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Structure Elucidation of Marker Glycolipids of Alloantigen-Activated Murine T Lymphocytes†

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ABSTRACT: DBA/2 mouse spleen cells were stimulated in vitro by (a) alloantigen (mitomycin-treated CBA/J splenocytestes), (b) the T cell mitogen concanavalin A (Con A), and (c) the B cell mitogen lipopolysaccharide (LPS). The cultures were pulsed for 10 h with ^{14}C-labeled galactose and glucosamine. Radiolabeled glycosphingolipids (GSL's) were extracted from the cells and the neutral GSL's isolated and analyzed by high-performance thin-layer chromatography. Two of the radioactive neutral GSL's, 9 and 12a, were found to be prominent in the alloantigen-stimulated cells but not in T cells stimulated by Con A. GSL 9 was also present as a minor component in LPS-stimulated B lymphocytes. GSL's 9 and 12a were purified by preparative column chromatography on Iatrobeads. The sequence and anomeric linkages of the carbohydrate moiety of these glycolipids were determined by successive degradation with exoglycosidases. The structures were shown to be Gal(α1-x)Gal(β1-x)GlcCer (glycolipid 9) and GalNAc(β1-x)Gal(α1-x)Gal(β1-x)GlcCer (glycolipid 12a), respectively. The latter glycolipid may serve as a marker for alloantigen-activated T cell subpopulations.

Glycosphingolipids (GSL's)[1] of cells involved in immune processes have recently become of interest because of their potential involvement in functional interactions between immune cells and/or accessory cells. A systematic study of the GSL's of these cells, particularly in an activated state, may eventually help to understand the possible functions of these cell surface molecules.

Antibodies directed against gangliotetraosylceramide (Gg_4Cer) have been reported to react specifically with murine

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[1] Abbreviations used: Con A, concanavalin A; C-M-W, chloroform-methanol-water; FCS, fetal calf serum; GSL, glycosphingolipid; GlcCer, glycosylceramide; *i*-Gb_3Cer, isoglobotriaosylceramide; *i*-Gb_4Cer, isoglobotetraosylceramide; Gb_4Cer, gangliotetraosylceramide; LPS, lipopolysaccharide.

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natural killer cells (Kasai et al., 1980; Young et al., 1980) or mature T cells of mice (Stein et al., 1978). Using a biochemical approach, we have shown in previous publications that GSL's of activated lymphoid cells can be conveniently analyzed after metabolic radiolabeling in the carbohydrate moiety (Rosenfelder et al., 1978, 1979). This method is useful in instances where only small amounts of cells are available or when activated subpopulations cannot be separated from the bulk of other cells. In principle, only those cells are labeled which are specifically activated, whereas "bystander" cells remain unlabeled and do not interfere with subsequent analysis. Using this method, we showed previously that mitogen-activated murine T and B lymphocytes differ in some of their neutral and acidic GSL's, which may serve as biochemical markers for these cells (Rosenfelder et al., 1979).

We report here that small subpopulations of T cells, which were activated and carbohydrate labeled during a secondary mixed lymphocyte reaction, exhibited mainly two radioactive neutral GSL's which were typical for allo-reactive T cells and were barley detectable in concanavalin A (Con A) stimulated T cells. The carbohydrate sequences of these compounds were determined by successive degradation with exoglycosidases.

Materials and Methods

Animals. Male mice of the inbred strains CBA/J ($H\text{-}2^k$) and DBA/2 ($H\text{-}2^d$) were used at 6–10 weeks of age.

