[2,3] Several types of tumour cells are known to shed abnormally high levels of gangliosides into the culture medium *in vitro*, in the form of both membrane fragments and micelles.[4] Ganglioside levels in serum or ascites fluid are also dramatically elevated in human cancers such as hepatoma,[5] neuroblastoma,[6,7] melanoma[8,9] and mammary carcinoma,[10] and mice with Ehrlich ascites tumours,[11] carcinoma[10] and several types of lymphomas.[12,13] Levels of tumour-specific gangliosides in the serum and ascites fluid of cancer-bearing patients and animals can be up to 100-fold normal, and the rate of ganglioside shedding appears to be related to the metastatic potential of the cells.[4,13,14] In addition, gangliosides are potent suppressors of both murine and human lymphoproliferative function *in vitro*, and it has been proposed that gangliosides shed from rapidly growing tumours may be responsible for the generalized immunosuppression often associated with cancer *in vivo*.[2,3] The inhibition of immune responses by tumour-derived gangliosides may thus be a significant factor in the escape of tumours from host immune surveillance and destruction.

The mitotic activation of T lymphocytes is a two-step signalling process. The initial signal is delivered by specific antigen together with major histocompatibility complex proteins on the surface of an antigen-presenting cell, or by a mitogenic lectin *in vitro*. Binding to the T-cell antigen receptor results in signal transduction to the cell interior, via generation of diacylglycerol and inositol phosphates, which activate protein kinase C and increase intracellular Ca^{2+}, respectively. The lymphocyte then moves from G_0 to G_1, and expresses receptors for the second mitotic signal, the lymphokine interleukin-2 (IL-2). The second stage of activation involves binding of IL-2 to its high-affinity cell surface receptors, leading to transduction of a proliferative signal.[15] IL-2, a 15,000 MW glycoprotein, is the primary T-cell growth factor which induces lymphoid differentiation and proliferation, and thus, to a large extent, regulates the magnitude and duration of the immune cell response.[16] The interleukin-2 receptor (IL-2R) is composed of two independent IL-2-binding molecules: IL-2Rα (p55) and IL-2Rβ (p75).[17] IL-2Rα binds IL-2 with a low affinity (K_d of 10 nM), whereas IL-2Rβ binds IL-2 with an intermediate affinity (K_d of 1 nM). Binding of IL-2 to IL-2Rα/IL-2Rβ is of high affinity (K_d of

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10 pM). Both IL-2Rα and IL-2Rβ are integral glycoproteins with a single transmembrane segment. IL-2Rα has a short cytoplasmic tail, whereas IL-2Rβ has a larger intracellular domain and belongs to the erythropoietin receptor superfamily.[18]

Recently, much attention has been given to the proposal that inhibition of T-lymphocyte immune responses by gangliosides arises from their ability to bind and sequester IL-2, thus preventing binding of IL-2 to its high-affinity cellular IL-2R.[19-22] In this report, we have examined the effects of ganglioside micelles on the interaction of [^{125}I]IL-2 with its high-affinity receptor complex, as well as the intermediate- and low-affinity receptors independently. We further demonstrate that IL-2 binds directly to gangliosides in thin-layer chromatography (TLC) overlay experiments. Taken together, these results suggest that inhibition of IL-2 binding and receptor-mediated functions provides a potential biochemical basis for the *in vivo* immunosuppressive effect of gangliosides on T lymphocytes.

MATERIALS AND METHODS

Isolation of gangliosides

Gangliosides were isolated from freeze-dried bovine brain by a modification of the method of Kanfer,[23] as previously described.[21] Gangliosides were analysed by TLC on silica gel G plates using $CHCl_3$/MeOH/0·25% $CaCl_2$ (60:35:8 v/v), and visualized by spraying with H_2SO_4/ethanol (30:70 v/v) or resorcinol–HCl reagent. Mixed bovine brain gangliosides consisted of mono-, di- and tri-sialo species. The individual ganglioside species GM_1, GM_2, GM_3, GD_{1a} and GT_{1b} were purchased from Sigma Chemical Co. (St Louis, MO), and were greater than 95% pure.

Lymphocyte cell lines

The murine cytotoxic T-lymphocyte line CTLL-2 and the helper T-cell line HT-2 were cultured in RPMI-1640 medium (Gibco, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Gibco), 2 mM L-glutamine, 2 mM penicillin–streptomycin and 50 μM β-mercaptoethanol. CTLL-2 and HT-2 were supplemented with 25% (v/v) Jurkat-derived supernatant (approximately 200–300 U/ml IL-2; see below for unit definition), prepared as described previously.[22] To synchronize CTLL-2 and HT-2 in G_0–G_1, cells were depleted of IL-2 prior to use by incubation in RPMI-1640/10% FBS for 14 hr (CTLL-2) or 24 hr (HT-2).[24] Synchronized cell populations were used for all cellular proliferation experiments.

MT-1β is an HTLV-I-infected human T-cell line expressing only the p55 (α-chain) and displays approximately 500,000 low-affinity IL-2 binding sites/cell.[25] YT 2C2 is derived from YT,[26] a human leukaemic natural killer-like cell line expressing only the p75 (β-chain), and exhibits 20,000 intermediate-affinity IL-2 binding sites/cell.[27] Both cell lines were maintained in RPMI-1640/10% FBS without IL-2 supplement.

