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Gangliosides are potent immunosuppressors of IL-2-mediated T-cell proliferation in a low protein environment

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SUMMARY

Gangliosides are immunosuppressive to many classes of immune cells, and shedding of these glycosphingolipids by tumour cells may regulate immune responses in cancer, and protect tumours from host immune destruction. One mechanism of immunosuppression by gangliosides *in vitro* involves competition with interleukin-2 receptors (IL-2R) for binding of IL-2. Previous studies on inhibition of IL-2-mediated events by gangliosides have been conducted in the presence of high levels of fetal bovine serum (FBS). However, gangliosides shed by tumours *in vivo* will encounter immune cells in the low protein microenvironment of the tissue fluid. In order to better mimic physiological conditions, we have examined immunosuppression by gangliosides towards IL-2-dependent HT-2 cells in a low serum–low protein medium. The ability of gangliosides to inhibit IL-2-stimulated DNA synthesis in HT-2 increased dramatically as the serum concentration in the culture medium was decreased; the 50% inhibitory concentration (IC_{50}) value for GM_1 was 13 μM under low serum conditions, 14-fold lower than the value obtained in 10% FBS. Further investigation revealed that the mechanism of immunosuppression by gangliosides in low serum–low protein medium involved interference with the IL-2/IL-2R system. Ganglioside-mediated inhibition was dependent on the continued presence of the glycolipids during the first few hours after IL-2 stimulation, and could be reversed by increasing levels of IL-2. Receptor binding experiments demonstrated that gangliosides blocked the interaction of IL-2 with high-affinity IL-2 receptors on HT-2. Taken together, these results support the view that gangliosides will act as much more potent suppressors of IL-2-dependent processes *in vivo* in the vicinity of a tumour.

INTRODUCTION

Several different tumour types, including melanomas, leukaemias, neuroblastomas, hepatomas and retinoblastomas, are associated with greatly increased levels of gangliosides in the circulation *in vivo*, in both animals and humans.[1–5] In many cases, only specific ganglioside species are shed by a particular tumour cell type. These gangliosides are often the same species overexpressed by the tumour cells, and can serve as tumour-specific markers. For example, the sera of hepatoma patients show 100-fold increases in GM_2 ganglioside, and sera of individuals with head and neck carcinomas have elevated levels of GM_3. Human melanomas overexpress GM_2, neuroblastoma tumours overexpress GM_2 and GD_2, and serum GD_2 levels in neuroblastoma patients are 50-fold greater than normal. The high circulating level of these specific ganglioside species in serum correlates with tumour burden, progression, and clinical responses to treatment.[1,6] In patients with retinoblastoma, the level of GD_2 could be a potential serum marker of the disease.[2] GD_2 levels are also useful clinical markers for human neuroblastoma tumour diagnosis and monitoring.[4,7] It has been suggested that a more rapid cancer progression and lower survival rate are associated with high circulating ganglioside levels at clinical diagnosis,[7,8] and several studies have confirmed that gangliosides can enhance tumour growth.[9,10]

In recent years, it has been widely recognized that gangliosides are immunosuppressive to many classes of immune cells, including lectin- and antigen-stimulated B and T lymphocytes, helper T cells, natural killer (NK) cells and accessory cells.[8,11,12] These immunosuppressive properties are clearly evident *in vitro*, and have also been demonstrated *in vivo*.[13] Tumour cells of the types known to generate high levels of gangliosides *in vivo* have been found to shed gangliosides into the culture medium *in vitro*, and previous studies have shown that such tumour-derived species are highly immunosuppressive.[14–17] Ganglioside levels in the serum and ascites fluid of tumour-bearing animals and humans are comparable to those required to suppress immune competence *in vitro*,[14] and it has been proposed that tumour-derived gangliosides are responsible for the immunosuppression observed in many cancer patients.

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Abbreviations: FBS, fetal bovine serum; IL-2R, interleukin-2 receptor; LSLP, low serum–low protein medium; N-SP, Nutridoma SP; rIL-1β, recombinant interleukin-1β.