Allogenic Stimulation. Spleen cell suspensions were prepared by gently teasing the spleens through a nylon screen. Erythrocytes were lysed with ammonium chloride (Boyle, 1968). CBA/J stimulator cells were treated at a cell concentration of 2×10^7/mL with 25 μg/mL mitomycin C for 30 min at 37 °C. They were then washed twice with RPMI 1640 medium with 5% (v/v) fetal calf serum (FCS). In vitro priming was done by cocultivating 2.1×10^9 DBA/2 responder cells with 1.3×10^9 CBA/J stimulator cells in a total volume of 1300 mL of RPMI 1640 medium with 5% (v/v) FCS distributed over 20 75-cm^2 plastic tissue culture flasks in a humidified atmosphere of 5% CO_2 in air at 37 °C. After 14 days the surviving 8×10^7 cells were harvested. These cells were subjected to a secondary stimulation by cocultivating them with 1.2×10^8 mitomycin-treated CBA/J stimulator cells in a total volume of 200 mL distributed over four 24-well flat-bottom tissue culture plates (Costar, Cambridge, MA) as above. The cultures were radiolabeled and harvested after 3 days.

Mitogen Stimulation. CBA/J or DBA/2 splenocytes, 5×10^6 cells/mL, were cultured in 2-mL volumes in Costar plates as described above. Mitogens were added at the beginning at the following concentrations: 25 μg/mL *Salmonella minnesota* R595 lipopolysaccharide (LPS) or 8 μg/mL Con A (Pharmacia, Frankfurt, Federal Republic of Germany).

Antisera. Rabbit anti-mouse T lymphocyte antiserum was prepared and absorbed as described (Rosenfelder et al., 1979). This antiserum killed 50% thymocytes at a dilution of 1:700. Monoclonal anti-thy 1.2 mouse antiserum was a generous gift of Dr. Opitz, Bayer AG, Wuppertal, West Germany. Fifty percent killing of thymocytes was effected at a dilution of 1:300 000. However, only 60% of Con A T blasts were killed by this serum even at a dilution of 1:100. Fluorescein-conjugated goat anti-rabbit IgG was purchased from TAGO Inc.

Fluorescent Antibody Staining. Cells, 3 days after restimulation, were incubated in 1:100 diluted rabbit anti-mouse T lymphocyte antiserum for 30 min at 0 °C, washed in the cold, incubated with fluorescent second antibody at a dilution of 1:75 for 30 min at 0 °C, washed again, and inspected under the fluorescence microscope. Only intact cells, fluorescent at the rim, were counted positive.

Treatnent of Alloantigen-Stimulated Cells with Anti-thy 1.2 Antiserum and Complement. Cells were harvested 3 days after secondary stimulation. Antiserum plus complement treatment was performed in microtiter wells in a total volume of 150 μL of RPMI 1640, 2% FCS, containing 5×10^5 cells, rabbit complement (Behring Werke, Marburg, Germany) at a final concentration of 1:15, and a serial dilution of anti-thy 1.1 monoclonal antibody. Incubation was for 1 h at 37 °C. The experiment was performed in duplicate. One set of samples served to determine cell vitality with the trypan blue exclusion test, and the other to determine the effect on carbohydrate incorporation. These cells were centrifuged, and the supernatant medium was replaced with 200 μL of fresh RPMI 1640 containing 2 μCi each of D-[1-^{3}H]galactose and D-[6-^{3}H]glucosamine. After an incubation period of 6 h, the cells were filtered onto glass fiber filters with an automatic harvesting machine and washed, and acid precipitable radioactivity was counted.

Labeling of Cells. Mitogen-activated cells were pulsed from 30 to 40 h with 1 μCi/mL each of D-[1-^{14}C]galactose (54 Ci/mol) and D-[1-^{14}C]glucosamine hydrochloride (55 Ci/mol). Alloantigen-activated cells were pulsed after secondary stimulation from 62 to 72 h as above.