IL-2

Human recombinant IL-2 (rIL-2) was obtained from Boehringer-Mannheim (Dorval, Québec, Canada). A low specific activity rIL-2 preparation (200 U/ml) was used for cellular inhibition studies. High specific activity rIL-2 (10,000 U/ml) was used for binding assays, and experiments involving high IL-2 concentrations; this allowed the addition of large amounts of IL-2 in a small volume. For these products, one unit is defined as the amount of IL-2 required to support half-maximal tritiated thymidine ([^{3}H]TdR) incorporation into CTLL-2. One Boehringer-Mannheim unit aproximates 1·8 NIBSC (National Institute for Biological Standards and Control) unit, which has been established by the WHO (World Health Organization) as an interim standard. Radioiodinated IL-2 (3-[^{125}I]iodotyrosyl-IL-2-[Met^0,Ala^{125}]; 800–850 Ci/mmol) was purchased from Amersham (Oakville, Ontario, Canada).

Measurement of DNA synthesis

Cell proliferation was monitored by [^{3}H]TdR incorporation into cellular DNA. Synchronized CTLL-2 and HT-2 (1×10^5 cells/ml) were seeded in flat-bottomed 96-well microplates (Nunc, Roskilde, Denmark) in RPMI-1640/10% FBS. After addition of rIL-2 and gangliosides as indicated, cells were incubated for 10 hr at 37°. Cells were pulse-labelled with 1 μCi/well [^{3}H]TdR (6·7 Ci/mmol; Amersham) for an additional 4 hr, followed by harvesting on glass fibre filter strips with a Titertek automatic cell harvester. The filter discs were dried and counted using anhydrous scintillant. Positive controls contained cells with rIL-2, whereas negative controls contained no rIL-2.

For determination of the time–course of ganglioside inhibition, synchronized CTLL-2 or HT-2 (1×10^5 cells/ml) were incubated with either 5 or 50 U/ml rIL-2 at time zero. Gangliosides (250 μg/ml) were added to the cultures at various times up to 14 hr after rIL-2 stimulation. The uptake of [^{3}H]TdR into cellular DNA was then measured as outlined above.

[^{125}I]IL-2 receptor binding assay

The binding of [^{125}I]IL-2 to lymphocyte cell surface receptors was determined as previously described.[22] Cells were first depleted of endogenous bound IL-2 by incubating twice for 1 hr at 37° in RPMI-1640/10% FBS, followed by washing. To measure the kinetics of IL-2 binding to receptors, cells (1×10^6/100 μl) were incubated under conditions where IL-2 binds primarily to high-affinity (200 pM for CTLL-2), intermediate-affinity (1 nM for YT 2C2), or low-affinity (5 nM for MT-1β) sites, either in the absence or presence of gangliosides. After 20 min (37°) or 30 min (4°), cells were sedimented through a cushion of *n*-butylphthalate at 15,000 ***g*** for 10 min. Tubes were frozen in liquid nitrogen, tube tips containing the cell pellet were cut off, and pellets and supernatants were counted. Non-specific binding of [^{125}I]IL-2 in the presence of a 200-fold molar excess of unlabelled IL-2 was less than 5% of the maximum level of [^{125}I]IL-2 binding. Surface [^{125}I]IL-2 binding was determined by washing with 10 mM citrate/0·14 M NaCl, pH 3·5.

[^{125}I]IL-2 cross-linking studies and SDS–PAGE analysis

[^{125}I]IL-2 was cross-linked to IL-2R as previously described,[28] with some modifications. Cells (3×10^7/200 μl) were incubated with [^{125}I]IL-2 in RPMI-1640/10% FBS under either high-affinity (200 pM for CTLL-2), intermediate-affinity (1 nM for YT 2C2), or low-affinity (5 nM for MT-1β) conditions at 4° for 30 min, in the presence or absence of gangliosides. Cells were then washed twice in phosphate-buffered saline (PBS; 10 mM phosphate/0·14 M NaCl, pH 7·4) containing 1 mM $MgCl_2$, and cross-linked using bis(sulphosuccinimidyl)suberate (BSS; Pierce Chemical Co., Rockford, IL), at a final concentration of 1 mM. After 30 min at 4°, the reaction was terminated by the addition

of 2 mM EDTA. Cells were pelleted, washed twice with cold PBS, and lysed in 2 × -concentrated Laemmli sample buffer containing 10% (v/v) β-mercaptoethanol.[29] The lysates were freeze thawed three times and centrifuged at 15,000 *g* for 5 min to pellet insoluble materials. Lysates were denatured by boiling for 5 min, and SDS–PAGE was carried out according to Laemmli[29] using a BioRad Mini-Protean II apparatus. After electrophoresis, gels were fixed for 1 hr in 40% MeOH/10% acetic acid, dried and autoradiographed at −70° for 3 weeks using Kodak X-Omat XAR-5 film with an intensifying screen. Over 90% of the radioactivity in the cross-linked [^{125}I]IL-2/IL-2R bands was eliminated when cross-linking was carried out in a 200-fold excess of unlabelled rIL-2.

