Biochimica et Biophysica Acta, 1028 (1990) 205–214
Elsevier

BBAMEM 75007

Interleukin-2 binds to gangliosides in micelles and lipid bilayers

Joseph W.K. Chu and Frances J. Sharom

Guelph-Waterloo Centre for Graduate Work in Chemistry, Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario (Canada)

(Received 28 November 1989)
(Revised manuscript received 29 June 1990)

Key words: Interleukin-2; Ganglioside; Micelle; Lipid bilayer

Gangliosides shed from the surface of tumour cells may be involved in tumour-induced immunosuppression. These anionic sialoglycolipids are known to be potent inhibitors of lymphocyte proliferation, and it has been suggested that they interfere with processes mediated by the growth factor interleukin-2 (IL-2). We have thus investigated the interaction of IL-2 with gangliosides in micelles and lipid bilayers. Gel filtration FPLC showed that ^{125}I-IL-2 can bind to micellar gangliosides in aqueous solution, and this interaction was strongly promoted by low concentrations of serum. Binding to ganglioside micelles was specific in that it required a native IL-2 molecule. IL-2 binding remained unchanged in the presence of 40% ethylene glycol, suggesting that it was not due to hydrophobic interactions. Ganglioside oligosaccharides alone were not able to bind to IL-2. Direct binding studies and gel filtration chromatography indicated that both multilamellar liposomes and 100 nm unilamellar vesicles containing gangliosides were able to interact with IL-2. Bilayers of lipid alone showed no binding. The interaction of IL-2 with bilayer gangliosides was highly dependent on the bilayer lipid composition, but appeared independent of lipid phase state. These results suggest that gangliosides may be a physiologically relevant target for IL-2 binding.

Introduction

Gangliosides form a family of structurally diverse sialoglycolipids found in the outer leaflet of the plasma membrane in mammalian cells. Over the years, these glycolipids have been implicated in such processes as differentiation, oncogenic transformation and adhesion. Evidence has recently been presented showing that gangliosides act as bimodal regulators of cell growth [1] and modulate the activity of certain growth factor receptors [2–5].

High levels of gangliosides have been observed in the serum and ascites fluid of animals and human patients with certain malignant tumours [6–11]. In such cases, a general loss of immunological competence is also observed, which correlates with high levels of circulating gangliosides. These findings have led to suggestions that tumour-derived gangliosides are responsible for immunosuppression, which may, in turn, play a crucial role in the escape of tumour cells from destruction by the immune system of the host [12]. Gangliosides have been found to inhibit the proliferation in vitro of several classes of immune cells, including lectin- and antigen-stimulated T and B-lymphocytes, helper T-cells, interleukin-1-stimulated monocytes, and natural killer cells (for reviews see Refs. 12 and 13). The spontaneous release of gangliosides and other glycolipids from the surface of tumour cells in vitro is a commonly observed phenomenon [11,14,15], and in many cases, tumour-specific ganglioside species are shed. Gangliosides shed by tumour cells are immunosuppressive, and reach in vivo concentrations that have been shown to inhibit lymphocyte responses to polyclonal mitogens and antigen in vitro [10,11].

Attention has recently been focussed on the possible mechanisms by which gangliosides inhibit the proliferation of immune cells, particularly T-lymphocytes. The activation of T-cells occurs in two stages. The first involves the interaction of the antigen with the T-cell antigen receptor, which then transmits a signal across the plasma membrane [16], resulting in the secretion of

Abbreviations: DMPC, L-3-dimyristoylphosphatidylcholine; DPPC, L-3-dipalmitoylphosphatidylcholine; FPLC, fast protein liquid chromatography; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IL-2, interleukin-2; IL-2r, interleukin-2 receptor; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PC, L-3-phosphatidylcholine; SDS, sodium dodecyl sulfate; V_e, elution volume.

Correspondence: F.J. Sharom, Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

IL-2 and the expression of IL-2r on the cell surface. The binding of IL-2 to its receptor generates another transmembrane signal, as yet unknown, that results in cell division [17]. The development of IL-2-dependent T-lymphocyte cell lines, which proliferate only in response to IL-2, has allowed the dissection of the two stages of activation. Gangliosides have been shown to inhibit the IL-2-mediated growth of several such IL-2-dependent T-cell lines [18–20], implying that the glycolipids interfere with one or more of the IL-2-driven events.

Other researchers have demonstrated retention of IL-2 by a ganglioside affinity support [20], and gangliosides blocked the binding of IL-2 to specific antibodies [21], suggesting the possibility of a direct interaction between the growth factor and the glycolipids. The present study was undertaken to determine whether such an interaction does in fact take place. Our results show that IL-2 binds directly to bovine brain gangliosides, both in micelles and in lipid bilayers. The nature of the interaction between the growth factor and the glycolipid has been examined, and may involve a lectin-like binding site on the IL-2 molecule.

Materials and Methods

Purified human recombinant IL-2 was purchased from Boehringer-Mannheim Canada (Dorval, Quebec). ^{125}I-labelled IL-2(3-[^{125}I]iodotyrosyl-IL-2[Met^{0},Ala^{125}]; 650–850 Ci/mmol) was obtained from Amersham Canada (Mississauga, Ontario).

Glycolipids

Individual purified ganglioside species (GM_1, GD_{1a} and GT_1) were purchased from Supelco Canada (Oakville, Ontario). Mixed gangliosides were isolated from bovine brain by a modification of the method of Kanfer [22]. The crude gangliosides obtained from the initial Folch extraction were purified by chromatography on Bio-Sil A (200–400 mesh, Bio-Rad Laboratories, Mississauga, Ontario), eluting with a gradient of CH_3OH in $CHCl_3$. The ganglioside fractions, consisting of mono-, di- and tri-sialo species as judged by thin layer chromatography, were pooled, dialysed, and lyophilized.

Bovine brain gangliosides were specifically labelled with ^{3}H on sialic acid residues by mild periodate oxidation and subsequent reduction with NaB[$^{3}H_4$] (228 mCi/mmol; Amersham) [23]. The final specific activity of the ^{3}H-labelled gangliosides was around 18 mCi/mmol. Intact sialyloligosaccharides were cleaved from ^{3}H-labelled bovine brain gangliosides by ozonolysis followed by base hydrolysis, and purified by ion-exchange chromatography on DEAE-Sephadex [24].