Isolation of Neutral GSL's. The following procedure was adapted for small amounts of GSL's and was found adequate for 5×10^7 radiolabeled cells. Unincorporated label was removed by washing the cells in physiological NaCl containing 1 mM galactose and glucosamine hydrochloride. Cell pellets were twice extracted with 5-mL portions of chloroform–methanol (2:1) and once with 5 mL of C–M (1:1 v/v). The combined extracts were evaporated and taken up in 20 mL of C–M–W (30:60:8) and passed over a 2-mL column of DEAE-Sepharose acetate (Iwamori & Nagai, 1978). The neutral GSL's are not retained. After evaporation of the solvent, the neutral GSL's were acetylated and further fractionated on a 2-mL Florisil column (Saito & Hakomori, 1971). Deacetylation was done by a 30-min treatment at room temperature in 200 μL of C–M (2:1) containing 0.05% sodium methoxide. The mixture was neutralized with 3 μL of acetic acid, 2 mL of C–M (2:1) was added, and the salt was removed by extraction with 200 μL of water. The organic phase contained the neutral GSL's.

Thin-Layer Chromatography and Autoradiography. Thin-layer chromatography on high-performance plates and autoradiography were performed as described (Rosenfelder et al., 1979). The solvent was C–M–W (120:70:17 v/v/v) containing 1 mM $CaCl_2$ (van den Eijnden, 1971). For the identification of the monohexosylceramides resulting from the glycosidase degradation, activated Merck high-performance plates were sprayed with borate and after drying developed with C–M–W (65:25:4 v/v/v) as described (Young & Kanfer, 1965). The relative migration distances from the solvent front are 0.62 and 0.68 for phrenosin and kerasin, respectively (both galactosylceramides), while that of glycocerebrosides from Gaucher's spleen was 0.48.

Preparative Separation of Neutral Glycopeptides from Allogenically Stimulated Lymphocytes. Neutral glycolipids were further fractionated by column chromatography using Iatrobeads silica gel (Iatron Laboratory, Inc., Tokyo, Japan) according to Ando et al. (1976). Briefly, a sample containing ~200 000 cpm of labeled neutral GSL's was applied to a column (2 × 100 cm) containing 183 g of Iatrobeads 6RS 8060. The column was eluted with a convex gradient of a mixture of C–M–W in a ratio changing from 80:18:2 to

40:55:5) (v/v/v). The ^{14}C radioactivity of each fraction (10 mL) was assayed from dried aliquots by liquid scintillation spectrometry. The samples were counted in emulsifier scintillator special MI96 (Packard Instruments, Frankfurt, Federal Republic of Germany). For characterization of components in the eluate, selected fractions were analyzed by thin-layer chromatography.

Treatments with Exoglycosidases (Li & Li, 1972, 1977). The incubations were carried out in 0.4-mL volumes of 0.05 M sodium citrate buffer containing 300 μg of sodium deoxytaurocholate, 20 μg of appropriate carrier GSL's, and ~ 10000–20000 cpm of the labeled GSL's. The samples were sonified for 5 min, 0.5 unit of the respective enzymes was added, and the mixtures were incubated in a shaking water bath at 37 °C. The treatments with α-galactosidase (EC 3.2.1.22) from *Aspergillus niger* and with β-galactosidase (EC 3.2.1.33) from jack beans were carried out at pH 4.0 for 1 and 3 h, respectively. Incubation with β-*N*-acetylhexosaminidase (EC 3.2.1.30) from jack beans was for 24 h at pH 5.0. All enzymes were purchased from Sigma, München, Federal Republic of Germany. Carrier lipids were globotriaosylceramide (Gb_3Cer) and tetraosylceramide (Gb_4Cer) (Supelco Inc., Bellefonte, PA) for pools 1 and 2, respectively, and lactosylceramide was prepared from frozen calf thymus (Laine et al., 1974) to be added as carrier for the degradation of the labeled dihexosylceramides.

Reisolation of GSL's after Glycosidase Treatment. Neutral glycolipids were recovered from the reaction mixture by a procedure designed to remove the detergent which interferes with the thin-layer chromatography. The incubations were stopped by the addition of 5.4 mL of C–M (2:1). After addition of 0.4 mL of water, the mixture was vigorously shaken and the phases were separated by centrifugation. GSL's and deoxytaurocholate in the lower phase were separated by anion-exchange chromatography on a 1-mL column of DEAE-Sepharose acetate. The neutral GSL's are eluted from the column by 10 mL C–M–W (15:60:4), while the acidic detergent is retained on the column.