Binding of [^{125}I]IL-2 to gangliosides using a TLC overlay technique

Overlay experiments with [^{125}I]IL-2 were performed using an adaptation of the technique of Kielczyski and Harrison.[30] Precoated analytical high-performance TLC (HPTLC) plates (10 × 10 cm, silica gel 60, aluminum-backed, E. Merck AG, Darmstadt, Germany) were first activated by heating to 100° for 30 min. Various ganglioside and lipid standards (5 μg) and bovine brain gangliosides (50 μg) were chromatographed, using $CHCl_3$/MeOH/0·25% KCl (5:4:1 v/v). After drying, developed plates were dipped in 0·1% poly(isobutyl methacrylate) (high molecular weight; Aldrich Chemical Co., Milwaukee, WI) in hexane for 90 seconds. Plates were then blocked by incubation in Petri plates with Tris-buffered saline (TBS; 50 mM Tris/0·15 M NaCl, pH 7·4) containing 1% BSA, for 1 hr at 4°, with continuous gentle agitation. [^{125}I]IL-2 in TBS/1% BSA was added to a final concentration of 5 μCi/μg of ganglioside. Plates were incubated for 1 hr at 4° in a covered Petri dish, washed four times (20 min each) in cold TBS, air dried and exposed to Kodak X-Omat XAR-5 film for 4 days at −70°.

RESULTS

Gangliosides inhibit IL-2-induced proliferation of synchronized T-lymphocyte populations

To examine the effects of gangliosides on IL-2-dependent processes, two murine IL-2-dependent cell lines, CTLL-2 and HT-2, were studied. These cell lines express IL-2R, and proliferate and divide in response to IL-2. We have previously reported that both micellar and bilayer gangliosides inhibited IL-2-induced proliferation of unsynchronized CTLL-2 and HT-2,[22] as measured by the incorporation of [^{3}H]TdR into cellular DNA. To obtain more information on the inhibitory effects of gangliosides during one complete cell cycle, CTLL-2 and HT-2 were synchronized in G_0–G_1 by IL-2 deprivation.[24] After 14 hr incubation for CTLL-2 and 24 hr for HT-2 without IL-2, cell viability remained high (> 80%), as assessed by trypan blue exclusion. Cells were then stimulated with rIL-2 in the presence of various concentrations of gangliosides for a further 10 hr, followed by a 4-hr pulse with [^{3}H]TdR. The 14-hr time-point was the first at which substantial IL-2 induced [^{3}H]TdR incorporation was detectable (data not shown), and represents entry of the cells into S-phase. In the absence of gangliosides, 5 U/ml rIL-2 induced maximal proliferation in both CTLL-2 and HT-2. Figure 1 demonstrates that IL-2-stimulated DNA synthesis in synchronized cell populations was inhibited by micellar bovine brain gangliosides in a dose-dependent manner. The ganglioside concentration required for half-maximal inhibition (IC_{50}) was 175–190 μg/ml for both cell lines, similar to that observed for unsynchronized cells.[22]

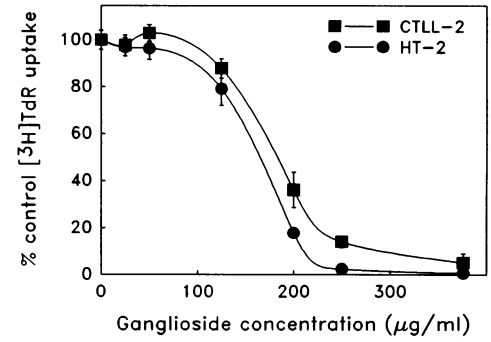

Figure 1. Inhibition of IL-2-stimulated DNA synthesis in synchronized cells by gangliosides. Synchronized CTLL-2 (■) and HT-2 (●) were incubated with rIL-2 (5 U/ml) and various concentrations of gangliosides. After 10 hr at 37°, cells were pulsed with 1 μCi/well [^{3}H]TdR for 4 hr. [^{3}H]TdR incorporation into cellular DNA was calculated as per cent control relative to cells stimulated in the absence of gangliosides. 100% control values for [^{3}H]TdR uptake were 15,000 ± 200 c.p.m. for CTLL-2, and 38,000 ± 2500 c.p.m. for HT-2. Each value represents the mean ± SEM of triplicate determinations.

Time–course of ganglioside inhibition in synchronized cell populations

To further examine the time frame within the cell cycle over which gangliosides are able to inhibit proliferation, synchronized CTLL-2 or HT-2 were stimulated with rIL-2 at time zero, and a maximally inhibitory concentration of gangliosides (250 μg/ml) was added at various times ranging from 0 to 14 hr. As shown in Fig. 2, the inhibition profile was similar for both

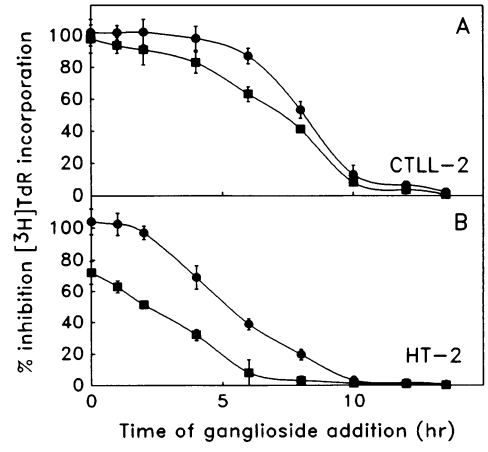

Figure 2. Time-course of ganglioside inhibition in synchronized cell populations. Synchronized CTLL-2 (A) or HT-2 (B) were incubated with 5 U/ml (●) or 50 U/ml (■) rIL-2 at time zero, and gangliosides (250 μg/ml) were added at various times after stimulation. Cells were pulsed with [^{3}H]TdR (1 μCi/well) at 10 hr, and harvested at 14 hr. The 14-hr time-point represents IL-2-induced [^{3}H]TdR incorporation in the absence of gangliosides. Results are presented as the mean ± SEM for triplicate determinations.