FPLC of gangliosides and IL-2

FPLC was carried out on a Superose 6 column (1 × 30 cm; Pharmacia, Dorval, Quebec) linked to a Gilson HPLC system and a Gilson 111B ultraviolet flow detector. The column was calibrated by chromatographing a series of standard proteins of known molecular mass [25], ranging from β-galactosidase (465 kDa) to β-lactoglobulin (36.8 kDa) (Pharmacia High Molecular Weight Gel Filtration Calibration kit, and Sigma Chemical Co., St. Louis, MO). The void volume and total included volume of the column were determined using Blue dextran (2000 kDa) and sodium azide, respectively. A calibration curve of log (molecular mass) vs. V_e was fitted to a straight line by linear regression, and the apparent molecular mass of eluting peaks was interpolated from this calibration curve.

To study the binding of ^{125}I-labelled IL-2 to micellar gangliosides, gangliosides alone (100 μg, 0.162 μCi), ^{125}I-IL-2 alone (42 fmol, 0.028 μCi), or a mixture of both were eluted through the Superose 6 column. Sample volume was usually 200 μl, in either Tris-buffered saline (50 mM Tris in 0.15 M NaCl, pH 7.4) or RPMI 1640 culture medium (Gibco Canada, Burlington, Ontario) containing heat-inactivated fetal bovine serum. Mixed samples were incubated for 30 min at 37°C before chromatography. Samples were passed through a 0.45 μm filter, injected onto the column, and eluted at a flow rate of 0.5 ml/min. Fractions (1.0 ml) were collected and counted for ^{125}I and ^{3}H in a γ- and liquid scintillation counter, respectively.

Preparation of lipid bilayer systems containing gangliosides

Egg PC, DMPC, DPPC and cholesterol were supplied by Sigma. All lipids were pure as determined by thin layer chromatography. Large multilamellar vesicles (liposomes) containing reconstituted gangliosides were prepared by vortexing dried lipid mixtures in Hepes-buffered saline (10 mM Hepes in 0.15 M NaCl, pH 7.4) [26]. A population of larger MLV was harvested by centrifugation at 15 600 × g for 10 min in a microcentrifuge, and washed once with Hepes-buffered saline to remove smaller vesicular structures.

Large unilamellar lipid vesicles (LUV) containing reconstituted gangliosides were prepared by freeze-thaw high pressure extrusion [27,28]. Dried lipid samples were dispersed in Hepes-buffered saline at concentrations of 2.5–25 mg/ml, subjected to five freeze-thaw cycles, and extruded through two stacked 100 nm pore size polycarbonate membrane filters (Nuclepore Canada, Toronto, Ontario). After ten extrusion cycles, the initially cloudy lipid dispersions were optically clear. ^{14}C-Labelled DPPC (1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine, 117 mCi/mmol, Amersham) was often included in the lipid mixtures as a tracer to enable the determination of lipid recoveries. Quasi-elastic light scattering analysis was used to determine the size distribution of LUV preparations as previously described

[29]. All LUV preparations were relatively homogeneous, with mean diameters equal to the filter pore size.

Pretreatment of ganglioside MLV with IL-2

MLV composed of DPPC/cholesterol/gangliosides (2:2:1 w/w, 10 mg total lipid) were harvested and washed as described above. The resulting large MLV were gently resuspended in 250 μl of Hepes-buffered saline, and incubated with IL-2 for 30 min at 4°C. MLV were then pelleted by ultracentrifugation in a Beckman Airfuge at 110000 × *g* for 15 min, to prevent carry-over of small vesicles. Serial dilutions of the IL-2-containing supernatant were made into Hepes-buffered saline, and IL-2 activity was assayed using the CTLL-2 DNA synthesis bioassay (see below). MLV containing no gangliosides were tested as a control.

IL-2 bioassay

The biological activity of IL-2 was assayed using the murine IL-2-dependent cytotoxic T-lymphocyte cell line CTLL-2. Cells were cultured in 96-well flat-bottomed microplates (Nunc) at 1×10^5 cells/ml in RPMI 1640/10% fetal bovine serum. Appropriate dilutions of IL-2-containing supernatant were added, and the cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C for 42 h. After a 6 h pulse with 1 μCi/well of ^{3}H-thymidine (6.7 Ci/mmol; Amersham), cellular DNA was collected on glass fibre filter discs using a Titertek automatic cell harvester. Dried filter discs were counted for ^{3}H using an anhydrous scintillant. Positive control wells contained CTLL-2 with 1 U/well IL-2, while negative control wells contained no IL-2. All determinations were carried out in triplicate.

Binding of ^{125}I-IL-2 to MLV containing gangliosides

MLV containing gangliosides in a variety of different host lipids were prepared as described above. MLV were mixed with 0.007 μCi (10 fmol) ^{125}I-IL-2 in Hepes-buffered saline containing 3.6% serum, in a total volume of 100 μl in 0.5 ml microfage tubes. After incubation on a gyrorotatory shaker at 37°C for 30 min, MLV with bound IL-2 were collected by centrifugation at 37000 × *g* for 30 min at 4°C. The supernatants were removed, the walls of the tubes washed with 100 μl of chilled buffer, and the tubes were re-centrifuged. The supernatant was pooled with that collected previously, and counted to determine unbound ^{125}I-IL-2. The tips of the microfuge tubes containing the liposomal pellets were cut off and counted for ^{125}I to determine bound ^{125}I-IL-2. Nonspecific binding was determined using MLV containing no gangliosides.

Gel filtration chromatography of LUV containing gangliosides

High pressure extrusion was used to produce homogeneous LUV (100 nm diameter; total lipid concentration 20–25 mg/ml) consisting of 20% w/w gangliosides in various host lipid mixtures, with ^{14}C-DPPC as a tracer. An aliquot (100 μl) of the vesicles was incubated with 42 fmol (0.028 μCi) ^{125}I-IL-2 for 30 min at 37°C, in the absence or presence of 3.6% v/v fetal bovine serum. LUV were then chromatographed on a Sepharose 2B-300 column (Sigma; 5 ml bed volume), equilibrated with Tris-buffered saline (pH 7.4). The column was eluted at a flow rate of 0.12 ml/min at room temperature, and fractions were collected and counted for both ^{14}C and ^{125}I.