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Over the last few years, work in our laboratory has focused on the immunosuppressive actions of gangliosides and sialoglycoproteins on T lymphocytes, using both natural T-lymphocyte populations[18] and various cultured T-lymphocyte cell lines.[19–21] We have shown previously that one important mechanism of T-lymphocyte immunosuppression by gangliosides *in vitro* involves interference with the interleukin-2 (IL-2)/IL-2 receptor (IL-2R) system,[18–20,22] which is central to T-cell proliferative responses.[23] Virtually all previous studies on the immunosuppressive properties of gangliosides *in vitro* have been carried out in the presence of high levels of serum. As relating such immunosuppression to the situation of tumours *in vivo* is an important goal, the use of serum presents two problems. First, the local microenvironment (the tissue fluid) in the vicinity of a ganglioside-shedding tumour is relatively protein-free. The protein content of the interstitial fluid is approximately 2·5 mg/ml,[24] which is only 3% of that reported for whole plasma (60–80 mg/ml). Second, gangliosides are known to interact with serum proteins,[25–28] especially albumin and low-density lipoproteins. Thus the use *in vitro* of gangliosides added to medium containing high levels of serum does not reflect the *in vivo* situation, and may lead to misleading conclusions.

In the present work, we examined the immunosuppressive action of gangliosides while more closely mimicking the conditions likely to prevail *in vivo*. The cell line HT-2 provides an excellent model for investigating the immunomodulation of T cells that express IL-2R and respond to IL-2. In order to better approximate a physiological environment, we adapted HT-2 cells to growth in a low serum–low protein medium. The immunosuppressive effects of gangliosides on cellular proliferation in this low protein environment were then explored. In this report, we demonstrate that gangliosides are much more potent immunosuppressive agents when presented to HT-2 cells in a low protein milieu. In addition, we have determined that the mechanism of ganglioside action under these conditions results from interference with the IL-2/IL-2R system. These findings have implications for the immunosuppressive actions *in vivo* of gangliosides shed from tumours, and also for the proposed use of gangliosides as clinical immunosuppressive agents.

MATERIALS AND METHODS

Materials

GM_1 (1545 MW), GM_2 (1360 MW), GM_3 (1210 MW), GD_{1a} (1836 MW), GD_2 (1674 MW) and GT_{1b} (2127 MW) (all >95% purity) were purchased from Sigma Chemical Co. (St Louis, MO). Mixed gangliosides (1830 MW) were isolated from bovine brain using a modification of the procedure of Kanfer.[22,29] Human recombinant (r)IL-1β and rIL-2 were obtained from Boehringer-Mannheim (Dorval, Quebec, Canada). Human recombinant [^{125}I]IL-2 (800–850 Ci/mmol) was purchased from Amersham (Oakville, Ontario, Canada).

Lymphocyte cell lines

The murine IL-2-dependent helper T-cell line HT-2 was routinely cultured in RPMI-1640 (ICN Biochemicals and Flow Laboratories, Mississauga, Ontario, Canada) supplemented with 0·5% fetal bovine serum (FBS, heat-inactivated; Gibco, Burlington, Ontario, Canada) and 1% Nutridoma SP (N-SP; Boehringer-Mannheim), which is referred to as LSLP (low serum–low protein medium). The protein content of LSLP was approximately 0·21 mg/ml. N-SP is a biochemically defined medium supplement containing no serum, growth factors, mitogens or hormones, except for insulin and transferrin. LSLP was also supplemented with 50 μM 2-mercaptoethanol and 2 mM each of non-essential amino acids, sodium pyruvate, L-glutamine, and penicillin–streptomycin, and 10% v/v Jurkat-derived IL-2[19] was added for routine maintenance. The cell lines A20 (murine acute B-cell lymphoma; ATCC no. TIB 208; Rockville, MD), K562 (human chronic myelogenous leukaemia; ATCC no. CCL 243) and EL-4 NOB-1 (a murine thymocyte line)[30] were cultured in RPMI-1640/1% N-SP in the absence of serum. Cells were counted using a Coulter Counter model ZM (Hialeah, FL), and cell viability was determined using trypan blue exclusion.

Measurement of DNA synthesis

Cell proliferation was monitored by [^{3}H]thymidine incorporation into cellular DNA, as described previously.[19–21] HT-2 cells (1×10^5/ml) were seeded in flat-bottomed 96-well microplates (Nunc, Roskilde, Denmark) in 200 μl of either LSLP or RPMI-1640/1% N-SP containing 0–10% FBS. After addition of 5 U/ml rIL-2 and gangliosides as indicated, cells were incubated for 20 hr at 37°. Cells were pulse-labelled with 1 μCi/well [^{3}H]thymidine (6·7 Ci/mmol; Amersham) for an additional 6 hr, followed by harvesting on glass fibre filter strips with a Titertek automatic cell harvester (Flow Laboratories, McLean, VA). The filter discs were dried and counted using anhydrous scintillant. Positive controls contained cells with rIL-2 only, whereas negative controls contained no rIL-2.

