Neutral Glycosphingolipids of Murine Myeloma Cells and Helper, Cytolytic, and Suppressor T Lymphocytes†

Bernhard Kniep, Thomas R. Hünig,‡ Frank W. Fitch,§ Joachim Heuer,‖ Eckehart Kölsch,‖ and Peter F. Mühlradt*

ABSTRACT: Functionally defined clones and lines of murine lymphocytes including myelomas, helper, suppressor and cytolytic T lymphocytes were analyzed for their glycosphingolipids (GSLs). GSLs were characterized by thin-layer chromatography and by high-performance liquid chromatography. Lymphocytes with different functions displayed, besides a number of common GSLs, some characteristic GSLs that may be regarded as markers. Globotriaosylceramide was found on myelomas and B blasts, whereas globotetraosylceramide was confined to helper T cells. All T cells including cytolytic T lymphocytes displayed gangliotetraosylceramide (asialo-GM_1) as a major GSL, which was further characterized by sequential degradation with exoglycosidases.

Several different subpopulations of lymphocytes and accessory cells participate in practically every immunological reaction. These subpopulations are customarily characterized by serological means. With the exception of anti-gangliotetraosylceramide (Gg_4Cer,[1] asialo-GM_1) serum, which was used to characterize certain murine T lymphocytes (Stein et al., 1978) and natural killer (NK) cells (Kasai et al., 1980; Young et al., 1980), the sera recognize epitopes on cell-surface components whose detailed chemical structures are unknown. It is of obvious interest to the cell biologist and immunologist to define population-specific cell-surface components of known structure.

We have shown in previous papers that murine lymphocytes, depending on whether stimulated by alloantigen in a mixed leucocyte reaction (MLR) or by mitogens specific for T or B lymphocytes, synthesize different glycosphingolipids (GSLs) (Gruner et al., 1981; Rosenfelder et al., 1979). These findings suggested that lymphocyte subpopulations differ in some of their cell-surface GSLs. Thus globotriaosylceramide (Gb_3Cer) was found on lipopolysaccharide-stimulated B lymphoblasts and alloantigen-stimulated T blasts, whereas a GSL with the carbohydrate sequence of globotetraosylceramide (Gb_4Cer, globoside) was confined to alloantigen-stimulated T blasts and was not exhibited by concanavalin A stimulated T cells. In this study, we report the analysis by high-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) of metabolically labeled GSL from two murine myelomas and several T cell clones with defined functions. The aim of the study was to clarify (a) whether Gb_3Cer, since it was found on B blasts, is also present on myelomas, which may be regarded as B cell derived, plasma cell-like leukemic cells, (b) which of the different T cell subpopulations participating in an MLR are carrying the Gb_4Cer, and (c) whether cytolytic T cells carry Gg_4Cer or whether this GSL is confined to NK cells as claimed by several groups (Kasai et al., 1980; Young et al., 1980). Our data indicate that Gb_3Cer is also found on myeloma cells, that Gb_4Cer (globoside) is a marker GSL for helper T cells, and that Gg_4Cer (asialo-GM_1) is a major GSL in all T cell clones studied, including killer T cells.

Materials and Methods

Animals. Male mice of the inbred strains CBA/J (H-2^k) and DBA/2 (H-2^d) were obtained from Gl. Bomholdgård (Ry, Denmark) and used at the age of 6–10 weeks.

Cell Lines and Clones. The lymphocyte cell lines and clones whose GSLs were studied, their genetic background, serological phenotype, and antigen specificity are listed in Table I.

Cell Culture Techniques and Carbohydrate Labeling. Stimulation of CBA/J and DBA/2 lymphocytes by mitogens or alloantigen in an MLR was done as described (Gruner et al., 1981). Viable blasts from the MLR were isolated 3 days after restimulation on a sodium metrizoate/ficoll density gradient (Lymphoprep. Nyegaard & Co., Oslo, Norway) as described for human leucocytes (Bøyum, 1968). Cells from T lymphocyte clones were harvested when growing exponentially. To metabolically label the T lymphocytes with carbohydrates, we adjusted the cells to a concentration of 2.5×10^6/mL in RPMI 1640 medium containing 5 mM glucose (instead of normally 11 mM), 2.5% v/v fetal calf serum, 2×10^{-5} M 2-mercaptoethanol, 10 units/mL of the T cell growth factor Interleukin-2, and 1.25 μCi each of D-[1-^{14}C]galactose and D-[1-^{14}C]glucosamine hydrochloride/mL (both 50 Ci/mol). The cells were cultured in a moist atmosphere containing 7.5% CO_2 in air for 6 h except for the helper cells that were cultured for 26 h. Murine Interleukin-2 was produced and purified as described (Bödeker et al., 1980). Units are defined in Bödeker et al. (1982). GlcCer, Gb_3Cer, and Gb_4Cer were purchased (Supelco, Bellefonte, PA). LacCer (human spleen) was a gift of U. Bethke from this laboratory. Gg_3Cer and Gg_4Cer were prepared from crude bovine brain gangliosides Catalog No. 34 57 95 (Calbiochem, Giessen, FRG) after hydrolysis with 1 N acetic acid for 2 h at 100 °C, and purification of the asialogangliosides was by HPLC as described