Results

Interaction of IL-2 with ganglioside micelles

In order to investigate the possible direct interaction of IL-2 with ganglioside micelles in aqueous solution, we employed gel filtration FPLC on a Superose 6 column. The column was first calibrated with a series of protein standards, selected for their behaviour on open column chromatography [25,30]. The apparent molecular size of eluted peaks was estimated by interpolation from a standard calibration curve. Ganglioside molecules aggregate to form large oblate ellipsoid micelles in aqueous solution, with a critical micelle concentration in the range 10^{-9}–10^{-3} M depending on the species [31,32]. Micelles of ^{3}H-labelled bovine brain gangliosides eluted from the column as a single peak at a V_e of 14–16 ml, with an apparent molecular mass of 440 kDa (Fig. 1A). An absorbance peak could also be detected at 280 nm, due to light scattering by the micelles. The

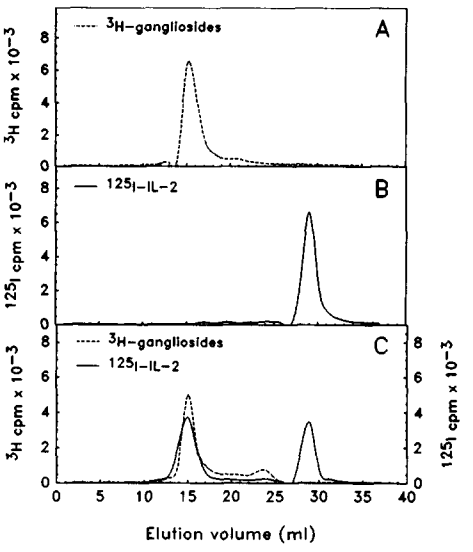

Fig. 1 Superose 6 FPLC elution profile of gangliosides, IL-2, and gangliosides mixed with IL-2, in the absence of serum. Samples (200 μl total volume) were eluted from the column with Tris-buffered saline at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and counted for ^{3}H and ^{125}I. (A) ^{3}H-gangliosides alone; (B) ^{125}I-IL-2 alone; (C) ^{3}H-gangliosides incubated with ^{125}I-IL-2 for 30 min at 37°C before chromatography.

ganglioside peak was well separated from ^{125}I-IL-2, which eluted as a single peak at a V_e of 28–30 ml (Fig. 1B). The 15 kDa IL-2 molecule eluted at a volume greater than the included volume of the column, suggesting some degree of interaction with the gel matrix. When ganglioside micelles were incubated with IL-2 in serum-free buffer prior to chromatography, two distinct peaks of IL-2 radioactivity were observed. Some free ^{125}I-IL-2 eluted at V_e 28–30 ml as expected, while a substantial fraction eluted in the high molecular mass region corresponding to the ganglioside peak (Fig. 1C). These results indicate that IL-2 may interact directly with ganglioside micelles. Approx. 4% of the ganglioside used in the FPLC separations was radiolabelled by periodate modification and reduction of the sialic acid side chain. Unmodified gangliosides, detected by light-scattering rather than ^{3}H counts, showed an identical IL-2 binding profile on FPLC.

The effect of serum on the interaction between ganglioside micelles and IL-2

The majority of glycosphingolipids found in human plasma, including gangliosides, are associated with serum proteins and lipoproteins [33,34]. It was thus important to establish whether IL-2 is capable of binding to ganglioside micelles in the presence of serum. The FPLC experiments described above were repeated in the presence of 3.6% v/v fetal bovine serum, and the results shown in Fig. 2 were obtained. The elution profile of ^{3}H-gangliosides remained very similar to that seen in buffer alone (Fig. 2A). The major portion of

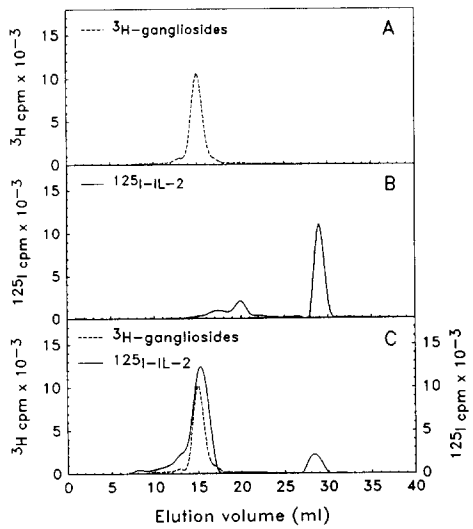

Fig. 2. Superose 6 FPLC elution profile of gangliosides, IL-2, and gangliosides mixed with IL-2, in the presence of serum. Samples (200 μl total volume, containing 3.6% fetal bovine serum) were eluted from the column with Tris-buffered saline at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and counted for ^{3}H and ^{125}I. (A) ^{3}H-bovine brain gangliosides alone; (B) ^{125}I-IL-2 alone; (C) ^{3}H-gangliosides incubated with ^{125}I-IL-2 for 30 min at 37°C before chromatography.

TABLE I

Effect of various concentrations of fetal bovine serum on binding of IL-2 to ganglioside micelles

Superose 6 gel filtration FPLC was used to assess IL-2 binding to micellar gangliosides in the presence of increasing concentrations of fetal bovine serum. ^{3}H-labelled gangliosides (100 μg) were mixed with ^{125}I-IL-2 (42 fmol) in the presence of fetal bovine serum (0.05–60% v/v), and then chromatographed on Superose 6. The fraction of ^{125}I-IL-2 eluting in the region of the ^{3}H-ganglioside peak (V_e 14–16 ml) and in the free IL-2 region (V_e 28–30 ml) was determined for each sample.

Fetal bovine serum (% v/v)	% total ^{125}I-IL-2	
	ganglioside region (V_e 14–16 ml)	free IL-2 region (V_e 28–30 ml)
no gangliosides/serum	7	93
0.0	53	47
0.05	56	44
0.1	59	41
0.5	72	28
1.0	78	22
2.5	89	11
5	89	11
10	90	10
20	90	10
60	91	9

^{125}I-IL-2 eluted as expected, however a minor peak of ^{125}I activity was also observed in the V_e range 15–20 ml (Fig. 2B). The 280 nm absorbance profile showed five minor peaks at V_e 12.5, 21.5, 22.5, 24.0 and 26.5 ml, and a major peak at 16.5 ml that corresponded exactly to the elution position for pure bovine serum albumin. It therefore seems likely that the minor IL-2 peak at 15–20 ml is due to co-elution of the growth factor with serum proteins, particularly serum albumin. When IL-2 was incubated with gangliosides in the presence of serum and then subjected to FPLC, over 90% of the ^{125}I-IL-2 co-eluted with the ^{3}H-ganglioside peak (Fig. 2C). Thus the interaction of IL-2 with ganglioside micelles is greatly enhanced in the presence of serum.