K562 and A20 cells were cultured at 1×10^5 cells/ml in RPMI-1640/1% N-SP medium in the presence of increasing concentrations of gangliosides. After incubation at 37° for 20 hr, cells were pulsed with 1 μCi/well [^{3}H]thymidine for a further 6 hr, followed by harvesting and counting as described above.

Synchronization of HT-2 cells

To synchronize HT-2 in G_0–G_1, cells were depleted of IL-2 prior to use[20,31] by incubation in LSLP alone for 6 hr, which was the longest period of IL-2 deprivation that maintained viability at >90%. For determination of the time–course of ganglioside inhibition, synchronized HT-2 (1×10^5 cells/ml) was incubated with rIL-2 at time 0, and gangliosides were added to the cultures at various times up to 14 hr later. The uptake of [^{3}H]thymidine into cellular DNA was then determined as outlined above.

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

In response to stimulation with IL-1, the murine thymocyte cell line EL-4 NOB-1 secreted large amounts of IL-2, which was measured using an HT-2 cell bioassay as described previously.[21,30] For experiments in which IL-1 stimulation was carried out in the presence of gangliosides, culture supernatants were diluted by four-, 10- or 20-fold, so that the effects of ganglioside carryover could be assessed. The final ganglioside concentration added to the HT-2 cells following 20-fold dilution was below the inhibitory range.[20,21]

Binding of [^{125}I]IL-2 to high-affinity IL-2 receptors

The interaction of IL-2 with its high-affinity cellular receptors

was determined by measuring the binding of [^{125}I]IL-2 to intact HT-2 cells.[19–21] Cells were depleted of endogenous bound IL-2 by incubating twice for 1 hr at 37° in LSLP, followed by washing. HT-2 (1×10^6 cells) was incubated with 200 pM [^{125}I]IL-2 in LSLP in a total volume of 200 μl. After incubation with shaking at 4° for 1 hr, cell-bound and free [^{125}I]IL-2 were separated by differential centrifugation through a cushion of prechilled *n*-butylphthalate at 15000 *g* for 10 min at room temperature. The 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.

RESULTS

Growth characteristics of HT-2 under LSLP conditions

HT-2 cells were adapted to growth in RPMI-1640/1% N-SP containing decreasing amounts of FBS. The lowest concentration of FBS compatible with acceptable growth of the cells in the presence of 1% N-SP was 0·5% (v/v). Growth curves for HT-2 in both LSLP and RPMI-1640/10% FBS were determined by cell counting, and the patterns of cell growth were found to be very similar, with the cell number increasing in a logarithmic fashion for the first 3 days and then reaching a plateau. The final cell numbers were generally two- to threefold lower in LSLP than in RPMI-1640/10% FBS. The IL-2 dose–response curve of HT-2 in LSLP was compared to that observed in RPMI-1640/10% FBS using the DNA synthesis bioassay. Maximal [^{3}H]thymidine uptake was obtained with 5 U/ml rIL-2, which was similar to that obtained for HT-2 in RPMI-1640/10% FBS.

Immunoinhibitory effect of gangliosides on HT-2 at different serum levels

To determine the effects of serum on immunosuppression of gangliosides, HT-2 cells were incubated, together with various concentrations of GM_1, in RPMI-1640/1% N-SP with 0·5%, 1%, 2%, 5% or 10% (v/v) FBS. As shown in Fig. 1, inhibition of DNA synthesis by GM_1 increased greatly as the FBS content of the medium decreased. For each level of FBS, the GM_1 concentration required to produce 50% inhibition of [^{3}H]thymidine uptake (IC_{50}) was measured, and the data are summarized in Table 1. As the concentration of FBS in the medium decreased from 10% to 0·5%, the IC_{50} value decreased approximately 14-fold. Thus the sensitivity of HT-2 to inhibition by GM_1 was much higher in LSLP than in medium containing FBS.