† From the Gesellschaft für Biotechnologische Forschung mbH., D-3300 Braunschweig-Stöckheim, Federal Republic of Germany. *Received July 26, 1982.*

‡ Present address: University of Würzburg, Institut für Virologie, D-8700 Würzburg, Federal Republic of Germany.

§ Present address: Department of Pathology, The University of Chicago, Chicago, IL 60637.

‖ Present address: Westfälische Wilhelmsuniversität, Lehrstuhl für Immunologie, D-4400 Münster, Federal Republic of Germany.

[1] Abbreviations: BSA, bovine serum albumin; C–M–W, chloroform–methanol–water; GSL, glycosphingolipid; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; KLH, keyhole limpet hemocyanin; MLC, mixed leucocyte culture; MLR, mixed leucocyte reaction; Forssman GL, globopentaosylceramide; Gb_3Cer, globotriaosylceramide; Gb_4Cer, globotetraosylceramide; Gg_3Cer, gangliotriaosylceramide; Gg_4Cer, gangliotetraosylceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; Lc_3Cer, lactotriaosylceramide; Con A, concanavalin A.

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Table I: Characteristics of Cell Lines from Which Glycosphingolipids Were Studied

cell line	cloned	function	genetic background	serological phenotype	antigen specificity	ref
T lymphocytes						
L2	+	amplifier	C57BL/6	thy 1.2$^+$, Lyt 1$^-$,2$^-$	M1s[a]	Glasebrook & Fitch, 1980
TKb/1	−	helper	(C57BL/6xDBA/2)F_1	thy 1.2$^+$, Lyt 1$^+$,2$^-$	KLH+H2[b]	T. R. Hünig, unpublished results[a]
L3	+	cytolytic	C57BL/6	thy 1.2$^+$, Lyt 1$^-$,2$^+$	H-2D^d	Glasebrook & Fitch, 1980
clone 3	+	cytolytic	CBA/nu/nu	thy 1.2$^+$, Lyt 1$^?$,2$^+$	H-2D^d	T. R. Hünig and Bevan, unpublished results[b]
HF 1 Ts	+	suppressor	CBA/J	thy 1.2$^+$, Lyt 1$^-$,2$^+$	BSA	Heuer et al., 1982
myelomas						
NSI/1-Ag4-1	+		Balb/c		none	Köhler et al., 1976
X63-Ag 8.6.5.3	+		Balb/c		none	Kearney et al., 1978

[a] TKb/1 was derived from spleen cells of (C57BL/6xDBA/2)F_1 mice immunized with KLH. It has been kept as a line since June 1981. It helps DNP-specific B cells in a secondary in vitro DNP-KLH response to produce anti-DNP antibodies. [b] Clone 3 was derived from H-2D^d-primed CBA/Tufts mice and is an IL-2-dependent cell clone that has been cultured since October 1979.

by Watanabe (Watanabe & Arao, 1981).

Isolation of Neutral GSLs, Thin-Layer Chromatography, and Autoradiography. GSLs were extracted from 2×10^7 cells. Isolation and analysis were described in a preceding paper (Gruner et al., 1981).

Sequential Degradation with Exoglycosidases. Identification of the Liberated N-Acetylhexosamine. These experiments were carried out as described previously (Gruner et al., 1981).