CTLL-2 (Fig. 2A) and HT-2 (Fig. 2B), although the early kinetics were slightly different, reflecting the longer generation time of CTLL-2. The most pronounced inhibition of [^{3}H]TdR incorporation occurred when gangliosides were added soon after rIL-2 stimulation; the first 4 hr for CTLL-2, and the first 2 hr for HT-2. With 5 U/ml of rIL-2 treatment, ganglioside addition up to 8 hr for CTLL-2, and 5 hr for HT-2, still resulted in over 50% inhibition. When gangliosides were added at later times after IL-2 stimulation, the inhibitory potency was greatly diminished. Previous work has shown that prolonged incubation with IL-2 (at least 6 hr) is required to induce IL-2-dependent cells to enter S-phase and become committed to cell division.[24] The data presented in Fig. 2 suggest that the presence of gangliosides during even the latter part of this period is sufficient to block further cell cycle progression.

Although 5 and 50 U/ml rIL-2 in the absence of gangliosides gave essentially identical maximal proliferative responses for both cell lines, some differences were observed in ganglioside inhibition at the higher concentration. As shown in Fig. 2, after stimulation with 50 U/ml rIL-2, proliferation of HT-2 was only 75% inhibited by addition of gangliosides at time zero, while maximal inhibition was still observed for CTLL-2. Half-maximal inhibition of DNA synthesis was also seen at a correspondingly earlier time-point for HT-2 (2 hr) compared to CTLL-2 (7 hr). These findings may perhaps be explained by the competitive nature of the ganglioside inhibition of IL-2-dependent proliferation. In the case of HT-2, we have shown that they have over threefold fewer high-affinity IL-2R than CTLL-2.[22] Sequestering of IL-2 by gangliosides will be less efficient at high IL-2 concentrations, and fractional receptor occupancy will probably be higher for cells with lower numbers of receptors. However, it should be noted that the relationship between IL-2R occupancy and extent of proliferation has not been established for these cell lines.

Reversal of ganglioside inhibition by high concentrations of IL-2

If the sole mechanism of ganglioside inhibition of proliferation is sequestration of IL-2 in the culture medium,[19 22] such inhibition should be readily and completely reversible at high IL-2 concentrations. Using synchronized CTLL-2 and HT-2, we added increasing concentrations of exogenous rIL-2 (1–1000 U/ml) to a fixed concentration of gangliosides (125 or 250 μg/ml). High rIL-2 levels alone had no negative effects on proliferation, and maximal DNA synthesis was reached at approximately 5 U/ml rIL-2. Ganglioside-induced inhibition of proliferation was completely overcome by high doses of rIL-2 (100 U/ml rIL-2 at 125 μg/ml gangliosides, and 1000 U/ml at 250 μg/ml gangliosides), in both CTLL-2 (Fig. 3A) and HT-2 (Fig. 3B). This observation would be expected if inhibition by gangliosides was due only to competition with IL-2R for available IL-2. Gangliosides significantly increased the concentration of rIL-2 required to give half-maximal proliferation (Fig. 3A and B). In the absence of gangliosides, half-maximal proliferation was achieved at 2·5 U/ml of rIL-2 in both CTLL-2 and HT-2 (not shown). At 125 μg/ml gangliosides, which gave 30% of maximal inhibition, IL-2-stimulated proliferation was completely recovered in the presence of 100 U/ml rIL-2. At 250 μg/ml gangliosides, 100 U/ml of rIL-2 was required to give half-maximal proliferation for HT-2, and 200 U/ml for CTLL-2. These results

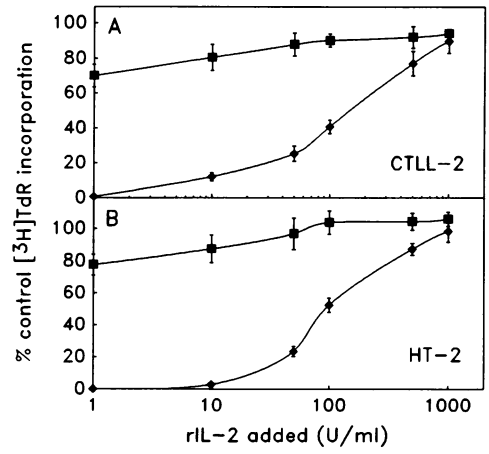

Figure 3. Reversal of ganglioside inhibition by high concentrations of IL-2. CTLL-2 (A) and HT-2 (B) were incubated for 10 hr with increasing concentrations of rIL-2, in the absence or presence of 125 μg/ml (■) or 250 μg/ml (◆) gangliosides. Uptake of [^{3}H]TdR into cellular DNA was measured following a 4-hr pulse. Each data point represents the mean ± SEM for triplicate determinations.

suggest that IL-2 can be 'titrated' against gangliosides, and give some indication of the affinity of their interaction.

