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Ganglioside alterations in stimulated murine macrophages

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A two-dimensional thin-layer chromatographic technique has been used to separate and display gangliosides from murine peritoneal macrophages in different functional states. Resident macrophages have a relatively simple ganglioside pattern with about 15 resorcinol-positive spots. Gangliosides from resident cells contained mostly (90%) *N*-glycolylneuraminic acid. Thioglycolate-elicited and *Corynebacterium parvum*-activated macrophages have much more complex patterns with about 40 resorcinol-positive spots. Although ganglioside sialic acid content of stimulated macrophages was only slightly higher than that of resident cells, it consisted of nearly equal amounts of *N*-acetyl- and *N*-glycolylneuraminic acid. The shift in the ganglioside sialic acid type and the expression of different gangliosides in macrophages upon stimulation may help explain some of the differences in function and responsiveness noted in these macrophage populations.

Introduction

Gangliosides are sialic acid-containing glycosphingolipids which occur in the plasma membrane of a variety of cells. They are found in highest concentration in neuronal tissue with lower amounts in visceral tissues such as lymphoid cells [1,2] and are concentrated in the outer portion of the cellular plasma membrane [3]. They are amphipathic molecules consisting of a hydrophobic ceramide region and a hydrophilic oligosaccharide portion. Gangliosides may form all or part of the receptor for immunomodulatory substances such as lymphokines [4,5], interferon [6], viruses [7], and glycoprotein hormones [8]. The monosialoganglioside G_{M1} has been convincingly demonstrated to be the membrane receptor for cholera toxin [9]. A fucose-containing ganglioside has been indicated to be the receptor for macrophage migration inhibition factor [4,5].

Studies on macrophages in varying states of stimulation have shown profound physiological and biochemical changes compared to unstimulated cells [10–12]. However, there is no information on macrophage ganglioside content or on possible ganglioside changes after macrophage stimulation. This information is pertinent because sialic acid changes, especially changes in gangliosides, have been observed in the process of cellular differentiation and in oncogenesis [12,13]. In addition, the expression of new and/or different gangliosides may enable activated macrophages to respond to soluble mediators of cell function.

In order to assess the role of macrophage membrane gangliosides as possible markers of macrophage differentiation, we have developed a gang-

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Abbreviations: G_{M3}, II^3NeuAc-LacCer; G_{M1}, II^3NeuAc-$GgOse_4Cer$; G_{D1a}, IV^3NeuAc,II^3NeuAc-$GgOse_4Cer$; G_{D1b}, $II^3(NeuAc)_2$-$GgOse_4Cer$; G_{T1b}, IV^3NeuAc,$II^3(NeuAc)_2$-$GgOse_4Cer$; G_{Q1b}, $IV^3(NeuAc)_2$,$II^3(NeuAc)_2$-$GgOse_4Cer$; HP-TLC, high-performance thin-layer chromatography; NeuAc, *N*-acetylneuraminic acid; TLC, thin-layer chromatography.

lioside isolation and thin-layer chromatographic (TLC) analysis procedure. This procedure has made it logistically feasible to examine complex ganglioside patterns from murine macrophages in different functional states.

Materials and Methods

Animals. C3H/HeN and Balb/c female mice (6–20 weeks) were obtained from the N.I.H. breeding colony (Bethesda, MD). All solvents were redistilled or HPLC grade.

Isolation of adherent peritoneal cells. Thioglycolate-elicited peritoneal macrophages were isolated as described previously [14] with minor modifications. Briefly, 5 to 10 ml aliquots of peritoneal exudate cells ($(25–40)\cdot 10^6$ cells), obtained four days after the intraperitoneal injection of thioglycolate, were placed in 15 mm × 100 mm glass petri dishes and were incubated for 90 min at 37°C with 5% CO_2 and 95% humidity to permit adherence of the macrophages. Nonadherent cells were removed by washing each dish four times with phosphate-buffered saline or 0.9% NaCl. In one experiment, adherent cells were then incubated in media without serum for an additional 18 h at 37°C with 5% CO_2 and 95% humidity. Media were removed by an additional four washes with 0.9% NaCl. Each dish was then washed once with 0.31 M pentaerythritol [15] to remove excess salts. Resident peritoneal macrophages were prepared using the same technique. *Corynebacterium parvum*-activated macrophages were also isolated by the above procedure. The mice were injected i.p. with 1.4 mg of killed bacteria (*C. parvum*, lot CA749; Wellcome Research Laboratories, Beckenham, U.K.) 6 days prior to being killed.