To further examine the effects of serum on the association of IL-2 with gangliosides, the two components were incubated in various concentrations of fetal bovine serum, and the percentage of ^{125}I-IL-2 co-eluting with ganglioside micelles was determined in each case. As shown in Table I, in the presence of buffer alone about 53% of the total IL-2 co-eluted with the ganglioside micelle peak. As the serum content of the buffer was increased, the association of IL-2 with gangliosides was greatly enhanced, reaching a maximum of 89–91% at 2.5% (v/v) serum. Further increases in the serum concentration up to 60% (v/v) did not produce any additional binding of IL-2. Bovine serum albumin alone could produce only some of the effect of whole serum. In the presence of 2 mg/ml albumin, 62% of the total IL-2 co-eluted with the ganglioside peak (not shown).

To examine the effects of IL-2 concentration on complex formation with ganglioside micelles, the concentration of ^{125}I-IL-2 was progressively lowered up to 16-fold, and FPLC analysis was carried out. As the IL-2 concentration dropped, the fraction of IL-2 co-eluting with the ganglioside peak in the absence of serum declined. In the presence of serum, the fraction of ^{125}I-IL-2 interacting with gangliosides remained essentially unchanged when the IL-2 concentration was decreased (data not shown). Different ganglioside species vary in their ability to inhibit IL-2-mediated lymphocyte proliferation in vitro, so it was of interest to determine whether they interact differentially with IL-2. The binding of the purified ganglioside species GM_1, GD_{1a}, and GT_1 to IL-2 in the presence of 3.6% serum was also examined using FPLC. The percentage of ^{125}I-IL-2 co-eluting with the ganglioside peak was very similar in each case, in the range 88–92%.

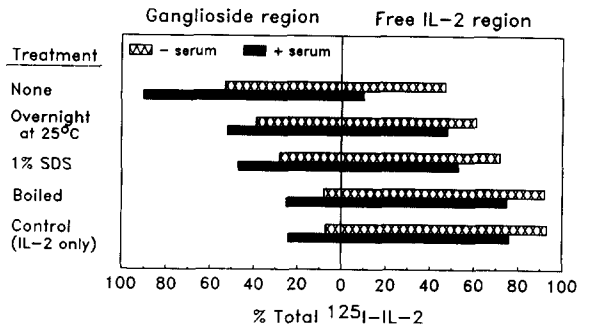

Fig. 3. Effect of denaturation of IL-2 on the interaction with gangliosides. ^{125}I-IL-2 was subjected to different treatments designed to induce various degrees of denaturation. Treated IL-2 was then co-incubated with ganglioside micelles in the absence or presence of serum for 30 min at 37°C before FPLC on Superose 6. The percentage of the total ^{125}I-IL-2 eluting in the ganglioside region (14–16 ml) and the free IL-2 region (28–30 ml) was determined. Controls consisting of native IL-2 (no treatment) and IL-2 in the absence of gangliosides are also presented for comparison.

The nature of the interaction between IL-2 and ganglioside micelles

It was of interest to determine whether binding of IL-2 to ganglioside micelles is nonspecific, or whether it requires a native, biologically active IL-2 molecule. Native IL-2 was therefore subjected to several different treatments that resulted in various degrees of denaturation, including overnight incubation at 25°C (mild denaturation), treatment with 1% SDS (moderate denaturation) and boiling (severe denaturation). Biological activity of the IL-2 following these treatments was determined using incorporation of [^{3}H]thymidine into cellular DNA of the IL-2-dependent indicator cell lines HT-2 and CTLL-2. Results showed that boiled IL-2 retained no biological activity. After overnight incubation at room temperature, 55% of the IL-2 activity was retained. The activity of SDS-treated IL-2 could not be tested due to toxic effects of SDS on the indicator cells. The various treated IL-2 samples were then incubated with ^{3}H-ganglioside micelles and chromatographed on Superose 6. As shown in Fig. 3, a high fraction of native IL-2 co-eluted in the ganglioside region, and this interaction was increased greatly in the presence of serum. As the severity of IL-2 denaturation increased, the fraction of ^{125}I-IL-2 co-eluting with the ganglioside peak decreased, both in the absence and presence of serum. IL-2 denatured by boiling showed no additional elution in the ganglioside region over that observed for the IL-2 control containing no gangliosides, indicating that binding to ganglioside micelles had been abolished. These results suggest that the interaction of IL-2 with ganglioside micelles is specific in that it requires the growth factor to be in its native state.

Previous work carried out in our laboratory suggests that the carbohydrate portion of gangliosides plays a central role in their ability to inhibit T-lymphocyte proliferation [19]. We thus investigated any possible interaction between ganglioside oligosaccharides and IL-2, again using FPLC. ^{3}H-labelled oligosaccharides were cleaved from bovine brain gangliosides by ozonolysis and base hydrolysis, and purified by ion-exchange chromatography. On FPLC, the oligosaccharides eluted as a single peak at a V_e of 20–21 ml (at the included volume of the column), as expected for their molecular size (0.45–0.98 kDa). When 100 μg of ^{3}H-oligosaccharides were incubated with 42 fmol of ^{125}I-IL-2 (in both the absence and presence of serum) followed by FPLC, no co-elution of the oligosaccharides with the IL-2 peak was observed (data not shown). Thus it appears that the oligosaccharide portion of gangliosides alone is unable to interact with IL-2.

The IL-2 molecule is known to be relatively hydrophobic, and it is possible that the growth factor interacts with the hydrophobic alkyl chains of the ganglioside molecule. To further investigate this possibility, the effect of ethylene glycol (a known disruptor of hydrophobic interactions) on IL-2 binding to gangliosides was tested. In the presence of 40% v/v ethylene glycol, gangliosides eluted as a major peak at V_e 14–16 ml, with a broad tail in the range of V_e 17–23 ml. This tailing phenomenon may be due to a hydrophobic interaction between ethylene glycol and gangliosides, resulting in alterations in micelle size. The ^{125}I-IL-2 peak was broadened over the region of V_e 25–36 ml, perhaps because the presence of ethylene glycol alters the interaction between IL-2 and the column matrix. When IL-2 and gangliosides were mixed in 40% ethylene glycol and chromatographed, almost all the IL-2 co-eluted with the gangliosides, and the ^{125}I profile exactly paralleled the tailing ganglioside peak. These results show that the interaction between IL-2 and gangliosides is not disrupted by 40% ethylene glycol, suggesting that the binding is not due to simple hydrophobic interactions.