High-Performance Liquid Chromatography (HPLC). HPLC was done on a LiChrosorb Si 100 RT 250-4 column with an average particle size of 7 μm (Merck, Darmstadt, FRG) with a Varian 5060 liquid chromatograph equipped with a Jasco Uvidec 100-III variable wavelength detector (Japan Spectroscopic Co., Tokyo, Japan). Underivatized GSLs were separated in a chloroform–methanol–water (C–M–W) system as follows: flow rate 0.4 mL/min; linear gradient from 15% to 50% v/v methanol in chloroform (30 min), followed by a linear gradient of 50% v/v methanol in chloroform to 55% v/v methanol and 5% v/v water in chloroform (15 min). Benzoylation was performed according to Ullman & McCluer (1977). Before this procedure the samples were dried in vacuo for 40 min at room temperature and 20 min at 45 °C. The benzoylated GSLs were separated with a gradient of 1-propanol–methanol (50:50 v/v) in *n*-hexane–dioxane (99:1 v/v). The program is shown in Figure 5. The flow rate was 1 mL/min. The UV response of benzoylated GSLs was monitored at 230 nm. Fractions were collected every minute directly into counting vials to monitor radioactivity. The solvents were evaporated before counting and replaced by a liquid scintillation cocktail (emulsifier scintillator 299, Packard).

Methylation Analysis. The Gb_3Cer-like and Gb_4Cer-like GSLs from [^{14}C]galactose- and [^{14}C]glucosamine-labeled MLC blasts [see Gruner et al. (1981)] were isolated in the underivatized form by preparative HPLC. Gb_4Cer-like material was degraded to the corresponding trihexosyl-Cer by β-hexosaminidase as described previously (Gruner et al., 1981). The product was isolated by HPLC and combined with the Gg_3Cer-like material from the first separation. To this material, containing 85 000 cpm, was added 200 μg of Gb_4Cer as carrier. The mixture was methylated and hydrolyzed after the method of Esselman et al. (1972). Ions were removed by ion-exchange resins, and the eluants were dried in vacuo. The methylated sugars were separated by HPTLC in acetone–5 N NH_4OH (50:0.9 v/v) (Stoffyn et al., 1972). Reference compounds were obtained from authentic LacCer (2,3,4,6-tetra-*O*-methylgalactose), Gg_4Cer (2,3,6-tri-*O*-methylgalactose), and Gb_4Cer (2,3,6- and 2,4,6-tri-*O*-methylgalactose).

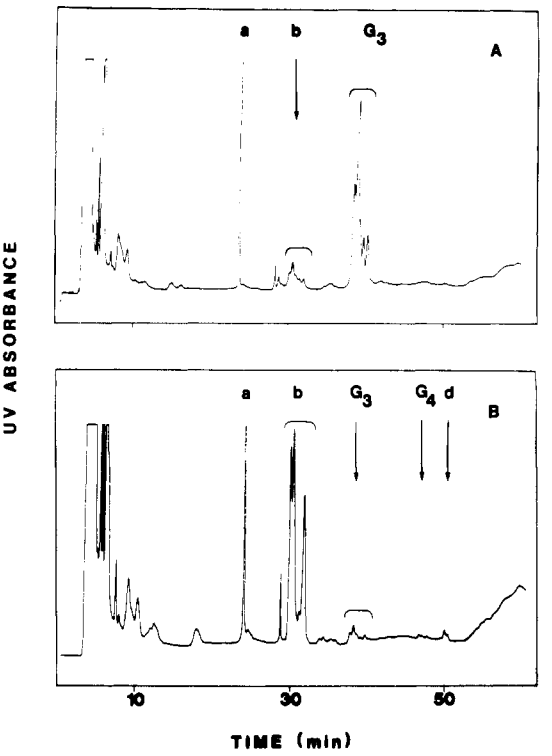

FIGURE 1: High-performance liquid chromatography of benzoylated neutral GSLs from myeloma NSI/1-Ag4-1 (A) and its subclone X63-Ag 8.6.5.3 (B). Column: LiChrosorb Si 100 RT 250-4 (7 μm). The solvent gradient is shown in Figure 5; a, b, and d denote the elution times of GlcCer, LacCer, and Gg_4Cer, respectively, and G_3 and G_4 the positions of Gb_3Cer and Gb_4Cer; the peak eluting before b is an unknown artifact from the solvent gradient.

Results

Neutral GSLs of Myelomas. Two myeloma lines that are commonly used for hybridoma techniques were analyzed for their neutral GSLs by HPLC. The separation patterns of their benzoylated derivatives are shown in Figure 1; the solvent gradient used for the separation is shown in Figure 5B. GSLs were identified on the basis of retention times in comparison to authentic standards including GSLs of known carbohydrate sequence such as Gb_3Cer and Gg_3Cer from mouse lymphocytes (compare also Figure 5). The system separates Gb_3Cer from Gg_3Cer, and both are eluted before Lc_3Cer.