Cell count was determined in a Levy chamber hemocytometer. Cell yields were $(2–3)\cdot 10^6$ cells per mouse for resident cells, $(15–20)\cdot 10^6$ cells per mouse for thioglycolate-elicited cells, and $(11–20)\cdot 10^6$ cells per mouse for *C. parvum*-activated cells. The percentage of phagocytic cells in the peritoneal exudate was approx. 57% for resident cells, 84% for thioglycolatle-elicited cells, and 82% for *C. parvum*-activated cells as estimated by ingestion of neutral red dye [14]. Cellular protein content of adherent cells was determined by the procedure of Lowry et al. [16].

Isolation of the total ganglioside fraction. The total lipid extract was obtained from adherent peritoneal cells ($(2.5–50)\cdot 10^7$ cells) by the following procedure. Immediately after washing as described above, petri dishes (100 mm) containing the cells were each flooded with 5 ml of chloroform/methanol (1 : 1, v/v) for 15 min at room temperature. Each petri dish was touched briefly to the surface of a bath sonicator after addition of the chloroform/methanol. The extracts were pooled and the dishes, in sets of five or less, were serially rinsed twice with 5 ml of chloroform/methanol (1 : 1, v/v). Variations in extraction schemes (e.g., increased water content, varied solvent ratios, differing extraction or sonication times) did not result in increased ganglioside yields. The pooled extracts and rinses were filtered through a scintered glass funnel overlaid with Whatman No. 1 filter paper to remove membrane fragments. The solvent was then removed by rotary evaporation.

The total ganglioside fraction was isolated from the total lipid extract by a procedure which represents a combination and modification of previously reported methods [17,18]. The total lipid extract was taken up in 20 ml of chloroform/methanol/water (30 : 60 : 8, v/v) and added to a 3 ml column of DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, NJ) prepared in the same solvent and packed in a disposable 10 ml glass pipette. The neutral lipid fraction was eluted in 12 column volumes (including the loading volume) of the above solvent. The acidic lipid fraction was then eluted using eight column volumes of chloroform/methanol/0.8 M sodium acetate (30 : 60 : 8, v/v). Organic solvent was removed from the acidic lipid fraction by rotary evaporation. The sample was redissolved in 5 ml of 0.1 M NaOH and heated to 37°C for 90 min in a shaking water bath to saponify the contaminating acidic phospholipids. Samples were chilled in an ice bath and neutralized to pH 4–4.5 using 0.1 M HCl. The sample was diluted to 20 ml using the appropriate volume of water and added to a 2 ml reverse-phase silica gel column (packing from Sep Paks; Water Associates, Milford, MA) for desalting. The column was packed in a 10 ml pipette fitted via a rubber stopper to a Fisher Filtrator (Fisher Scientific, Pittsburgh, PA). The vacuum

was regulated to produce a flow rate of 1–2 ml/min. The use of vacuum rather than pressure to elute the column allowed continuous addition of sample and rinses without interupting the flow. The flask was rinsed twice with 5 ml of 0.1 M NaCl. Following elution of the rinses, the column was eluted with 30 ml of H_2O at a flow rate of 5 ml/min. The desalted lipid fraction was collected by eluting the column with 7 ml of methanol followed by 35 ml of chloroform/methanol (1 : 2, v/v). The organic solvent was removed by rotary evaporation, the sample was frozen, and any residual traces of water were removed by lyophilization. The lyophilized residue was taken up in 5 ml of chloroform/methanol (85 : 15, v/v) utilizing a bath sonicator. The sample and 2 × 5 ml rinses of the same solvent were added to a 2 ml Iatrobead 6RS-8060 column (Iatron Laboratories, Inc. Tokyo, Japan). The column was eluted with an additional 10 ml of chloroform/methanol (85 : 15, v/v). The total ganglioside fraction was eluted with 20 ml of chloroform/methanol (1 : 2, v/v). The ganglioside fraction was dried by rotary evaporation and transferred to a small conical screw-cap test tube via 3 × 1.5 ml chloroform/methanol (1 : 1, v/v) rinses, and taken to dryness under N_2. Ganglioside sialic acid was determined by resorcinol assay [19] or by gas-liquid chromatographic assay [20]. Ganglioside recovery using brain ganglioside or $^3H\text{-}G_{T1b}$ was estimated to be ≥ 90% with no selective loss of any ganglioside.