Identification of the N-Acetylhexosamine Released from Band 12a Material by Hexosaminidase. Ten nanomoles of unlabeled *N*-acetylglucosamine and *N*-acetylgalactosamine was added as carriers to the upper phase resulting from the reisolation of the degraded glycolipids. Ionic components were removed with small batches of Amberlite MB3 ion-exchange resin. The remaining solution was dried in vacuo, redissolved in a small volume, and, in parallel with authentic standards of radioactive *N*-acetylglucosamine and *N*-acetylgalactosamine, subjected to high-voltage paper electrophoresis in 0.2 M borate buffer, pH 10.0 (Spiro & Spiro, 1965). A voltage gradient of 30 V/cm was applied for 6 h at 4 °C. Radioactivity was detected in a radiochromatogram spark chamber (Birchover Instruments, Ltd., Letchword, U.K.).

Results

Allogenic Stimulation. DBA/2 mouse spleen cells were primed in vitro with mitomycin-treated DBA/2 stimulator cells. On the third day after restimulation with fresh stimulator cells (secondary mixed lymphocyte reaction), maximal incorporation of carbohydrate was observed (R. V. W. van Eijk, unpublished experiments). This time was chosen to label stimulated DBA/2 cells with [^{14}C]galactose and [^{14}C]-glucosamine.

Antiserum Treatment of Alloantigen-Activated Cells. In a separate experiment cells were reisolated 3 days after the secondary stimulation and treated with antiserum prior to labeling to obtain information about the nature of the responding cells. Practically all cells obtained were viable. They were nonadherent cells of various sizes, the largest ones having the appearance of typical lymphoblasts. Ninety percent of all cells fluoresced when treated with anti T cell antibodies and fluorescein-conjugated second antibody. Treatment with anti-thy 1.2 monoclonal antibody and complement resulted in 70% specific reduction of viability and in 53% inhibition of carbohydrate uptake (see Table I). This seeming descrepancy is due to the enormous stability of blast cells toward complement lysis, which we have also observed with Con A T blasts (P. F. Mühlradt, unpublished experiments). The total viable cell number is therefore more extensively reduced than that of the blast cells incorporating the carbohydrates.

Neutral GSL's of Allogenically Stimulated Lymphocytes. Lipids were extracted from labeled cells. Neutral glycolipids were separated from other lipids and analyzed by high-per-

Table I: Influence of Anti-thy 1.2 Monoclonal Antibody and Complement Treatment on Number of Viable Cells and Carbohydrate Incorporation of Cells after Secondary Alloantigen Stimulation

dilution of antibody	% viable cells[a]	cpm[b]
1:100	29	955
1:200	31	948
1:400	30	1223
1:800	35	1160
1:1600	38	1234
1:3200	37	1422
1:6400	53	1397
1:12800	60	1680
complement alone	95	2022
no complement	100	2484

[a] Antibody complement treatment was performed in microtiter wells containing 5×10^5 cells in a total volume of 150 μL. Rabbit complement was present at 1:15 final dilution. Treatment was for 1 h at 37 °C. [b] Antibody complement treatment was performed as in footnote *a*. Cells were sedimented, resuspended in fresh medium, and pulsed for 6 h with 2 μCi each of [^3H]galactose and [^3H]glucosamine.