Response of HT-2 to different ganglioside species under LSLP conditions

It has been reported that in RPMI-1640/10% FBS the inhibitory potency of specific ganglioside species depends on the sialic acid content and complexity of the oligosaccharide structure.[18,19,32] To examine whether the immunoinhibitory effect of gangliosides is related to their structure in a similar way in a low protein environment, different species of purified gangliosides were incubated with HT-2 in LSLP, and IL-2-mediated proliferation was assessed. As shown in Fig. 2, the relative ability of various gangliosides to suppress IL-2-induced DNA synthesis in LSLP was the same as that in RPMI-1640/10% FBS,[18,19] i.e. $GT_{1b} > GD_{1a} \approx$ mixed gangliosides > GM_1. For each ganglioside species, the IC_{50} value was measured, and the results are summarized in Table 1, together

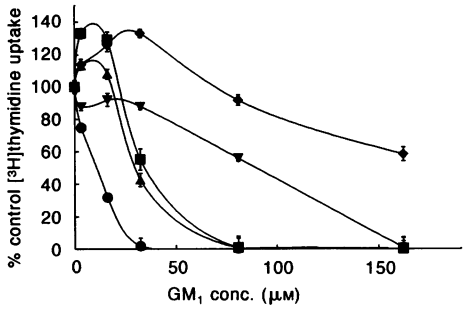

Figure 1. Inhibition of IL-2-stimulated DNA synthesis in HT-2 by GM_1 in RPMI-1640 containing increasing concentrations of serum. HT-2 (2×10^4 cells/200 μl) was cultured in RPMI-1640/1% N-SP containing 5 U/ml rIL-2 and various concentrations of GM_1, with different FBS levels in medium: 0·5% (●); 1% (▲); 2% (■); 5% (▼); and 10% (◆). After incubation for 20 hr at 37°, cells were pulsed with [^{3}H]thymidine (1 μCi/well) for an additional 6 hr, and then harvested. Percentage control [^{3}H]thymidine uptake into cellular DNA was calculated relative to that of cells grown in the absence of GM_1 (135000 ± 4500 c.p.m. for 10% FBS). Individual data points represent means ± SD for triplicate determinations. Results of a representative experiment are shown.

Table 1. Inhibition of HT-2 DNA synthesis in medium with different serum levels and species of gangliosides

Serum concentration (%)*	IC_{50} for GM_1 (μM)†
0·5	13
1	27
2	35
5	87
10	180‡

Ganglioside species	IC_{50} (μM) LSLP	IC_{50} (μM) 10% FBS§
GM_1	13	107
GM_2	12	—
GM_3	17	—
GD_{1a}	8·2	71
Mixed	8·7	77
GT_{1b}	4·7	31

*Culture medium was RPMI-1640/1% N-SP containing various concentrations of FBS.

†IC_{50} represents the ganglioside concentration producing 50% inhibition of DNA synthesis, obtained from the average of two separate experiments.

‡Value estimated from Fig. 1 by extrapolation.

§Data taken from ref. 18. Cell culture conditions differed slightly from those used in the present work, and cannot be compared directly with the data above.

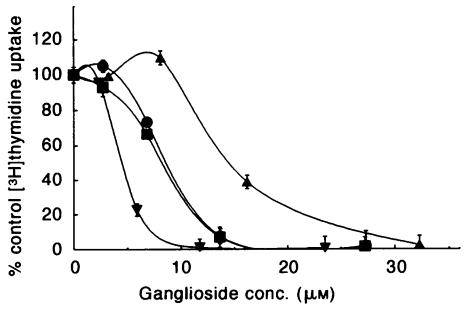

Figure 2. Inhibition of HT-2 DNA synthesis in LSLP medium by various species of gangliosides. HT-2 (2×10^4 cells/200 μl) was cultured in LSLP (RPMI-1640/1% N-SP/0·5% FBS) containing 5 U/ml of rIL-2 and increasing concentrations of mixed bovine brain gangliosides (●), GM_1 (▲), GD_{1a} (■) and GT_{1b} (▼). Percentage control uptake of [^{3}H]thymidine into cellular DNA was calculated relative to that of cells grown in the absence of gangliosides (150 000 ± 5600 c.p.m.). Individual data points represent means ± SD for triplicate determinations. Results of a representative experiment are shown.

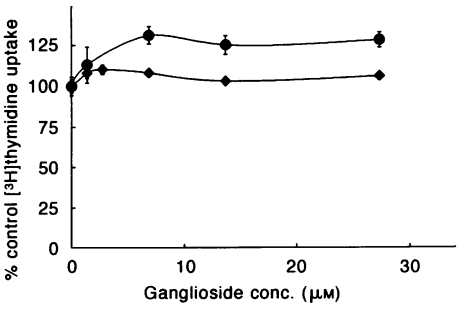

Figure 4. Effect of gangliosides on DNA synthesis in K562 and A20 cells in serum-free medium. A20 (●) and K562 (◆) were cultured at 1×10^5 cells/ml in RPMI-1640/1% N-SP, with increasing concentrations of mixed gangliosides. After incubation for 20 hr at 37°, cells were pulsed with 1 μCi/well [^{3}H]thymidine. Incorporation into cellular DNA was calculated as percentage control relative to untreated cells (72 000 ± 700 c.p.m. for K562, and 42 000 ± 1400 c.p.m. for A20). Each data point represents the mean ± SD for triplicate determinations. Results of a representative experiment are shown.