Analysis of the total ganglioside pattern by two-dimensional HP-TLC. Ganglioside patterns were examined by a modification of the two-dimensional thin-layer chromatographic technique developed for brain gangliosides by Ledeen et al. [21]. 10 cm × 10 cm HP-TLC Silica gel 60 plates (E. Merck, Darmstadt, F.R.G.) were used. All plates were stored desiccated prior to use. Ganglioside samples equivalent to $(2\text{–}15) \cdot 10^7$ cells extracted were spotted on the left margin 15 mm in and 15 mm up from the bottom of the plate. As a marker, an aliquot of human brain ganglioside (0.4 μg sialic acid) was spotted on the right margin 5 mm in and 15 up from the bottom. TLC tanks were lined with filter paper and equilibrated with solvent before use. In order to minimize variable vapor leakage, tank covers were held under pressure using cabinetmaker's cam clamps (Klemsia, F.R.G.). All tanks used were of the same size and type. The plates were first developed for 45 min in chloroform/methanol/0.25% aqueous KCl (50 : 45 : 10, v/v) and then dried in a vacuum desiccator in the presence of P_2O_5 for 90 min. The dry plates were rotated 90° counter-clockwise and a second marker of brain ganglioside was spotted 5 mm from the left margin and 15 mm up from the bottom. The plate was developped in the second dimension in chloroform/methanol/2.5 M aqueous NH_3 in 0.25% KCl (50 : 40 : 10, v/v). After drying the TLC plates under forced air, the gangliosides were visualized by resorcinol spray [19].

Results

Ganglioside sialic acid content of macrophages

Sialic acid analyses of macrophage gangliosides are given in Table I. The data indicate that resident cells had slightly less ganglioside sialic acid than either thioglycolate-elicited or *C. parvum*-activated cells. The resident cell gangliosides contain 90% *N*-glycolylneuraminic acid. Upon stimulation, the macrophage ganglioside sialic acid became 50 to 58% *N*-acetylneuraminic acid. Neither thioglycolate broth nor *C. parvum* contained sialic acid when examined by the resorcinol assay.

Ganglioside patterns of resident macrophages

The ganglioside pattern of resident macrophages is shown in Fig. 1. When compared with mouse brain (Fig. 2), resident macrophages appear to contain fewer major gangliosides and virtually

TABLE I

SIALIC ACID ANALYSIS OF PERITONEAL MACROPHAGE GANGLIOSIDES

Sample source	ng sialic acid per μg protein [a]	% NeuAc [b]
Resident	2.40–3.23	10.1 ± 0.1
Thioglycolate-elicited	4.56–5.49	48.6 ± 1.2
C. parvum-activated	3.44–4.09	58.2 ± 0.4

[a] Values are reported as the range of the lowest ratio of sialic acid to protein to the highest ratio of duplicate (resident) or quadruplicate analyses of a single preparation.

[b] Value given is for *N*-acetylneuraminic acid as a percentage of the total sialic acid, the remainder being *N*-glycolylneuraminic acid.

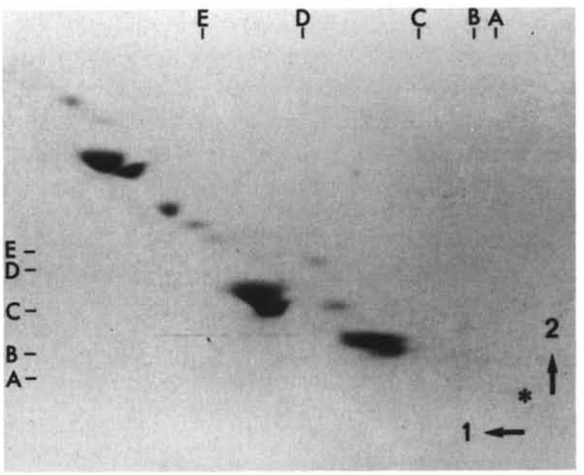

Fig. 1. Ganglioside pattern of C3H/HeN resident macrophages. Gangliosides spotted are from $1 \cdot 10^8$ peritoneal cells, and contain approx. 2 μg of lipid-bound sialic acid. The plate was heavily loaded in an attempt to visualize minor gangliosides. Origin is indicated by an asterisk with arrows and numbers indicating the direction of 1st and 2nd solvent runs as described in the text. Duplicate preparations gave similar TLC patterns. The mobility of brain ganglioside standards are indicated for the first solvent at the top of the plate and for the second solvent at the left side of the plate. The indicators show the positions of A, G_{Q1b}; B, G_{T1b}; C, G_{D1b}; D, G_{D1a}; and E, Gl_{M1}.