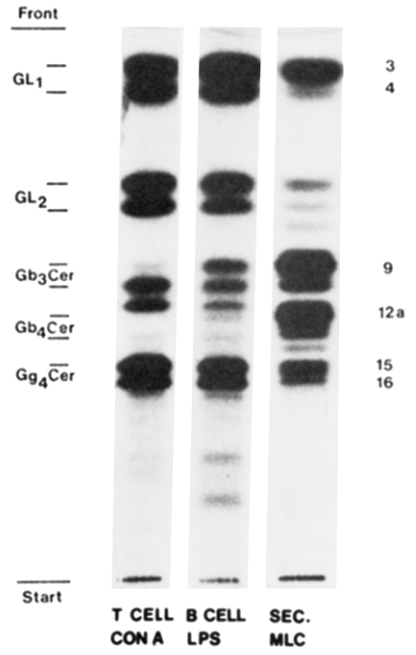

FIGURE 1: Neutral GSL's of mitogen- and alloantigen-activated lymphocytes. Autoradiogram of a thin-layer chromatogram of ^{14}C-labeled neutral GSL's. Solvent: C–M–W (120:70:17 v/v/v). From left to right: glycolipids of Con A-stimulated T cells, of LPS-stimulated B cells, and of alloantigen-stimulated T cells. The markers indicate the positions of authentic human GSL's.

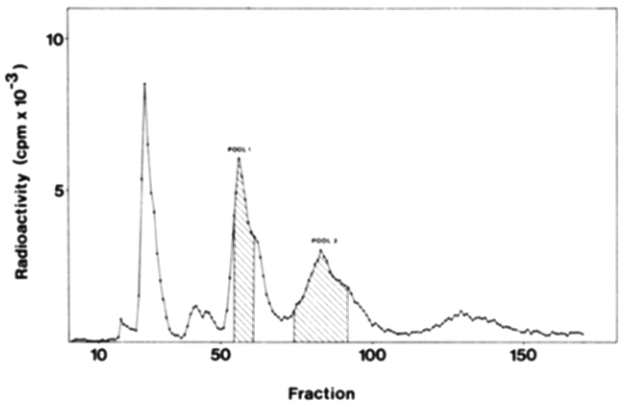

FIGURE 2: Iatrobead column chromatography of neutral GSL's from alloantigen-stimulated T lymphocytes. Neutral GSL's (200000 cpm) were applied to a 2 × 120 cm column. Elution by convex gradient of C–M–W [80:18:2 to 40:55:5 (v/v/v)]. 10-mL fractions. Radioactivity was determined from 1-mL aliquots.

formance thin-layer chromatography. The labeled neutral GSL's of allogenically stimulated T cells are compared with those from Con A stimulated T cells and LPS-stimulated B cells in the autoradiogram shown in Figure 1. The radioactive bands are numbered as in a previous publication (Rosenfelder et al., 1979). The two most prominent GSL's of the allogenically activated T cells, 9 and 12a, migrate in the range of human Gb_3Cer and Gb_4Cer, respectively. A band corresponding to Gb_3Cer is also seen in the separation pattern of the B cell GSL's but not in that of T cells stimulated with Con A. The slower migrating band 12a in the Gb_4Cer range appears to be confined to the allogenically stimulated T cell pattern.

Isolation of the Bands 9 and 12a from Allogenically Stimulated T Cells. Neutral GSL's from spleen cells activated in a secondary mixed lymphocyte reaction and radiolabeled with [^{14}C]galactose and [^{14}C]glucosamine were applied to an Iatrobead column and sequentially eluted with a C–M–W gradient. The radioactive profile from such a separation is shown in Figure 2. Three main peaks of radioactivity are eluted. The first peak contained the monohexosylceramide bands 3 and 4. Bands 9 and 12a were found in the second and third peak, respectively. On closer inspection bands 9 and 12a both consisted of a series of bands which, however, all behaved alike during consecutive degradation, indicating heterogeneity of the lipid moiety. For simplicity's sake we refer to them as bands 9 and 12a. Fractions containing band 9 were combined in pool 1, and those containing band 12a in pool 2 as indicated in Figure 2. These pools served as the starting material for subsequent degradation studies.