with those values obtained from previous work for HT-2 cultured in RPMI-1640/10% FBS.[19] Each ganglioside species had a greater inhibitory effect on IL-2-stimulated HT-2 proliferation in LSLP than in RPMI-1640/10% FBS, with IC_{50} values seven- to ninefold lower. Ganglioside species overexpressed by certain tumours (e.g. GM_2 and GM_3) were also potent immunoinhibitors in a low protein environment.

Effect of gangliosides on other lymphocyte cell lines

In order to determine the specificity of ganglioside-mediated immunosuppression, an IL-1-stimulated and two lymphokine-independent lymphocyte cell lines were employed. The mouse thymocyte line EL-4 NOB-1 responds to IL-1 by secreting IL-2, which can be quantified by using the HT-2 proliferation bioassay. EL-4 NOB-1 cells were cultured in RPMI-1640/1% N-SP in the presence or absence of gangliosides, and stimulated with rIL-1β. After 24 hr, IL-2-containing supernatants from EL-4 NOB-1 were diluted four-, 10- and 20-fold before addition to HT-2 cells, to account for the effects of ganglioside carryover. The data in Fig. 3 show that 14 and 27 μM ganglioside (concentrations sufficient to completely inhibit IL-2-stimulated HT-2 proliferation in LSLP) had little effect on IL-1-stimulated production of IL-2 by EL-4 NOB-1 in serum-free medium. This was true at each of the three supernatant dilutions tested. Thus gangliosides do not interfere with an IL-1-driven process under low protein conditions.

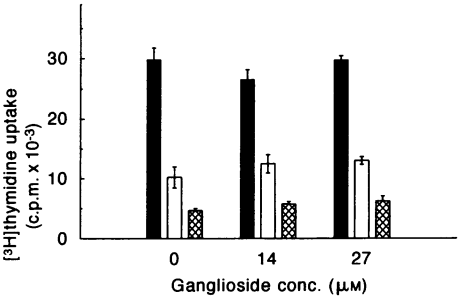

Figure 3. Effect of gangliosides on IL-1-dependent secretion of IL-2 by EL-4 NOB-1 in serum-free medium. EL-4 NOB-1 cells (2×10^5/200 μl) were cultured in RPMI-1640/1% N-SP and stimulated with 50 U/ml rIL-1β in the absence or presence of mixed gangliosides (14 and 27 μM). After incubation for 24 hr at 37°, IL-2-containing supernatants were removed, and diluted with medium, 20-fold (filled bar), 10-fold (open bar) or fourfold (hatched bar), to account for carryover of the gangliosides to the tester HT-2 cell line. The diluted supernatants were then assayed for their ability to stimulate DNA synthesis in HT-2. Individual data points represent means ± SD for triplicate determinations. Results of a representative experiment are shown.

To investigate the possibility of non-specific effects of gangliosides on lymphocyte proliferation, two growth factor-independent cell lines, murine acute B-cell lymphoma A20 and human chronic myelogenous leukaemia K562, were employed. As shown in Fig. 4, gangliosides in serum-free medium had no inhibitory effect on DNA synthesis in either A20 or K562. This observation indicates that the glycolipids are not non-specifically inhibitory to the proliferation of growth factor-independent lymphocyte cell lines.

Mechanism of immunosuppression by gangliosides under LSLP conditions

It is known that micellar gangliosides bind rapidly to the lymphocyte membrane, with the bulk of the association occurring during the first 90 min. Some of the bound gangliosides insert into the outer leaflet, and become molecularly dispersed in the lymphocyte plasma membrane.[33] Permanent alteration of the HT-2 cell membrane, as well as IL-2R, might result from ganglioside binding and insertion at the cell surface. To determine whether responsiveness of HT-2 to IL-2 in LSLP was changed by insertion of gangliosides, cells were preincubated with an inhibitory concentration (27 μM) of mixed gangliosides, then washed before addition of rIL-2. As discussed previously, the maximal period of IL-2 deprivation that maintained HT-2 cell viability over 90% was 6 hr. Pretreatment of HT-2 with gangliosides for 6 hr did not affect

Table 2. Effect of ganglioside pretreatment on IL-2-mediated proliferation of HT-2 cells