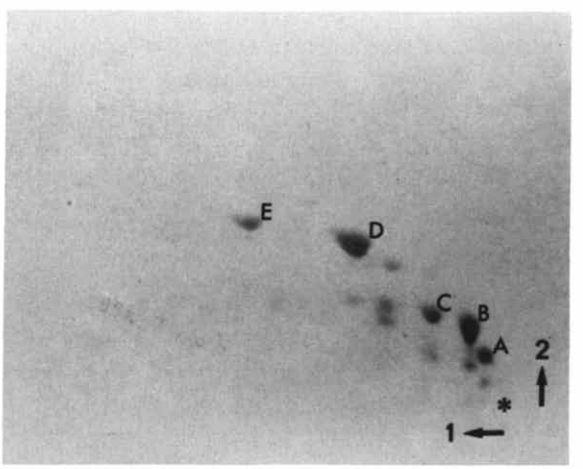

Fig. 2. Ganglioside pattern of mouse brain. These gangliosides were derived from whole mouse brain by the method described in the text. Ganglioside sialic acid content spotted is approx. 4 μg of lipid-bound sialic acid. Origin is indicated by an asterisk with arrows and numbers indicating the direction of first and second solvent runs as described in the text. The major gangliosides are identified as follows: A, G_{Q1b}; B, G_{T1b}; C, G_{D1b}, D, G_{D1a}; and E, G_{M1}.

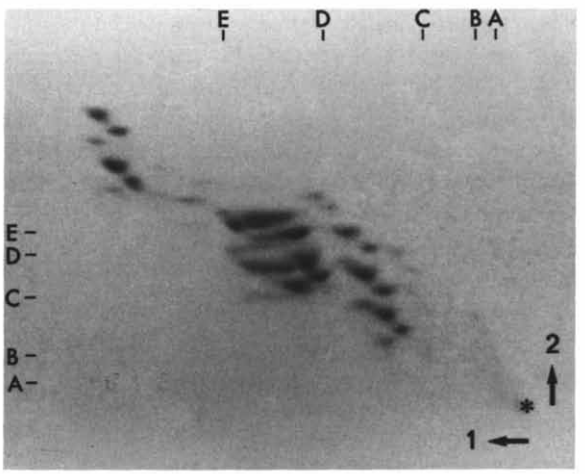

Fig. 3. Ganglioside pattern of C3H/HeN thioglycolate-elicted macrophages. Gangliosides spotted are from $3 \cdot 10^7$ peritoneal cells and contain approx. 8 μg of lipid bound sialic acid. Origin is indicated by an asterisk with the arrows and numbers indicating the direction of first and second solvent runs as described in the text. Five preparations gave similar TLC patterns. The mobility of ganglioside standards are indicated as described for Fig. 1.

no very polar polysialogangliosides (i.e., tri- or tetrasialo species). Approximately fifteen resorcinol-positive spots are seen on the chromato-

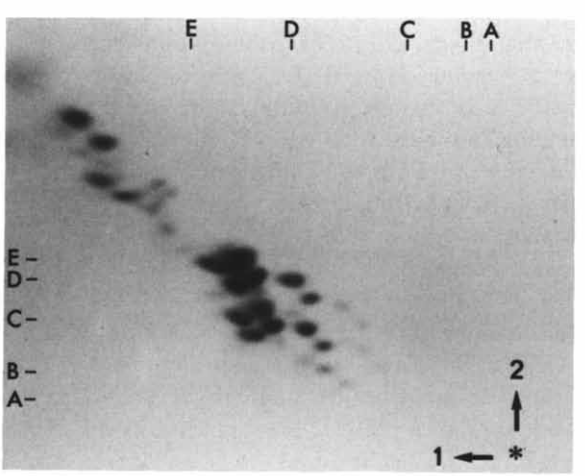

Fig. 4. Ganglioside pattern of C3H/HeN *C. parvum*-activated macrophages. Gangliosides spotted are from $1 \cdot 10^8$ peritoneal cells and contain approx. 7 μg of lipid-bound sialic acid. Origin is indicated by an asterisk with arrows and numbers indicating the direction of first and second solvent runs as described in the text. Duplicate preparations gave similar TLC patterns. The mobility of ganglioside standards are indicated as described for Fig. 1.

gram with six major spots, all migrating with polarities normally associated with mono- and disialo species.