GSLs with more than four carbohydrates were either missing (NSI) or present in only trace amounts (X63). Both myeloma lines have monohexosylceramide, lactosylceramide, and Gb_3Cer in common, although great quantitative differences were noted. In the case of myeloma X63, the peak in the area of Gb_3Cer is rather small. We still think it significant

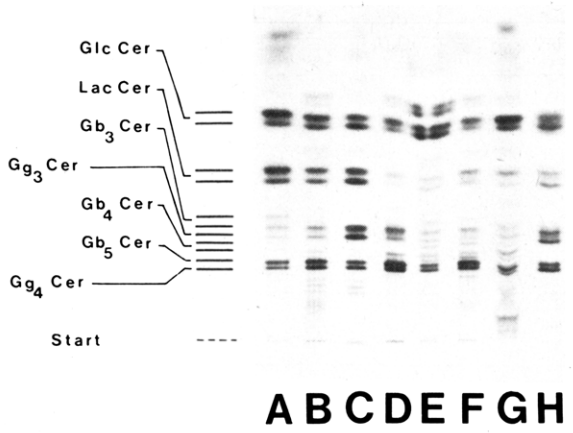

FIGURE 2: Autoradiogram of ^{14}C-labeled neutral GSLs separated on high-performance thin-layer plates (Merck) in C–M–W (120:70:17 v/v/v). The GSLs were derived from the following: (A) lipopolysaccharide-stimulated B blasts; (B) concanavalin A stimulated T blasts; (C) cytotoxic T cell clone 3; (D) cytotoxic T cell clone L 3; (E) alloantigen-stimulated T blasts; (F) helper-amplifier T cell clone L 2; (G) helper T cell line TKb/1; (H) suppressor T cell clone HF 1. The markers indicate the position of authentic standards.

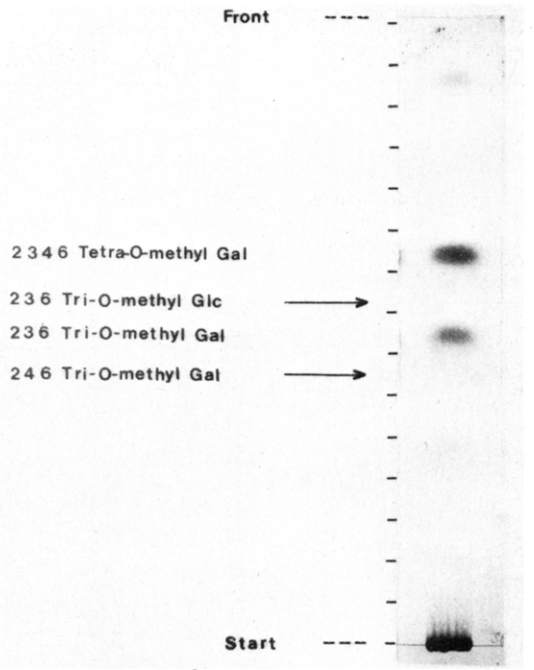

FIGURE 3: Autoradiogram of ^{14}C-labeled permethylated carbohydrates from Gb_3Cer-like material obtained from MLC blasts. The high-performance thin-layer plate (Merck) was developed with acetone–5 M NH_4OH (50:0.9 v/v). The positions of authentic standards are indicated. 2,3,6-Tri-*O*-methylglucose was not radiolabeled under our conditions.

because, when cells were radiolabeled with carbohydrate, radioactivity peaked in the fractions with retention times of Gb_3Cer. This seems to exclude that the UV signal recorded is due to substances other than a GSL. A third myeloma (FO) exhibited a similar pattern of GSLs with intermediate quantities of LacCer and Gb_3Cer (data not shown). The presence of Gb_3Cer and concomitant lack of tetraosylceramides may therefore represent a GSL pattern typical for myelomas.