	[^{3}H]thymidine uptake (c.p.m. $\times 10^{-4}$)	
Time of pretreatment	Medium alone	With gangliosides
None	8·75 ± 0·17	
2 hr	6·69 ± 0·38	5·75 ± 0·18
6 hr	7·73 ± 0·22	5·64 ± 0·16

HT-2 cells (4×10^6/ml) were precultured in 4 ml LSLP in either the absence or presence of an inhibitory concentration of mixed gangliosides (27 μM). After incubation for 2 or 6 hr, cells were washed three times with medium, and cultured at 1×10^5 cells/ml in the presence of 5 U/ml rIL-2. Proliferation responses were determined by uptake of [^{3}H]thymidine into cellular DNA. Results from a representative experiment are presented, as the means ± SD for triplicate determinations.

cell viability, and had only small effects on IL-2-induced DNA synthesis compared to preincubation with medium alone (Table 2). Accordingly, exposure of HT-2 to exogenous gangliosides had no significant deleterious effects on the ability of the cells to proliferate. Gangliosides which may spontaneously incorporate into the HT-2 plasma membrane during the 6 hr preincubation period do not, therefore, cause immunosuppression.

To examine the time-frame within the cell cycle over which gangliosides are able to inhibit proliferation, synchronized HT-2 cells were stimulated with rIL-2 at time 0. A highly inhibitory concentration of gangliosides (40 μM) was added at various times, ranging from 0–14 hr. The most pronounced inhibition of [^{3}H]thymidine incorporation occurred when gangliosides were added soon after rIL-2 stimulation. Addition of gangliosides 7 hr after IL-2 stimulation resulted in over 50% inhibition, but when gangliosides were added more than 10 hr after stimulation, there was no inhibitory effect. A similar time–course was reported previously for ganglioside effects on HT-2 grown in RPMI-1640/10% FBS.[20] These findings demonstrate that gangliosides must be present at a very early phase of the IL-2 signalling cascade to cause inhibition of proliferation.

If the inhibitory effects of gangliosides are mediated by interference with the IL-2/IL-2R system in low protein conditions, we would expect this inhibition to be reversible at high IL-2 concentrations. To test this hypothesis, synchronized HT-2 cells were incubated in LSLP with increasing concentrations of exogenous rIL-2 (1–1000 U/ml), in the presence of a fixed concentration of gangliosides (14 μM). High IL-2 levels alone had no negative effects on proliferation, and maximal DNA synthesis was reached at approximately 5 U/ml rIL-2. On the other hand, ganglioside-induced inhibition of proliferation was almost entirely overcome at 125 U/ml rIL-2 (Fig. 5). This observation is consistent with the proposal that inhibition by gangliosides is due only to competition with IL-2R for available IL-2. Gangliosides greatly increased the concentration of rIL-2 required to give half-maximal proliferation. In their absence, half-maximal proliferation was achieved at 1 U/ml, whereas at 14 μM ganglioside 65 U/ml rIL-2 was required.

If the mechanism of the inhibitory effect of gangliosides in a low protein environment is the same as in FBS-containing

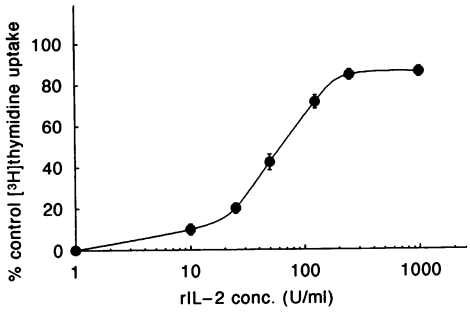

Figure 5. Reversibility of ganglioside inhibition by exogenous IL-2 in low serum medium. HT-2 (1×10^5 cells/ml) was synchronized in G_0, and cultured in LSLP with various concentrations of rIL-2 at time 0, in the presence of mixed gangliosides (14 μM). After 10 hr, cells were pulsed with [^{3}H]thymidine for an additional 4 hr. Incorporation of [^{3}H]thymidine into cellular DNA was calculated as percentage control relative to cells incubated in the absence of gangliosides (the 100% control value was 78 000 ± 3500 c.p.m.). Individual data points represent means ± SD for triplicate determinations. Results of a representative experiment are shown.

medium, it should be possible to demonstrate direct competition between gangliosides and IL-2R for binding to IL-2. The characteristics of IL-2 binding to intact HT-2 cells in LSLP have not been determined previously. Equilibrium binding was measured over a range of IL-2 concentrations (0–800 pM) in which the growth factor should bind to both high- and low-affinity IL-2R. At concentrations up to 200 pM, IL-2 binds mainly to high-affinity receptors, and at higher concentrations low-affinity IL-2R begins to be occupied.[19] [^{125}I]IL-2 bound to IL-2R with similar characteristics in both LSLP and RPMI-1640/10% FBS. The overall level of [^{125}I]IL-2 binding to IL-2R in LSLP was 50–100% higher over the concentration range tested than binding in RPMI-1640/10% FBS, probably because there was less interference from IL-2 binding to protein components in LSLP. An IL-2 concentration of 200 pM was used for further experiments, as at this concentration only high-affinity receptors were occupied.