Binding of IL-2 to gangliosides in multilamellar liposomes

Since FPLC had demonstrated binding of IL-2 to ganglioside micelles, it was of interest to examine whether IL-2 would interact similarly with gangliosides in lipid bilayers. Initial experiments were designed to measure IL-2 binding indirectly, by assaying the unbound IL-2 remaining after pre-incubation with lipid bilayers containing gangliosides. Vortexed multilamellar liposomes containing gangliosides were prepared, the larger structures collected by centrifugation, and the supernatant (containing smaller vesicles) discarded. It was important to remove small vesicles that might be transferred in the supernatant at the subsequent step, since we had previously determined that they were toxic to the tester cell line used for the IL-2 bioassay. Resuspended MLV, with or without incorporated gangliosides, were incubated with IL-2 for 30 min at 4°C before being sedimented by ultracentrifugation. The high speed supernatant was tested for IL-2 activity by its ability to stimulate ^{3}H-thymidine uptake into cellular DNA in the IL-2-dependent T-lymphocyte cell line CTLL-2. As shown in Fig. 4, after IL-2 was pre-treated with MLV (DPPC/cholesterol, 1 : 1 w/w) containing no gangliosides, the supernatant stimulated the proliferation of CTLL-2 in a dose-dependent fashion, indicating the presence of substantial amounts of free IL-2. When the amount of ganglioside-containing MLV in the incubation mixture was increased, resulting in an increase in the glycolipid : growth factor ratio from 50 μg per U of IL-2 to 200 μg per U of IL-2, a substantial decrease in DNA synthesis was observed (Fig. 4). When the IL-2 was pre-treated with 200 μg of MLV gangliosides per U of IL-2, DNA synthesis was completely abolished. These results indicate that liposomal gangliosides can cause significant depletion of IL-2 from aqueous solution, suggesting that membrane-bound

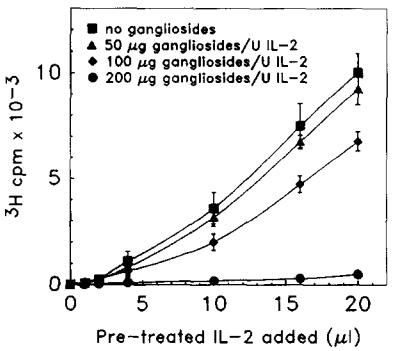

Fig. 4. Pre-treatment of IL-2 with multilamellar vesicles containing gangliosides. MLV of DPPC/cholesterol (1 : 1, w/w) containing 20% w/w gangliosides were incubated with IL-2 for 30 min at 4°C, at three different ratios of liposomal gangliosides to IL-2 (50–200 μg gangliosides/U of IL-2). The MLV were pelleted by ultracentrifugation and the supernatant was tested for IL-2 activity by measuring the incorporation of ^{3}H-thymidine into DNA in the IL-2-dependent cell line CTLL-2. Control MLV contained no gangliosides. The data are presented as the means ± S.E. (n = 3).

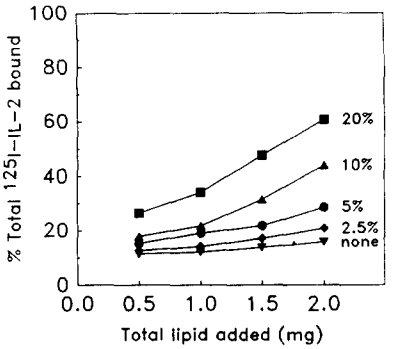

Fig. 5. Binding of ^{125}I-IL-2 to MLV containing gangliosides. MLV of DPPC/cholesterol (1 : 1 w/w) with ganglioside contents ranging from 0 to 20% w/w were incubated with ^{125}I-IL-2 in buffer containing 3.6% serum. After 30 min at 37°C, the MLV were sedimented and counted for ^{125}I.

gangliosides can interact directly with IL-2. A 200 μg quantity of gangliosides, of which only about 10 μg (5%) is exposed on the outer surface of multi-layered liposomes, was able to almost completely bind 1 U of IL-2.

To further study the interaction of bilayer gangliosides with IL-2, direct binding of ^{125}I-IL-2 to MLV containing 2.5–20% w/w gangliosides was measured. As shown in Fig. 5, ^{125}I-IL-2 shows substantial binding to MLV of DPPC : cholesterol : gangliosides in the presence of 3.6% serum. The amount of IL-2 binding increased with both the ganglioside content of the MLV and the total amount of lipid. MLV containing no gangliosides showed only a small amount of IL-2 binding. As in the case of ganglioside micelles, IL-2 binding to liposomal gangliosides was appreciably reduced in the absence of serum. To rule out the possibility of nonspecific IL-2 binding to the liposome surface as a result of the overall negative charge, ^{125}I-IL-2 binding to liposomes containing negatively-charged lipid species was measured. No significant binding of ^{125}I-IL-2 to MLV containing 20% w/w of phosphatidylserine or sulfatides could be detected (data not shown). The presence of sialic acid appeared to be necessary for IL-2 binding to glycosphingolipids. MLV containing 20% w/w of globoside, a neutral glycosphingolipid with a tetrasaccharide headgroup, showed little binding of ^{125}I-IL-2 (data not shown). These data are in agreement with studies we have carried out on the inhibition of proliferation of IL-2-dependent T-cell lines (CTLL-2 and HT-2) by various glycolipid and lipid species. Negatively charged glycolipids such as sulfatides, and neutral glycolipids such as cerebrosides and globoside, show little or no inhibition of proliferation, whereas gangliosides are potent inhibitors at 165–200 μg/ml (J.W.K. Chu and F.J. Sharom, unpublished data).

Three different types of host lipid, namely egg PC, DMPC and DPPC/cholesterol (1 : 1 w/w), were used to examine the effects of bilayer composition on IL-2

TABLE II

IL-2 binding to multilamellar ganglioside liposomes with various lipid compositions

Gangliosides were reconstituted into large MLV (egg PC, DMPC and DPPC/cholesterol 1:1, w/w) by vortexing in Hepes-buffered saline. Five sets of MLV with ganglioside contents ranging from 0 to 20% (w/w) were prepared. Increasing amounts of the liposomal dispersions (0.5–2.0 mg total lipid) were mixed with 0.007 μCi of ^{125}I-IL-2 in a total incubation volume of 100 μl, in the presence of 3.6% v/v serum. Separation of liposomal-bound and free ^{125}I-IL-2 was carried out as described in Materials and Methods. Results are expressed as the mean ± S.E. ($n = 2$).