Degradation of Bands 9 and 12a by Exoglycosidases. Since several GSL's with different carbohydrate moieties might migrate in the Gb_3Cer and Gb_4Cer range, we attempted sequential degradation of bands 9 and 12a by exoglycosidases of known specificity in order to elucidate the carbohydrate sequence of these GSL's.

The GSL's resulting from each degradative step were reisolated and separated from the detergent present in the reaction mixture before further treatment with the next glycosidase. As shown Figure 3, band 12a could be converted to band 9 by treatment with β-hexosaminidase. For identification of the liberated radioactive *N*-acetylaminohexose, it was isolated and subjected to electrophoresis in borate buffer. It comigrated with *N*-acetylgalactosamine (9.3 cm), whereas *N*-acetylglucosamine migrated 4.3 cm. Band 9 GSL either from pool 1 or derived from β-hexosaminidase-treated band 12a from pool 2 could be degraded by α-galactosidase to a dihexosylceramide. This could in turn be degraded to monohexosylceramide by β-galactosidase treatment. The now weakly radioactive monohexosylceramide comigrated with glucosylceramide (GlcCer) on thin-layer chromatography on

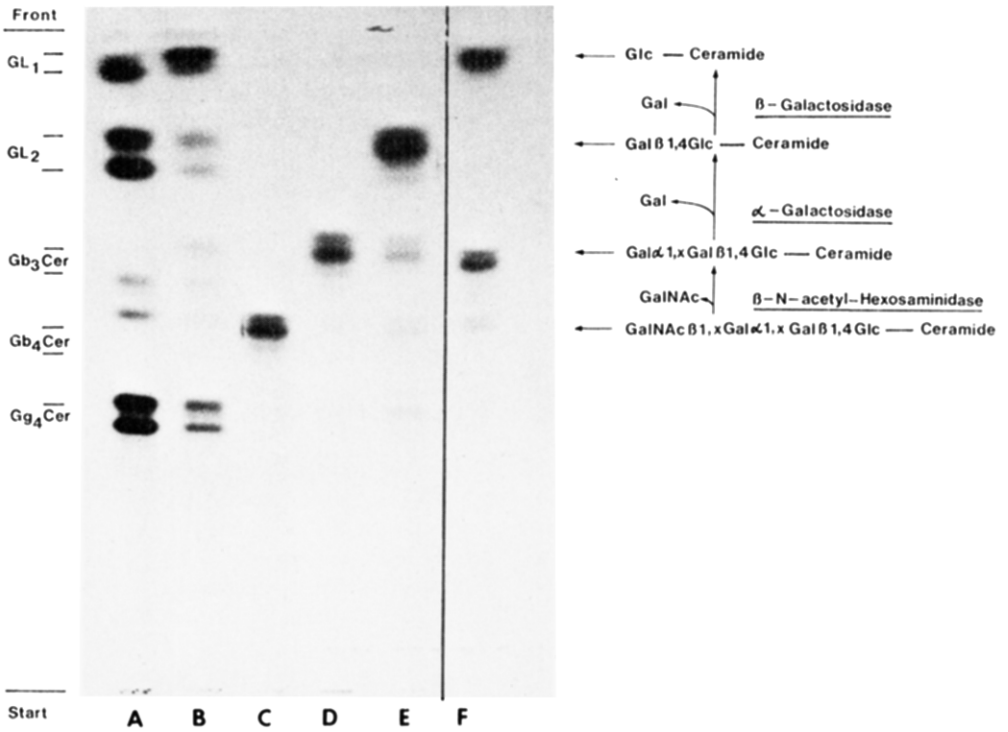

FIGURE 3: Successive degradation of GSL 12a by exoglycosidases. (Left side) Autoradiogram of a thin-layer chromatogram. Solvent as in Figure 1. Lane A, neutral GSL's from Con A stimulated T cells; lane B, the same from LPS-stimulated B cells; lane C, pool 1 material from Figure 2 (GSL 12a); lane D, pool 1 material after β-*N*-acetylhexosaminidase treatment; lane E, the same as lane D after α-galactosidase treatment; lane F, the same as lane E after β-galactosidase treatment. Whereas the substances in lanes A–E have all been applied to the same plate, lane F stems from a different one. The samples contained similar amounts of radioactivity which leads to a seeming enrichment of minor components (compare lanes E and F). (Right side) Schematic interpretation of degradation.