Inhibition of [^{125}I]IL-2 binding to high-affinity receptors by gangliosides

In order to investigate the effects of gangliosides on the interaction of IL-2 with high-affinity IL-2R, [^{125}I]IL-2 receptor binding assays were performed under high-affinity binding conditions (200 pM), in the presence of increasing concentrations of gangliosides. Experiments were conducted at 4° to prevent internalization of the IL-2/IL-2R complexes over the time of the experiment. After pretreatment of CTLL-2 with [^{125}I]IL-2 at 4° for 60 min, a low pH wash resulted in a >90% decrease in bound [^{125}I]IL-2, demonstrating that the bulk of [^{125}I]IL-2 remained on the cell surface. As shown in Fig. 4, gangliosides prevented binding of [^{125}I]IL-2 to IL-2R on CTLL-2 in a dose-dependent fashion. Inhibition was most effective when gangliosides and [^{125}I]IL-2 were simultaneously added to CTLL-2 at time zero. The IC_{50} value for inhibition of [^{125}I]IL-2 binding was 0·5 mg/ml gangliosides, and binding was reduced by >90% at 2 mg/ml. Preincubation of CTLL-2 with gangliosides for 30 min at 4° prior to addition of IL-2 also reduced [^{125}I]IL-2 binding to a similar extent. These obervations indicate that gangliosides are able to compete effectively with high-affinity IL-2R to prevent binding of free IL-2. However, if cells were preincubated with [^{125}I]IL-2 for 30 min prior to the addition of gangliosides, [^{125}I]IL-2 binding was decreased by a maximum of only 50% (Fig. 4), indicating that gangliosides were less efficient at displacing bound IL-2 than at preventing binding.

CTLL-2 were preincubated with various concentrations of gangliosides for 30 min at 4°, to examine whether gangliosides altered the binding properties of IL-2R. Cells were either not washed (as above), or subsequently washed three times to remove gangliosides present in the culture medium, or loosely associated with the cell surface. [^{125}I]IL-2 binding was then determined at 4°. Without washing, gangliosides (2 mg/ml) reduced [^{125}I]IL-2 binding by >95% (Table 1). After washing,

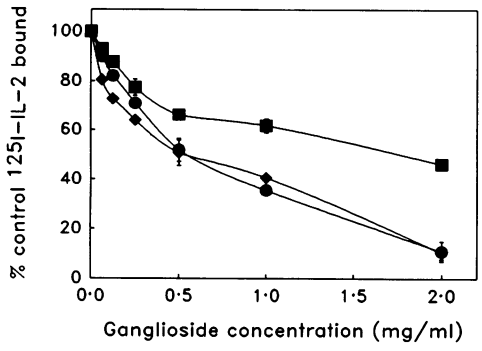

Figure 4. Inhibition of [^{125}I]IL-2 binding to high-affinity IL-2R by gangliosides. CTLL-2 were incubated with [^{125}I]IL-2 under high-affinity conditions (200 pM), and increasing concentrations of gangliosides were added as indicated. (a) [^{125}I]IL-2 and gangliosides were added simultaneously to cell cultures at time zero (●), followed by incubation for 30 min at 4°. (b) Cells were preincubated with [^{125}I]IL-2 for 30 min at 4°, followed by addition of gangliosides for a further 30 min (■). (c) Cells were preincubated with gangliosides for 30 min at 4°, followed by addition of [^{125}I]IL-2 for a further 30 min (◆). Cells were then sedimented through an *n*-butylphthalate cushion, and [^{125}I]IL-2 binding was determined. Per cent control [^{125}I]IL-2 bound was calculated relative to cells in the absence of gangliosides (9800 ± 180 d.p.m.). Data are shown as the mean ± SEM for duplicate determinations.

Table 1. Effect of washing on [^{125}I]IL-2 binding to ganglioside-treated CTLL-2

Ganglioside concentration (mg/ml)	% control [^{125}I]IL-2 bound	
	After washing	No washing
0	100·00 ± 1·28	100·00 ± 0·20
0·125	100·13 ± 0·89	73·73 ± 7·03
0·25	94·06 ± 0·93	68·23 ± 0·50
0·5	93·10 ± 2·16	53·70 ± 3·54
1·0	92·68 ± 0·75	37·40 ± 4·92
2·0	85·57 ± 5·17	4·99 ± 2·57

CTLL-2 (1×10^6 cells/200 μl) were preincubated with gangliosides for 30 min at 4°, and either not washed, or washed three times with RPMI-1640/10% FBS. [^{125}I]IL-2 (200 pM) was then added to the cells for 30 min, and binding was measured as described in Materials and Methods. Results are expressed as means ± SEM for triplicate determinations.

[^{125}I]IL-2 binding was decreased by < 15% at 2 mg/ml gangliosides. This marked recovery from the effect of gangliosides following washing suggests that the glycolipids have little effect on the IL-2R itself, and is consistent with their proposed role in IL-2 binding.