Weight % gangliosides	Total lipid (mg)	% total ^{125}I-IL-2 bound			
		egg PC 25 °C	DMPC 15 °C	DMPC 37 °C	DPPC/cholesterol 37 °C
none	0.5	1.6 ± 0.2	2.1 ± 0.1	2.4 ± 0.5	11.4 ± 0.4
	1.0	1.9 ± 0.8	2.7 ± 0.4	3.5 ± 0.1	12.0 ± 0.5
	1.5	1.7 ± 0.2	3.5 ± 0.3	4.9 ± 0.2	13.7 ± 0.4
	2.0	1.2 ± 0.1	3.7 ± 0.2	5.5 ± 0.3	15.7 ± 0.7
2.5	0.5	2.1 ± 0.6	3.2 ± 0.4	3.2 ± 0.5	12.6 ± 0.5
	1.0	1.3 ± 0.1	3.3 ± 0.2	3.6 ± 0.1	14.0 ± 0.4
	1.5	1.9 ± 0.4	3.8 ± 0.1	4.6 ± 0.3	17.0 ± 0.5
	2.0	1.5 ± 0.3	5.7 ± 0.1	5.1 ± 0.1	20.8 ± 0.3
5	0.5	1.6 ± 0.1	3.1 ± 0.3	4.9 ± 0.8	15.2 ± 0.8
	1.0	2.7 ± 0.5	3.5 ± 0.2	6.6 ± 0.3	19.0 ± 0.5
	1.5	2.5 ± 0.1	4.5 ± 0.1	7.1 ± 0.6	21.8 ± 0.9
	2.0	2.6 ± 0.5	4.9 ± 0.1	10.1 ± 0.9	27.6 ± 0.3
10	0.5	2.3 ± 0.7	4.5 ± 0.1	5.9 ± 0.5	16.8 ± 0.3
	1.0	2.1 ± 0.5	5.3 ± 0.1	8.6 ± 0.3	21.8 ± 0.5
	1.5	2.5 ± 0.4	6.4 ± 0.1	10.0 ± 0.1	31.5 ± 1.2
	2.0	1.3 ± 0.1	6.6 ± 0.4	11.5 ± 1.6	44.0 ± 4.3
20	0.5	2.3 ± 0.6	6.6 ± 0.3	6.5 ± 0.1	26.5 ± 1.6
	1.0	3.0 ± 0.1	6.9 ± 0.1	9.7 ± 0.5	34.1 ± 1.4
	1.5	2.4 ± 0.1	8.4 ± 0.6	12.9 ± 0.5	47.7 ± 0.5
	2.0	3.1 ± 0.3	14.8 ± 0.1	15.9 ± 0.9	60.7 ± 1.1

binding. In contrast to the results for the 1:1 DPPC/cholesterol mixture, very little IL-2 binding was seen for MLV composed of egg PC alone (Table II). Addition of cholesterol to egg PC in a ratio of 1:1 did not produce any increase in IL-2 binding, while DPPC alone showed substantially less binding than mixtures of DPPC with cholesterol (data not shown).

To investigate the possibility that lipid phase state may modulate IL-2 binding to bilayer gangliosides, we used MLV composed of DMPC (T_m = 22°C), and measured IL-2 binding to gangliosides in these DMPC liposomes at two different temperatures, 15°C (gel phase) and 37°C (liquid crystalline phase). As indicated in Table II, some IL-2 binding was observed for DMPC liposomes containing 20% gangliosides, reaching a maximum of around 15% of the total IL-2 present at both temperatures. This is somewhat higher than the binding seen for egg PC, but the extent of IL-2 binding was not dependent on the lipid phase state of the DMPC, and the amount bound was about four-fold lower than for MLV of 1:1 DPPC/cholesterol. The binding of IL-2 to gangliosides in MLV is therefore modulated by the composition of the host bilayer, but does not appear to depend on lipid phase state.

Binding of IL-2 to gangliosides in large unilamellar vesicles

Since a substantial portion of the gangliosides shed by tumour cells are in the form of small membrane vesicles, it was important to determine whether IL-2 is also able to bind to small lipid vesicles containing gangliosides. Gangliosides were incorporated into LUV composed of various host lipid mixtures by freeze-thaw high pressure extrusion. Sepharose 2B gel filtration chromatography was then carried out on LUV alone, ^{125}I-IL-2 alone, and mixtures of the two. As shown in Fig. 6A, LUV alone eluted as a narrow peak at the void volume of the column, with a V_e of 1.4–2.0 ml as shown by the [^{14}C]DPPC tracer. There was no difference in the elution profile of LUV with and without gangliosides, either in the absence or presence of serum. Fig. 6B shows a typical elution profile for ^{125}I-IL-2, which eluted as a broad peak with a V_e of 4.1–6.1 ml. When ^{125}I-IL-2 was pre-incubated with LUV of DPPC/cholesterol/gangliosides, 2:2:1 (w/w) before chromatography, a substantial amount of the growth factor co-eluted with the vesicle peak (Fig. 6C), indicating binding of ^{125}I-IL-2 to the LUV. In the absence of serum, this binding was considerably reduced. Vesicles composed of DPPC/cholesterol alone showed no binding of ^{125}I-IL-2, either in the absence or presence of serum. Interestingly, when LUV were composed of either egg PC or DMPC containing 20% w/w gangliosides, little ^{125}I-IL-2 co-eluted with the vesicle peak. These observations support the results shown in Table II, where only a very small amount of IL-2 binding to gangliosides in MLV of these

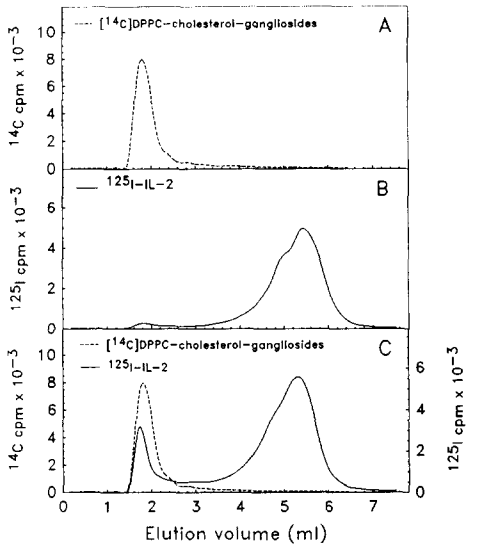

Fig. 6. Gel filtration chromatographic profiles of LUV containing gangliosides, IL-2, and LUV mixed with IL-2, in the presence of serum. LUV of [^{14}C]DPPC/cholesterol/gangliosides, 2:2:1 (w/w) (A), and ^{125}I-IL-2 (B) were chromatographed separately on a Sepharose 2B column. LUV were also incubated with ^{125}I-IL-2 in buffer containing 3.6% serum for 30 min at 37°C before chromatography (C). Fractions were collected and counted for both ^{14}C and ^{125}I.

lipids was measured. Binding of IL-2 to gangliosides in LUV is thus also highly dependent on the lipid composition of the vesicles.