Globoside Is Found on T Helper Cells. A brief description of the cells from which the GSLs were studied is given in Table I. With the aim of metabolically labeling the GSLs of the cells, viable, growing lymphocytes from cell lines or alloantigen-stimulated lymphoblasts from a secondary MLC were transferred to a special low-glucose labeling medium containing the T cell growth factor Interleukin-2 and ^{14}C-labeled galactose and glucosamine. Depending on their doubling time, the cells were further cultured between 6 and 26 h. Neutral GSLs were isolated and separated by HPTLC or by HPLC. An autoradiogram of the neutral GSLs of several T cell lines and of secondary MLC blasts is shown in Figure 2. While the GSL patterns of cytolytic and suppressor T cells resembled those from Con A blasts, GSLs from T helper cells were similar to those from MLC blasts. Accordingly, a GSL with migratory properties like Gb_4Cer is seen in the patterns from T helper lines and in those from MLC blasts. In a previous paper, the isolation and carbohydrate sequence of this globoside-like material from MLC blasts have been described, and the sequence was found to be GalNAc(β1-x)Gal(α1-x)Gal(β1-x)-GlcCer (Gruner et al., 1981). It had remained unclear at the time whether this GSL belonged to the globo or isoglobo series. Therefore, more of this material was isolated from cells labeled with [^{14}C]galactose and [^{14}C]glucosamine and degraded by hexosaminidase treatment to yield the trihexosylceramide. This was permethylated and hydrolyzed, and the radioactive methylated monosaccharides were identified by HPTLC as 2,3,4,6-tetra-*O*-methylgalactose and 2,3,6-tri-*O*-methylgalactose, respectively (Figure 3). This indicates that the GSL in question belongs to the globo series.

Contrary to our previous finding, Gb_4Cer did not appear as a major prominent GSL beside a rather weak double band of Gg_4Cer in MLC blasts, but in this more recent preparation Gg_4Cer is the more prominent GSL. We attribute this quantitative difference in the labeling intensity of the GSL to the presence of β-galactosidase in the culture medium of a secondary MLC. The stimulator cells in these cultures are mitomycin treated, and the dying cells give rise to cell debris including degrading enzymes. The glycosidases that partially degraded Gg_4Cer and LacCer in our earlier preparation were removed in the present experiment by the transfer of the MLC blasts to fresh labeling medium.

Identification of Gangliotetraosylceramide (asialo-GM_1) in GSLs from Cloned Cytolytic T Cells. GSLs isolated from all murine lymphocytes labeled by us including the cloned cytolytic T cells contained double bands with a migration behavior on HPTLC characteristic for Gg_4Cer (Figure 2). Since the presence of Gg_4Cer on cytolytic T cells is a point of controversy, the GSLs from the cytolytic T cell clone 3 were further characterized by HPLC in their underivatized form (Figure 4A) and after benzoylation (Figure 5). Distribution of radioactivity among the GSLs went approximately parallel to the absorbancy at 230 nm (Figure 5), indicating that no particular GSL species was synthesized or turned over at an enhanced rate. Had this occurred, there would have been an unusually high specific radioactivity of the GSL in question. Treatment of the GSLs from clone 3 with β-galactosidase followed by HPLC analysis (Figure 4B) showed complete degradation of the LacCer and disappearance of peak d, which contained the GSL with the migration properties like Gg_4Cer on HPTLC and HPLC. The LacCer peak was already degraded after 3 h whereas it took 24 h to degrade peak d material. This suggests a 1→3 bond of galactose in peak d material vs. the 1→4 bond in LacCer (Kobata & Ginsburg, 1972). Peak c material after reisolation could be degraded by β-*N*-acetylhexosaminidase to LacCer (Figure 4C). The free *N*-acetylhexosamine was isolated and identified by paper electrophoresis in borate buffer as *N*-acetylgalactosamine. All data point to the presence of Gg_4Cer on cytolytic T cells.

Discussion

The analysis of GSLs from lymphocyte clones or lines with

Table II: Occurrence of GSL on Murine Lymphocyte Subpopulations[a]

	myeloma	B blast	T cytolytic	T helper	T suppressor
GlcCer	+	+	+	+	+
LacCer	+	+	+	+	+
Gb_3Cer	+	+	−	t	−
Gb_4Cer	t	−	−	+	−
Forssman GL	−	−	t	+	−
Gg_3Cer	−	+	+	+	+
Gg_4Cer	t	+	+	+	+

[a] t = traces or inconsistent.