Effect of gangliosides on [^{125}I]IL-2 binding and cross-linking to p55 and p75

[^{125}I]IL-2 binding studies were carried out at 37° and 4° with MT-1β and YT 2C2, which express IL-2Rα (p55) and IL-2Rβ (p75), respectively.[25,27] At 37°, the ganglioside concentration required for half-maximal inhibition of binding was 190 μg/ml for YT 2C2, and 280 μg/ml for MT-1β, compared to an IC_{50} of 120 μg/ml for CTLL-2 (which expresses both p55 and p75) (Table 2). Maximal inhibition at 37° was reached at 1, 2 and 10 mg/ml for CTLL-2, YT 2C2, and MT-1β, respectively. Since a higher [^{125}I]IL-2 concentration is used in measurement of IL-2 binding to YT 2C2 (p75) and MT-1β (p55) than to CTLL-2 (p55/p75), a correspondingly higher ganglioside concentration is required for inhibition of [^{125}I]IL-2 binding in these two cell lines. IC_{50} values at 4° were substantially higher for all three cell lines. These results indicate that gangliosides can block binding of IL-2 not only to the high-affinity IL-2Rα/IL-2Rβ complex, but also to both IL-2Rα and IL-2Rβ expressed independently.

To confirm these findings, [^{125}I]IL-2 was incubated with YT 2C2 and MT-1β under intermediate-affinity (1 nM) or low-affinity (5 nM) conditions, and cross-linked to IL-2-binding proteins using the non-reducible homobifunctional cross-linking reagent BSS. After cell lysis, proteins were separated on reducing SDS–PAGE. Two radiolabelled protein bands with approximate molecular masses of 70,000 and 90,000 were observed (Fig. 5), corresponding to [^{125}I]IL-2 cross-linked to p55 and p75, respectively. Incubation of cells with gangliosides abrogated labelling of the 70,000 MW band on MT-1β, and the 90,000 MW band on YT 2C2. These results demonstrate that gangliosides inhibit IL-2 binding to both IL-2Rα and IL-2Rβ independently. To verify that this is also true for cells expressing the high-affinity p55/p75 complex, [^{125}I]IL-2 was cross-linked to CTLL-2 under high-affinity conditions (200 pM). In the presence

Table 2. Effect of gangliosides on binding of [^{125}I]IL-2 to CTLL-2, YT 2C2 and MT-1β

Ganglioside concentration (mg/ml)	% control [^{125}I]IL-2 bound (cell line)					
	CTLL-2		YT 2C2		MT-1β	
None	100	(100)*	100	(100)	100	(100)
0·0625	75	(90)	82	(ND)	89	(ND)
0·125	48	(82)	58	(88)	69	(ND)
0·25	40	(71)	47	(83)	55	(90)
0·5	24	(52)	24	(62)	27	(72)
1·0	2	(36)	17	(51)	26	(ND)
1·25	ND†	(ND)	ND	(ND)	ND	(49)
2·0	ND	(10)	7	(23)	22	(ND)
2·5	ND	(ND)	ND	(ND)	ND	(42)
4·0	ND	(ND)	ND	(22)	16	(ND)
5·0	ND	(ND)	ND	(ND)	ND	(38)
10·0	ND	(ND)	ND	(ND)	10	(29)
IC_{50} (mg/ml)‡	0·12	(0·5)	0·19	(0·75)	0·28	(1·25)

Cells (1×10^6/200 μl) were incubated with [^{125}I]IL-2 (200 pM for CTLL-2, 1 nM for YT 2C2 and 5 nM for MT-1β), in the presence of various concentrations of mixed gangliosides at 37° for 20 min, or at 4° for 30 min. Cell-bound and free [^{125}I]IL-2 were separated by differential centrifugation through an *n*-butylphthalate cushion, and pellets and supernatants were counted.

* Results for 4° are shown in parentheses.

† ND, not determined.

‡ IC_{50} represents the ganglioside concentration producing 50% inhibition of binding.

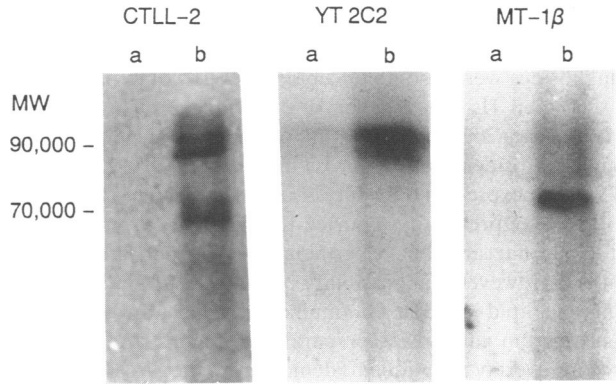

Figure 5. SDS–PAGE of [^{125}I]IL-2 cross-linked to IL-2R on CTLL-2, YT 2C2 and MT-1β. Cells were incubated with [^{125}I]IL-2 under high-affinity (200 pM for CTLL-2), intermediate-affinity (1 nM for YT 2C2), or low-affinity (5 nM for MT-1β) conditions, in the presence (a) or absence (b) of gangliosides (2 mg/ml). After 30 min at 4°, cells were cross-linked with BSS for an additional 30 min, and lysed. After freeze thawing, protein lysates were analysed on a 7·5% acrylamide gel under reducing conditions, followed by autoradiography. Two distinct bands are visible; the lower 70,000 MW band represents p55 cross-linked to IL-2, and the upper 90,000 MW band represents p75 cross-linked to IL-2.

of gangliosides, [^{125}I]IL-2 binding to both p55 and p75 was abolished, as indicated by the disappearance of both the 70,000 and 90,000 MW radiolabelled bands (Fig. 5). Maximal inhibition required 2 mg/ml of gangliosides, consistent with [^{125}I]IL-2 binding experiments.