Discussion

The FPLC studies described above have shown that ^{125}I-IL-2 co-elutes with bovine brain gangliosides in aqueous solution, suggesting the existence of a direct interaction between the growth factor and ganglioside micelles. This interaction is substantial in the absence of serum, with about 50% of the IL-2 co-eluting with ganglioside micelles. However, the interaction is strongly promoted by serum, with over 90% of the ^{125}I-IL-2 bound to gangliosides in the presence of serum. Binding of IL-2 was dependent on serum concentration, reaching a maximum at 2.5% v/v. It is not clear what role serum proteins might play in promoting the interaction of the growth factor and glycolipid micelles. Serum albumin is known to adsorb to the surface of lipid bilayers, where it forms a monomolecular coat on the membrane surface [35], and it may interact with ganglioside micelles in a similar fashion. However, we noted that serum albumin alone could produce only part of the enhancement of binding seen for whole serum. These observations suggest that some additional factors in serum, perhaps lipoproteins, can promote IL-2 binding to gangliosides.

Binding of IL-2 to ganglioside micelles was specific in that it was only observed if the growth factor was in its native state; the extent of IL-2 binding decreased as the protein was exposed to progressively harsher denaturing conditions. Muchmore and Decker [36] have demonstrated that the lymphokine IL-1 exhibits binding activity towards the N-linked oligosaccharides of the 85 kDa immunosuppressive Tamm-Horsfall glycoprotein (also known as uromodulin). It was suggested that IL-1 behaves as an endogenous lectin, and that uromodulin serves as a biologically relevant glycoprotein target. Studies on the immunosuppressive properties of ganglioside micelles [19] have shown that the carbohydrate portion of gangliosides plays an important role in their ability to inhibit the proliferation of T-lymphocytes. The results of our denaturation experiments suggest that IL-2 may also contain a lectin-like binding site for ganglioside oligosaccharides. However, no binding of ganglioside carbohydrate chains to IL-2 could be detected by FPLC. Thus the oligosaccharide chain alone is not sufficient for IL-2 binding; some other factors, perhaps a hydrophobic moiety or a charged surface, are required. The chemical nature of the binding interaction between IL-2 and gangliosides remains to be established. When FPLC was carried out in the presence of 40% ethylene glycol, binding of IL-2 to ganglioside micelles still occurred, suggesting that the interaction is unlikely to be due to simple hydrophobic interactions.

It has recently been reported that IL-2 binds with high affinity to uromodulin via a lectin-like site [37]. This site is clearly distinguishable from the putative IL-2 site responsible for ganglioside binding, since the former is specific only for certain high mannose *N*-linked oligosaccharides. Binding of IL-2 to mannose oligosaccharides is apparently not relevant at neutral pH, since binding only occurs under acidic conditions, and mannose oligosaccharides do not block the binding of IL-2 to its cell surface receptors [37]. On the other hand, gangliosides prevent the interaction of IL-2 with IL-2r on the lymphocyte cell surface under physiological conditions, resulting in complete inhibition of cellular proliferation [21,38,39]. The results we have obtained suggest that the IL-2 binding site for gangliosides is active under physiological conditions of pH, ionic strength, serum concentration etc., and tumour-derived gangliosides could thus modulate IL-2-driven responses in vivo.

The mole ratio of gangliosides to IL-2 in the FPLC experiments described above is around 10^6 (10^5 on a weight basis), so that at most a single molecule of IL-2 is bound per ganglioside micelle under our experimental conditions. Because of limited availability of the growth factor, it has not yet been possible to determine the mole ratio of bound IL-2 to gangliosides at saturating IL-2 concentrations. At this point, we cannot rule out the possibility that a minor ganglioside contaminant is responsible for IL-2 binding. However, our informal observations indicate that the ability of various ganglioside preparations (from crude mixtures to highly purified individual species) to inhibit IL-2-mediated

T-lymphocyte proliferation does not change significantly with the level of purity.

Several studies have shown that gangliosides are shed from tumour cell surfaces as components of membrane vesicles, as well as micelles. If these membrane vesicles can bind IL-2, they could also play a role in the suppression of immune responses in the vicinity of a tumour. Multilamellar liposomes and large unilamellar lipid vesicles are both effective model systems for studying the interactions of proteins such as lectins with membrane-bound glycolipids. We therefore examined the interaction of IL-2 with gangliosides incorporated into these two types of structures. A bioassay for IL-2 demonstrated that MLV containing gangliosides, but not MLV of lipid alone, were able to almost completely deplete IL-2 from the medium in a dose-dependent fashion. Further studies using ^{125}I-IL-2 revealed that MLV containing gangliosides were able to bind IL-2 directly, while those of lipid alone showed no binding. The amount of IL-2 bound was dependent on both the ganglioside content of the bilayers, and the composition of the host lipid. A 1 : 1 mixture of DPPC/cholesterol containing 20% w/w gangliosides was able to bind IL-2 much more effectively than gangliosides in bilayers of DMPC, DPPC or egg PC, which bound only small amounts of IL-2. IL-2 binding did not seem to be affected by the physical state of the lipid bilayers, with both gel phase and liquid crystalline phase DMPC showing poor binding. Thus it appears that the interaction of IL-2 with membrane gangliosides is modulated by the composition of the host bilayer. The molecular basis of the enhanced IL-2 binding displayed by DPPC/cholesterol mixtures is currently being investigated. The binding of specific antibody to glycolipids in lipid bilayers also shows a dramatic dependence on bilayer composition, with maximum antibody binding requiring phospholipid, cholesterol and a certain glycolipid density, with DPPC/cholesterol mixtures being particularly effective [40]. The cholesterol molecule may play a role in regulating IL-2 binding by promoting certain surface glycolipid distributions, such as ganglioside clusters, which may be the preferred sites for binding of the growth factor.

Gangliosides incorporated into 100 nm diameter LUV prepared by high pressure extrusion were also tested for their ability to interact with IL-2. Gel filtration chromatography on Sepharose 2B showed that these structures were also able to bind ^{125}I-IL-2. Modulation of binding by the host lipid was again observed, with little or no binding seen for LUV composed of DMPC or egg PC compared to DPPC/cholesterol. Our results suggest that ganglioside-containing micelles and membrane vesicles shed from tumour cell surfaces may both be targets for binding of IL-2. Further work will be needed to establish whether such structures are able to sequester IL-2 in vivo, and the potential contribution this may make to tumour-induced immunosuppression.