Ganglioside patterns of thioglycolate-elicited macrophages

Thioglycolate-elicited macrophages from C3H/HeN mice manifest dramatic differences in the complexity of their ganglioside patterns compared with resident cells. Examination of the chromatogram (Fig. 3) indicates approximately 40 resorcinol-positive spots with a marked increase in the number of gangliosides migrating as disialo species. No ganglioside pattern differences were noted for cells adherent for 90 min versus 18 h.

No ganglioside differences were observed (data not shown) across the age span of our mouse colony (6 to 20 weeks). In one experiment, gangliosides from thioglycolate-elicited macrophages of Balb/c mice were also examined and no differences were observed compared to C3H/HeN elicited macrophage gangliosides mice (data not shown).

Ganglioside pattern of Corynebacterium parvum-activated macrophages

The ganglioside pattern of C3H/HeN *C. parvum*-activated macrophages (Fig. 4) is also qualitatively different from the resident cell pattern shown in Fig. 1. In comparison with the elicited macrophage pattern shown in Fig. 3, ganglioside patterns from the *C. parvum*-activated cells have corresponding resorcinol-positive spots with different relative intensities. A few distinct gangliosides, however, appear to be associated with each pattern.

Discussion

We have used a two-dimensional thin-layer chromatographic technique to display ganglioside changes in elicited or activated macrophages. The entire isolation procedure has been applied to samples of $2.5 \cdot 10^7$ cells containing as little as 0.5 μg of ganglioside sialic acid. The chromatographic technique can detect from 1 to 10 μg of ganglioside sialic acid, depending on the complexity of the ganglioside pattern. The quantity of sialic acid required for ganglioside analysis is comparable to the two-dimensional system of Chigorno et al. [22] and utilizes less sialic acid than that required for other two-dimensional chromatographic systems [21,23]. The propanol/ammonia/water solvent system of Chigorno et al. [22] was inadequate for the resolution of macrophage gangliosides.

Previous studies on changes in endogenous macrophage gangliosides focused on G_{M1} [32]. Lymphocytes also increase endogenous ganglioside synthesis upon stimulation with concanavalin A [24]. The addition of exogenous ganglioside has also been shown to alter the stimulation of lymphocytes in a variety of systems [25–27]. Recent evidence from our laboratory indicates that addition of specific exogenous gangliosides can abrogate the lipopolysaccharide-induced stimulation of macrophages (Ryan, J.L., Brown, D.E., Yohe, W.B., Gobran, L., Morrison, D.C., Modulation of macrophage metabolism by gangliosides (submitted)).

In the work reported here, we have shown changes in both the quantity and complexity of macrophage gangliosides upon macrophage stimulation. Studies on murine thymus gangliosides [2] have indicated that, while thymus gangliosides contain the same gangliotetraosylceramide base, the sialic acid moiety varies considerably in regards to structure and linkage. We have found both *N*-acetyl- and *N*-glycolylneuraminic acid in murine macrophage gangliosides. The *N*-acetylneuraminic acid level is very low in resident cells but increases dramatically in stimulated macrophages. The ganglioside pattern of the macrophages suggests that these gangliosides may also vary in ceramide structure since some gangliosides appear to migrate as doublets (two closely migrating spots of nearly equal intensity). This type of TLC pattern can be due to differences in fatty acid composition of the ceramide portion [28]. In addition, the work of Liu et al. [4,5] clearly indicates the presence of a fucose-containing ganglioside in macrophages. We are currently attempting to analyze the structural parameters of macrophage gangliosides utilizing chemical and enzymatic methods.

The growing evidence of gangliosides as membrane receptors for immunomodulatory substances and the profound change of gangliosides in stimulated macrophages suggests a possible biochemical

mechanism by which stimulated macrophages have enhanced responsiveness to immunoregulatory agents [9–11]. This is further suggested by the observation that serotonin increases the phagocytic capability and interleukin-1 production of bone marrow macrophages after the macrophages have been grown in the presence of concanavalin A-stimulated spleen cell supernatants [29]. Sturgeon and Sturgeon [30] have shown serotonin will bind *N*-acetyl- but not *N*-glycolylneuraminic acid. *N*-Acetylneuraminic acid-containing gangliosides also greatly increase serotonin binding to serotonin-binding proteins [31]. These observations suggest that the expression of new gangliosides, particularly *N*-acetylneuraminic acid-containing gangliosides, upon macrophage stimulation may increase macrophage responsiveness to soluble factors.