Binding of IL-2 to gangliosides on TLC

TLC overlay has become a powerful technique to demonstrate the direct interaction of toxins and lectins with glycosphingolipids.[1,30,31] We have employed a TLC overlay technique to establish further the direct interaction of IL-2 with gangliosides. Various gangliosides and other lipids were separated on HPTLC plates, which were then coated with poly(isobutyl methacrylate), blocked, and incubated with [^{125}I]IL-2 at 4°. Bound [^{125}I]IL-2 was detected by autoradiography. Figure 6 indicates that [^{125}I]IL-2 bound to several different ganglioside species on the HPTLC plate, including GM_3, GM_2, GM_1, GD_{1a} and GT_{1b}. [^{125}I]IL-2 also bound to many ganglioside components in a bovine brain mixture. These results were further confirmed by spraying the chromatogram with resorcinol–HCl, which is specific for sialic acid. The resorcinol-stained purple spots matched the radioactive bands (not shown). These findings strongly support the idea that IL-2 interacts directly with gangliosides. [^{125}I]IL-2 did not bind to neutral glycolipids (under these conditions), or anionic lipids such as phosphatidylserine and sulphatides. When less stringent washing conditions, or a longer autoradiogram exposure time, were used, [^{125}I]IL-2 binding to complex neutral glycolipids (asialo-GM_1, globoside) was also observed. We previously noted[20,21] that these glycolipids were able to inhibit T-cell proliferation, although less effectively than gangliosides.

DISCUSSION

Gangliosides have been found to be effective suppressors of both murine and human T-lymphocyte proliferation *in vitro*.[2,3,19,20,22] We have proposed that these glycosphingolipids interfere with lymphocyte proliferation by depleting available IL-2 in the extracellular medium, thus reducing the amount of IL-2 available for binding to IL-2R.[21,22] In order to examine further the ability of gangliosides to interfere with the interaction of IL-2 with IL-2R, we have employed two IL-2-dependent murine T-lymphocyte cell lines, CTLL-2 and HT-2, which proliferate in response to IL-2. In the present study, we show that gangliosides inhibit the IL-2-mediated proliferation of synchronized CTLL-2 and HT-2 populations *in vitro*, in a dose-dependent fashion. We previously reported that IL-2 concentrations of up to 10^4 U/ml were able to only partially restore the proliferative responses of ganglioside-treated unsynchronized CTLL-2 and HT-2, giving up to 40% recovery for HT-2, and $<12\%$ for CTLL-2.[22] In unsynchronized populations, cells completing one round of cell division and entering G_1 of the next

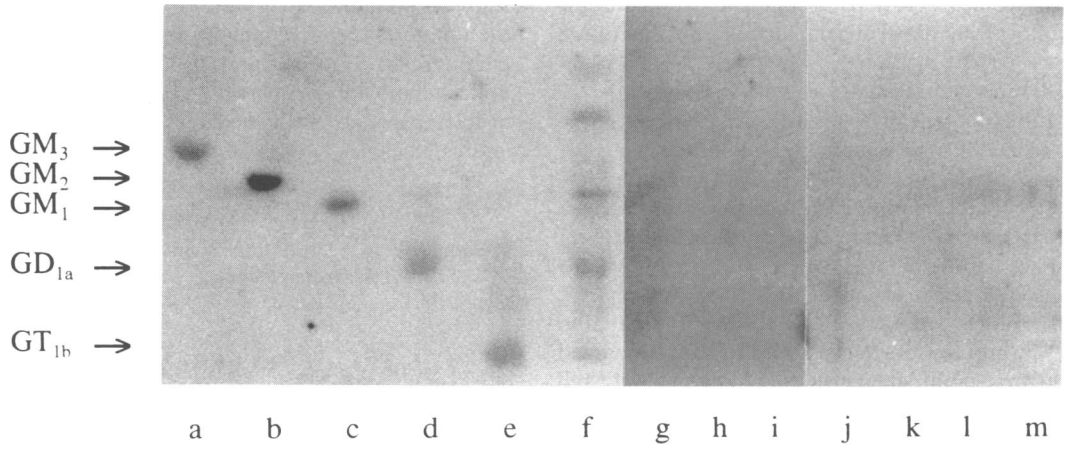

Figure 6. Binding of [^{125}I]IL-2 to gangliosides on HPTLC chromatograms. Gangliosides and other lipids were separated in $CHCl_3$/MeOH/0·25% KCl (5:4:1 v/v) on HPTLC plates, which were dipped in 0·1% poly(isobutylmethacrylate). After blocking with 1% BSA in TBS (pH 7·4) for 1 hr at 4°, the plate was overlaid with [^{125}I]IL-2 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 trihexoside, (h) asialo-GM_1, (i) globoside, (j) lactosyl ceramide, (k) galactosyl ceramide, (l) phosphatidylserine, and (m) sulphatides.