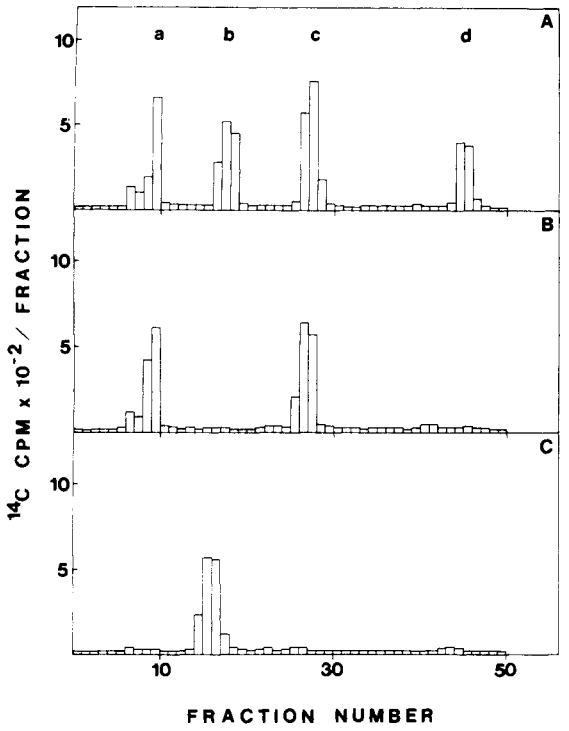

FIGURE 4: High-performance liquid chromatography of underivatized ^{14}C-labeled, neutral GSLs from cytotoxic T cell clone 3 before and after degradation by glycosidases. Column: as in Figure 1. The solvent gradient was as follows: (0–30 min) M–C (15:85) to M–C (50:50); (30–45 min) M–C (50:50) to M–C–W (55:40:5); (45–60 min) M–C–W (55:40:5). The solvent ratios are given in v/v. a, b, c, and d denote the elution times of GlcCer, LacCer, Gg_3Cer, and Gg_4Cer, respectively. (A) Starting material; (B) GSLs after β-galactosidase treatment; (C) peak c material after β-*N*-acetylhexosaminidase treatment.

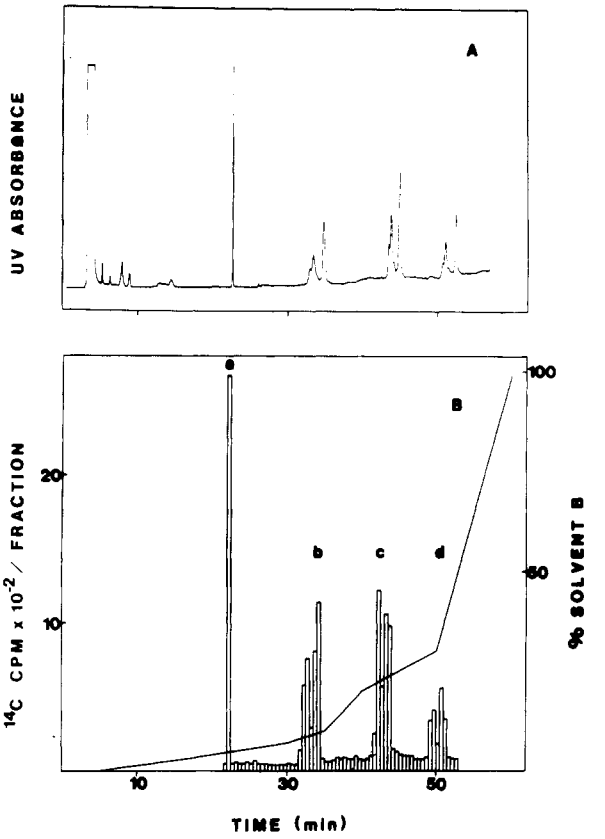

FIGURE 5: High-performance liquid chromatography of ^{14}C-labeled, benzoylated, neutral GSLs from cytotoxic T cell clone 3. Column: as in Figure 1. A solvent program of two mixtures was run as follows: (mixture I) *n*-hexane–dioxane (99:1); (mixture II) *n*-hexane–methanol–1-propanol (70:15:15). (0–11 min) 100% I to I–II (97:3); (11–25 min) I–II (97:3) to I–II (93:7); (25–30 min) I–II (93:7) to I–II (90:10); (30–35 min) I–II (90:10) to I–II (80:20); (35–45 min) I–II (80:20) to I–II (70:30); (45–55 min) I–II (70:30) to I–II (1:99). The solvent ratios are given in v/v. (A) UV tracing; (B) radioactivity profile.