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References

1 Ledeen, R.W. and Yu, R.K. (1978) in Research Methods in Neurochemistry (Marks, N. and Rodnight, R., eds.), pp. 371–410, Plenum Publishing, New York
2 Schwarting, G.A. and Gajewski, A. (1983) J. Biol. Chem. 258, 5893–5898
3 Hakomori, S.I. (1981) Annu. Rev. Biochem. 50, 733–764
4 Liu, D.Y., Petschek, K.D., Remold, H.G. and David, J.R. (1980) J. Immunol 124, 2042–2047
5 Liu, D.Y., David, J.R. and Remold, H.G. (1982) Nature 296, 78–80
6 Besanson, F., Ankel, H. and Basu, S. (1976) Nature (London) 259, 576–578
7 Bergelson, L.D., Bukrinskaya, A.G., Prokazova, N.V., Shaposhnikova, G.I., Kocharov, S.L., Shevchenko, V.P., Kamlaeva, G.V. and Fomina-Ageeva, E.V. (1982) Eur. J. Biochem. 128, 467–474
8 Kohn, L.D., Consiglio, E., DeWolf, M.J.S., Grollman, E.F., Ledley, F.D., Lee, G. and Morris, N.P. (1980) Adv. Exp. Med. Biol. 125, 487–503
9 Fishman, P.H. (1982) J. Membrane Biol. 69, 85–87
10 North, R.J. (1978) J. Immunol. 121, 806–809
11 Karnovsky, M.L. and Lazdins, J.K. (1978) J. Immunol. 121, 809–813
12 Cohn, Z.A. (1978) J. Immunol. 121, 813–816
13 Srinivas, L. and Colburn, N.H. (1982) J. Natl. Cancer Inst. 68, 469–473
14 Coleman, D.L., Root, R.K. and Ryan, J.L. l(1983) J. Immunol. 130, 2195–2199
15 Barton, N.W. and Rosenberg, A. (1973) J. Biol. Chem. 248, 7353–7358
16 Lowry, O.H., Rosebrough, N.J., Farr, A.I. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Yohe, H.C. and Yu, R.K. (1980) J. Biol. Chem. 255, 608–613
18 Kundu, S.K. and Suzuki, A. (1981) J. Chromatogr. 224, 249–256
19 Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604–611
20 Yu, R.K. and Ledeen, R. (1970) J. Lipid Res. 11, 506–516
21 Ledeen, R.W., Haley, J.E. and Skrivonek, J.A. (1981) Anal. Biochem. 112, 135–142
22 Chigorno, V., Sonnino, S., Ghidoni, R. and Tettamanti, G. (1982) Neurochem. Int. 4, 397–403
23 Hunter, G.D., Wiegant, V.M. and Dunn, A.j. (1981) J. Neurochem. 37, 1025–1031
24 Yates, A.J., Mattison, S.L. and Whisler, R.L. (1980) Biochem. Biophys. Res. Commun. 96, 211–218
25 Spiegel, S., Ravid, A. and Wilchek, M. (1979) Proc. Natl. Acad. Sci. USA 76, 5277–5281
26 Sela, B.A. (1981) Eur. J. Immunol. 11, 347–349
27 Krishnaraj, R., Lin, J. and Kemp, R.G. (1983) Cell. Immunol. 78, 152–160
28 Seyfried, T.N., Ando, S. and Yu, R.K. (1978) J. Lipid Res. 19, 538–543
29 Sternberg, E.M., Trial, J. and Parker, C.W. (1984) Fed. Proc. 43, 1744
30 Sturgeon, R.J. and Sturgeon, C.M. (1982) Carbohydr. Res. 103, 213–219
31 Tamir, H. (1981) in Gangliosides in Neurological and Neuromuscular Function, Development, and Repair (Rapport, M.M. and Gorio, A., eds.), pp. 67–76, Raven Press, New York
32 Mercurio, A.M., Schwarting, G.A. and Robbins, P.W. (1984) J. Exp. Med. 160, 1114–1125