cycle are not inhibited by gangliosides until some time after initiation. This results in a time-course of ganglioside inhibition that extends over a period of ~18 hr for HT-2, and ~44 hr for CTLL-2,[22] which has a longer generation time. The lifetime of exogenously added IL-2 in the culture system is likely limited to a few hours, so that only the fraction of cells entering G_1 during this early time period would escape ganglioside inhibition. This would result in only a partial reversal of inhibition, and recovery would be expected to be lower for the cell line with the longer generation time. However, in the case of synchronized cell populations, proliferation in the presence of gangliosides was completely restored by high levels of exogenous IL-2. In this case, all cells enter G_1 together at time zero, and gangliosides are only inhibitory for a few hours (Fig. 2). High levels of exogenous IL-2 should thus be completely effective at preventing ganglioside inhibition in synchronized cell populations. Our observations are consistent with the proposal that the anti-proliferative effect of gangliosides is due only to competitive inhibition of IL-2 binding over the first few hours after stimulation. Synchronized cell populations initiated at the G_0–G_1 phase of the cell cycle are thus an important tool to examine the molecular basis of immunosuppressive processes.[32]

Since ganglioside inhibition is predicted to be of a competitive nature, a binding assay was carried out to study the effect of gangliosides on the interaction of IL-2 with its high-affinity IL-2R. If gangliosides were added to CTLL-2 either before, or simultaneously with [^{125}I]IL-2, the amount of [^{125}I]IL-2 associated with cells was dramatically decreased, to less than 10% of control. However, if [^{125}I]IL-2 was allowed to bind to cells prior to addition of gangliosides, a lesser degree of inhibition was observed (Fig. 4). These results indicate that gangliosides can very effectively prevent IL-2 from binding to IL-2R, but they are less effective at removing previously bound growth factor. Washing the cells free of gangliosides before addition of [^{125}I]IL-2 fully restored binding to high-affinity IL-2R (Table 1), consistent with the view that the glycolipids interact with IL-2 in the extracellular medium. Ganglioside treatment thus does not appear to have a significant effect on the binding properties of IL-2R themselves. This is in agreement with our previous report that the inhibitory effects of gangliosides on IL-2-stimulated [^{3}H]TdR incorporation are completely reversible for at least 12 hr following removal of gangliosides from the culture medium.[22]

Based on a time-course study, gangliosides appeared to block proliferation of synchronized cells in the G_1 phase of the cell cycle, via an inability to generate the requisite signals for progression into S-phase. Interaction of IL-2 with its receptor is rapid, and a series of biochemical changes occurs within seconds of addition of IL-2, including increases in intracellular pH and tyrosine phosphorylation.[15,33–35] However, IL-2 treatment for less than 6 hr is insufficient to induce cells to enter S-phase,[24] indicating that prolonged IL-2/IL-2R occupancy is essential to promote cell cycle progression. When added to IL-2-stimulated cells at the 6-hr time-point, gangliosides (250 μg/ml) were still able to inhibit proliferation by 40–90% (Fig. 2), which suggests that sufficient bound IL-2 is removed from the cell surface to prevent entry into S-phase.

Both α- and β-subunits of IL-2R can independently bind IL-2 at (non-physiological) nanomolar IL-2 concentrations. Cell lines expressing exclusively the α-subunit (MT-1β) or the β subunit (YT 2C2) are useful tools to dissect IL-2R binding processes. We used these cell lines to show that, in addition to preventing binding of IL-2 to the high-affinity p55/p75 complex, gangliosides were also able to block [^{125}I]IL-2 binding to both IL-2Rα and IL-2Rβ independently (Table 2). This was confirmed using chemical cross-linking studies, which indicated that gangliosides abolished cross-linking of [^{125}I]IL-2 to p55 and p75, when expressed either separately, or together (Fig. 5).

We have previously reported that IL-2 co-chromatographs with micellar and bilayer gangliosides on gel filtration columns.[21] However, the possibility remained that binding was to non-glycolipid peptide contaminants, which are difficult to remove from ganglioside preparations. In this study, we have used an overlay technique to demonstrate the direct binding of IL-2 to gangliosides on TLC. The overlay method is highly sensitive and specific, and has been used to study the interaction of gangliosides with ^{125}I-labelled cholera toxin and *Limax flavus* agglutinin. Previous work in our laboratory showed that IL-2 binding to gangliosides was abolished by denaturation of the lymphokine, suggesting that a native protein is required.[21] In addition, the interaction required both the ganglioside carbohydrate headgroup and the lipid moiety, and did not appear to involve hydrophobic interactions. We previously proposed that IL-2 may contain a lectin-like site capable of interacting with gangliosides.[21] In support of this view, a recent report indicates that inhibition of T-lymphocyte proliferation by gangliosides involves a complex structure–function relationship at the level of the oligosaccharide headgroup.[36] We suggest that the relationship between immunosuppressive potency and headgroup structure may reflect the affinity of the interaction between the various ganglioside species and IL-2. This TLC overlay procedure may prove useful in the rapid screening of various glycolipid species for IL-2 binding.

The results presented in this paper indicate that ganglioside-mediated inhibition of IL-2-induced proliferation is a direct result of competition between gangliosides and IL-2R for IL-2 binding. Rapidly growing tumours shed substantial quantities of membrane gangliosides into the extracellular medium. These shed components may transfer into nearby cells and alter their proliferative responses. In addition, shed gangliosides enter the blood circulation, where they may sequester IL-2 and thus suppress immune responses both close to, and far away from, the tumour site. Such immunosuppression may contribute, in part, to the uncontrolled growth of malignant tumours. A better understanding of the interaction between IL-2 and gangliosides at the molecular level is necessary before this problem can be addressed clinically.

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