In addition, the ability of membrane gangliosides to bind IL-2 opens up the possibility that endogenous gangliosides may be somehow involved in binding of the growth factor to its cell surface receptor. The existence of complexes between gangliosides and integral membrane proteins has been known for some time, and gangliosides are intimately associated with the glycoprotein receptors for botulinum and diphtheria toxins [41]. Endogenous gangliosides have also been shown to regulate both ligand binding and tyrosine kinase activity of the epidermal growth factor and platelet-derived growth factor receptors, via a proposed specific interaction with the receptor itself [42]. It has been proposed that the mechanism of IL-2r signalling involves receptor tyrosine kinase activity, and it is possible that regulation of such activity by glycolipids is a general phenomenon.

Acknowledgements

This investigation was supported by a grant from the National Cancer Institute of Canada. J.W.K. Chu received a University of Guelph Graduate Visa Student Scholarship.

References

1 Spiegel, S. and Fishman, P.H. (1987) Proc. Natl. Acad. Sci. USA 84, 141–145.
2 Bremer, E.G., Hakomori, S.-I., Bowen-Pope, D.F., Raines, E. and Ross, R. (1984) J. Biol. Chem. 259, 6818–6825.
3 Bremer, E.G., Schlessinger, J. and Hakomori, S.-I. (1986) J. Biol. Chem. 261, 2434–2440.
4 Hanai, N., Dohi, T., Nores, G.A. and Hakomori, S.-I. (1988) J. Biol. Chem. 263, 6296–6301.
5 Hanai, N., Nores, G.A., MacLeod, C., Torres-Méndez, C.-R. and Hakomori, S.-I. (1988) J. Biol. Chem. 263, 10915–10921.
6 Ladisch, S., Wu, Z-L., Feig, S., Ulsh, L., Schwartz, E., Flousis, G., Wiley, F., Lenarsky, C. and Seeger, R. (1987) Int. J. Cancer 39, 73–76.
7 Portoukalian, J., Zwingelstein, G., Abdul-Malak, N. and Doré J.F. (1978) Biochem. Biophys. Res. Commun. 85, 916–920.
8 Kloppel, T.M., Keenan, T.W., Freeman, M.J. and Morré D.J. (1977) Proc. Natl. Acad. Sci. USA 74, 3011–3013.
9 Saha, S. and Chattopadhyay, U. (1988) Int. J. Cancer 41, 432–435.
10 Krishnaraj, R., Lengle, E.E. and Kemp, R.G. (1982) Eur. J. Cancer Clin. Oncol. 18, 89–98.
11 Ladisch, S., Gillard, B., Wong, C. and Ulsh, L. (1983) Cancer Res. 43, 3808–3813.
12 Dyatlovitskaya, E.V. and Bergelson, L.D. (1987) Biochim. Biophys. Acta 907, 125–143.
13 Marcus, D.M. (1984) Mol. Immunol. 21, 1083–1091.
14 Young, W.W., Borgman, C.A. and Wolock, D.M. (1986) J. Biol. Chem. 261, 2279–2283.
15 Ladisch, S., Kitada, S. and Hays, E.F. (1987) J. Clin. Invest. 79, 1879–1882.
16 Isakov, N., Scholz, W. and Altman, A. (1986) Immunol. Today 7, 271–277.
17 Smith, K.A. (1988) Science 240, 1169–1176.
18 Merritt, W.D., Pluznik, D.H. and Bailey, J.M. (1984) Fed. Proc. 43, 1829.

19 Sharom, F.J., Chiu, A.L.H. and Ross, T.E. (1990) Biochem. Cell Biol. 68, 735–744.
20 Parker, J., Caldini, G., Krishnamurti, C., Ahrens, P.B. and Ankel, H. (1984) FEBS Lett. 170, 391–395.
21 Robb, R.J. (1986) J. Immunol. 136, 971–976.
22 Kanfer, J.N. (1969) Methods Enzymol. 14, 660–664.
23 Veh, R.W., Corfield, A.P., Sander, M. and Schauer, R. (1977) Biochim. Biophys. Acta 486, 145–160.
24 Smith, D.F., Magnani, J.L. and Ginsburg, V. (1981) Arch. Biochem. Biophys. 209, 52–56.
25 Loe, D.W., Glover, J.R., Head, S. and Sharom, F.J. (1989) Biochem. Cell Biol. 67, 214–223.
26 Sharom, F.J. and Grant, C.W.M. (1978) Biochim. Biophys. Acta 507, 280–293.
27 Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55–65.
28 Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Biochim. Biophys. Acta 858, 161–168.
29 Chicken, C.A. and Sharom, F.J. (1985) Biochim. Biophys. Acta 814, 125–134.
30 Le Maire, M., Aggerbeck, L.P., Monteilhet, C., Andersen, J.P. and Moller, J.V. (1986) Anal. Biochem. 154, 525–535.
31 Corti, M., Degiorgio, V., Ghidoni, R., Sonnino, S. and Tettamanti, G. (1980) Chem. Phys. Lipids 26, 225–238.
32 Ulrich-Bott, B. and Wiegandt, H. (1984) J. Lipid Res. 25, 1233–1245.
33 Prokazova, N.V., Mikhailenko, I.A., Preobrazhensky, S.N., Ivanov, V.O., Pokrovsky, S.N., Timofeeva, N.G., Martinova, M.A., Repin, V.S. and Bergelson, L.D. (1986) Glycoconj. J. 3, 273–286.
34 Venerando, B., Roberti, S., Sonnino, S., Fiorilli, A. and Tettamanti, G. (1982) Biochim. Biophys. Acta 692, 18–26.
35 Rehfeld, S.J., Eatough, D.J. and Hansen, L.D. (1975) Biochem. Biophys. Res. Commun. 443, 587–600.
36 Muchmore, A.V. and Decker, J.M. (1987) J. Immunol. 138, 2541–2546.
37 Sherblom, A.P., Sathyamoorthy, N., Decker, J.M. and Muchmore, A.V. (1989) J. Immunol. 143, 939–944.
38 Chu, J.W.K. (1989) M.Sc. Thesis, University of Guelph, Guelph, Ontario, Canada.
39 Sharom, F.J. and Chu, J.W.K. (1989) J. Cell Biol. 107, 446a.
40 Stewart, R.J. and Boggs, J.M. (1990) Biochemistry 29, 3644–3653.
41 Montecucco, C. (1986) Trends in Biochem. Sci. 11, 314–317.
42 Schlessinger, J., Schreiber, B., Levi, A., Lax, I., Libermann, T. and Yarden, Y. (1983) Crit. Rev. Biochem. 14, 93–115.