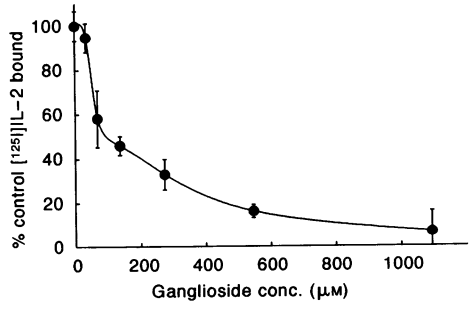

Figure 6. Inhibition of [^{125}I]IL-2 binding to high-affinity IL-2R by gangliosides in low serum medium. HT-2 (1×10^6 cells/ml) in LSLP was incubated for 1 hr at 4° with [^{125}I]IL-2 under conditions for high-affinity receptor occupancy (200 pM), in the presence of increasing concentrations of mixed gangliosides. Cells were then sedimented through a cushion of *n*-butylphthalate, and [^{125}]IL-2 association with the cells was determined by gamma counting. Percentage control [^{125}I]IL-2 bound was calculated relative to cells in the absence of gangliosides. Data are shown as the mean ± range for duplicate determinations. Results of a representative experiment are shown.

HT-2 cells were simultaneously incubated with 200 pM [^{125}I]IL-2 and increasing concentrations of gangliosides in LSLP. Figure 6 shows that [^{125}I]IL-2 binding to high-affinity IL-2R was decreased to less than 20% of the control at $\sim 400\,\mu$M ganglioside; the IC_{50} value for inhibition of IL-2 binding was $\sim 100\,\mu$M. These results indicate that gangliosides are able to block the high-affinity binding of [^{125}I]IL-2 to IL-2R in a dose-dependent fashion.

DISCUSSION

Gangliosides shed by tumour cells will be present at different sites *in vivo*; thus immunosuppression must be considered under different conditions. Close to a tumour, lymphocytes will encounter high levels of gangliosides in the relatively protein-free environment of the tissue fluid. As lymphokines are produced in very small quantities, and usually act locally, tumour cell membrane shedding is likely to have the greatest effect on lymphocytes in the microenvironment of a tumour. The experiments carried out in this study were designed to explore the immunosuppressive effects of these glycolipids in a milieu which more closely approximates that in the immediate vicinity of a tumour.

To achieve this goal, the HT-2 cell line was chosen as an appropriate cell model, adapted to growth in a LSLP medium, and used to examine the effect of gangliosides on IL-2/IL-2R activation. This simple model system was selected for several reasons. First, both high-affinity (9400/cell) and low-affinity (187 000/cell) IL-2R are expressed on this helper T-cell line. Second, IL-2-dependent cells do not require mitogen or accessory cells to progress through the cell cycle; thus, HT-2 cells will proliferate when supplied solely with IL-2. Third, helper T lymphocytes form the major IL-2-responsive T-cell population induced during mitogenesis. Finally, this cell line is well-characterized and has been used extensively by many research groups studying IL-2.

The ability of gangliosides to inhibit IL-2-stimulated DNA synthesis in HT-2 increased dramatically as the serum concentration in the medium decreased. In general, the IC_{50} values for ganglioside inhibition of proliferation were about an order of magnitude lower under LSLP conditions. This finding was true for several different ganglioside species tested, and the general relationship between carbohydrate headgroup structure and immunosuppressive ability in a low protein environment was similar to that noted previously in medium containing 10% FBS. Ganglioside species overexpressed by certain tumours, such as GM_2 and GM_3, were also highly immunoinhibitory in a low protein environment.