defined functions confirmed and extended our earlier results obtained with primary cell cultures that consisted of less well defined subpopulations and could only be analyzed after specifically labeling the subpopulations in question. On the other hand, one has to be cautious in the study of cloned cell lines alone, since it was frequently observed that chromosome abnormalities and thus loss of genes, e.g., those coding for glycosyltransferases, may occur after prolonged passages. This must in fact have occurred with the myelomas, since X63 was derived from NSI and already shows a different GSL pattern. Such effects are unlikely in our study with the T cells since, except for the suppressor T cells, two functional subpopulations of different origin were analyzed and no fundamental differences between these and primary cells were observed.

The presence of Gb_3Cer on B blasts and also the plasma cell-like B cell derived myelomas is compatible and to be expected. Interestingly, Gb_3Cer was also found on MLC blasts (Gruner et al., 1981) but not on cytolytic or suppressive T cell clones. Globoside, the product of the next biosynthetic step in the globo series, was identified in the GSLs from MLC blasts, and a GSL with identical migratory properties was found in the GSLs from helper-amplifier T cells. Since these are the lymphocytes that primarily proliferate in response to alloantigen (Simon & Eichmann, 1980) and one of the helper clones (L2) was actually derived from alloantigen-stimulated lymphocytes (Glasebrook & Fitch, 1980), it is reasonable to assume that the globoside-like GSL from helper T cells is indeed globoside. A further step in the biosynthesis would lead to Forssman antigen. This GSL was only tentatively identified to be present on helper T cells and on one of the cytotoxic clones (L3). Interestingly, the products of two different biochemical pathways, the globo and the ganglio series, may both be present on helper T cells.

In studies with anti-Gg_4Cer (asialo-GM_1) antisera, Gg_4Cer was reported to be present on mature murine T cells (Stein et al., 1978) and nylon column enriched T cells and other

immunocytes of the rat (Momoi et al., 1982). Schwarting & Summers (1980) detected Gg_4Cer in murine splenic T cells also by HPLC. However, this GSL was not detected by antisera on cytolytic T cells prepared in an MLR (Kasai et al., 1980; Young et al., 1980), and T cells labeled in an MLR showed indeed only a weak double band of Gg_4Cer when analyzed by us (Gruner et al., 1981). As became apparent later, the low amounts of Gg_4Cer on MLR-reactive lymphocytes were due to degradative glycosidases in the medium, and transfer of the cells to fresh medium with subsequent labeling led to an intense double band of Gg_4Cer (see Figure 2, lane E). It was therefore not surprising to detect appreciable amounts of Gg_4Cer in both cytolytic T cell clones analyzed. In the case of clone 3, labeling intensity and actual chemical quantities went parallel, thus excluding an exceptionally high turnover of Gg_4Cer as cause for extensive labeling of this compound. Thus, according to our data, a distinction between natural killer cells and cytotoxic T lymphocytes on the basis of the presence of Gg_4Cer on the former and the absence of it on the latter (Kasai et al., 1980; Young et al., 1980) may not be feasible.

It is difficult to draw conclusions from the analysis of just one suppressor T cell clone available to us. Its GSL pattern appears almost undistinguishable from that of a cytotoxic T cell, despite the fact that on the basis of functional studies its cytotoxicity can be distinguished from the one of typical cytotoxic T cells (Heuer et al., 1982; B. Opalka, J. Heuer, and E. Kölsch, unpublished data). The presence of Gg_4Cer in this clone is in good agreement with the serological data of Nakano et al. (1980), who were able to eliminate concanavalin A activated murine suppressor T cells with antiserum to this GSL and complement.

In Table II we have listed the GSLs detected on the murine lymphocyte subpopulations analyzed by us and have attempted to assign some GSLs to distinct subpopulations. Lymphocytes apparently vary their cell-surface carbohydrates as they diversify from common precursor cells by expressing or shutting of different glycosyltransferase genes (Mühlradt et al., 1982). Similar phenomena have been observed also in completely different differentiating tissue such as early mouse embryos (Willison et al., 1982) and intestinal tissue (Suzuki & Yamakawa, 1981) and could be a general feature of differentiating cells.

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Registry No. LacCer, 4682-48-8; Gb_3Cer, 71965-57-6; Gb_4Cer, 11034-93-8; Forssman GL, 60267-39-2; Gg_3Cer, 35960-33-9.

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