The large increase in the immunosuppressive capability of gangliosides at low protein concentration probably arises from the fact that they are known to interact with various serum components. When exogenous gangliosides are added to serum-containing medium (as is often done in experiments carried out *in vitro*), they associate primarily with albumin,[25] whereas circulating gangliosides *in vivo* are transported exclusively in association with serum lipoproteins, predominantly low-density lipoproteins.[27,28] Such interactions will reduce the amount of free gangliosides available to suppress IL-2-stimulated cell growth. Taken together, these observations suggest that gangliosides will be very powerful immunosuppressive agents in the local microenvironment of a tumour, where there are no serum proteins and the overall protein concentration is low. The local ganglioside concentration in the vicinity of the tumour is also likely to be higher than that in the circulation.

We also found that DNA synthesis in HT-2 was often stimulated by up to 40% at low concentrations of GM_1 (Fig. 1). This effect has been noted previously in our laboratory for murine splenocytes,[18] and may be related to the fact that membrane-inserted gangliosides (see below) appear to modulate IL-2-mediated proliferation of T lymphocytes.[34,35]

In contrast to their effects on the IL-2-dependent HT-2 cell line, gangliosides had little effect on IL-1-stimulation of the thymocyte cell line EL-4 NOB-1, and did not inhibit DNA synthesis in the lymphocyte cell lines A20 and K562 in serum-free medium. Gangliosides are, therefore, not non-specifically inhibitory to either a process mediated by IL-1, or the growth of lymphokine-independent lymphocyte cell lines.

Previous work in our laboratory has established that a major mechanism of inhibition of IL-2-mediated proliferation in serum-containing medium *in vitro* involves interference with the IL-2/IL-2R system. As the immunoinhibitory potency of gangliosides is an order of magnitude greater in a low protein environment, the question arises as to whether other mechanisms come into play under these conditions. Several types of experiments addressed the issue of the mechanism of immunosuppression by gangliosides in a low protein environment. First, ganglioside pretreatment did not permanently affect the ability of HT-2 to proliferate, and inhibition of cell growth was dependent on the continued presence of the glycolipids in the culture medium. Thus gangliosides have no negative effects on the integrity of either the cells themselves or IL-2R in the plasma membrane. Further, inhibition of synchronized HT-2 cells by gangliosides could also be completely overcome by high levels of IL-2, which is consistent with the idea that they compete with IL-2R for available IL-2. Finally, gangliosides are able to inhibit binding of [^{125}I]IL-2 to high-affinity IL-2R in a dose-dependent fashion. The concentration of gangliosides needed for blocking of IL-2R binding is substantially higher than the IC_{50} for inhibition of DNA synthesis. This reflects the fact that the ratio of IL-2R to gangliosides in the binding assay is much higher than in the DNA synthesis experiments (the cell concentration is 50-fold higher, with comparable IL-2 levels). The equilibrium between receptor-bound and ganglioside-bound IL-2 will thus be greatly shifted in the direction of the receptor in the binding assays.

Gangliosides exerted their immunosuppressive effects most strongly at early times after IL-2 stimulation, which would be expected based on their ability to compete with IL-2R for IL-2 binding. Gangliosides are probably also able to displace previously bound IL-2 from IL-2R in LSLP, as was previously shown in medium containing 10% FBS.[20] As IL-2 must remain bound to IL-2R for > 6 hr in order to induce progression into S phase, gangliosides still inhibit proliferation significantly when added 7 hr after IL-2 stimulation.

Taken together, these data support the proposal that the major mechanism of immunosuppression by gangliosides in a low protein environment is interference with the IL-2/IL-2R system. It seems likely that this mechanism of action will also be in effect in the vicinity of a ganglioside-shedding tumour *in vivo*. In addition, the results presented here suggest that gangliosides will be an order of magnitude more immunosuppressive close

to a tumour *in vivo* than was previously expected based on *in vitro* assays in serum-containing medium. The *in vitro* HT-2 cell model developed in the present work has proved invaluable in our laboratory for detecting the immunosuppressive action, under low protein conditions, of tumour cell supernatants containing shed gangliosides (P. Lu & F. J. Sharom, unpublished data).

We have recently determined that gangliosides also interfere with IL-4-driven proliferation by interacting directly with the lymphokine, and preventing the binding of IL-4 to its high-affinity receptor.[36] The ability of gangliosides to block both IL-2- and IL-4-dependent processes helps to explain the diverse immunosuppressive actions on a wide variety of immune cells that have reported for these glycolipids. Presumably, gangliosides will also block IL-4-dependent processes much more efficiently in a low protein environment. Recent work indicates that a ganglioside mixture (GAMIX) is useful clinically as an immunosuppressant in combination with cyclosporin A.[37] Our results imply that gangliosides may be much more effective immunosuppressants when used in the absence of serum/serum proteins, and if direct administration into the circulation is avoided.

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