borate plates. The sequential degradation and our interpretation of the results are shown in Figure 3. All samples except the one in lane F were run on the same thin-layer plate. Because of this a seemingly different migratory behavior of the GSL's is observed in lane F. Since approximately the same radioactivity was applied to each lane, the small contamination of the dihexosylceramides with trihexosylceramides is seemingly enriched in lane F after removal of the radioactive galactose from the starting material in lane E.

Discussion

Our results show that mouse T cells activated during a secondary mixed lymphocyte reaction biosynthesize a series of mainly three neutral GSL's. These are GlcCer, formerly detected also in thymocytes of mice (Rosenfelder et al., 1978), and two other GSL's which, as determined by sequential degradation with exoglycosidases, bear tri- and tetrasaccharide moieties belonging to the globo or isoglobo series. Different GSL's, among these bands 15 and 16, which according to their migratory properties and carbohydrate sequence (K. R. Gruner and P. F. Mühlradt, unpublished experiments) are ganglio-tetraosylceramides (Gg_4Cer), are synthesized by mitogen-activated T and B cells.

Since lymphocytes proliferate in response to mitogen or alloantigen and, in doing so, replicate their membrane material, it is likely that the GSL's which they synthesize during activation also exist on the resting precursor cells. It is also conceivable, however, that some GSL's are only synthesized and exposed in the activated blast state. Such may be the case with Gg_4Cer which is synthesized by LPS-stimulated B lymphocytes but is not "seen" by specific antibodies on resting B cells (Stein et al., 1978). Our experiments do not distinguish between these possibilities. The relative distribution of the radioactivity between the GSL's gives only an approximate indication of the biosynthetic rate but no information concerning the relative amounts of these compounds. A quantitative chemical determination of the GSL's from murine alloreactive cells is hardly feasible because these cells represent at best a few percent of the total lymphocyte population.

Information from other laboratories about the structure and occurrence of GSL's in mouse lymphoid cells is scarce. A GSL with a retention value on high-performance liquid chromatography like that of Gg_4Cer (Asialo GM_1) has been reported to be present in splenic T cells (Schwarting & Summers, 1980). Other evidence is largely confined to work with antibodies directed against GSL's in which cross-reactivity with carbohydrates linked to glycoprotein has not always been excluded. Thus it was reported that anti-Gg_4Cer antibodies, while staining mature T cells (Stein et al., 1978), did not, in a complement lysis experiment, interfere with cytotoxic killing mediated by alloimmune T cells (Kasai et al., 1980; Young et al., 1980).

When these data are combined with our own, the following picture emerges. A glycolipid likely to be identical with Gg_4Cer is detectable by various techniques on resting (Stein et al., 1978) and Con A activated (Rosenfelder et al., 1979) T cells, on activated (Rosenfelder et al., 1979) but not on resting B cells (Stein et al., 1978), and on natural killer cells (Kasai et al., 1980; Young et al., 1980). Gb_3Cer or *i*-Gb_3Cer is found on alloantigen-activated T cells and, as a minor component, in activated B cells (Rosenfelder et al., 1979) whereas Gb_4Cer or *i*-Gb_4Cer is specifically detected in alloantigen-activated T cells, in which Gg_4Cer seems to be a minor glycolipid.

As reviewed recently (Katz, 1977), at least two different T cell subpopulations, the T amplifier and the T cytotoxic effector cells, can be expected to react during an allogenic stimulus. Although we have chosen experimental conditions which should primarily lead to labeling of the cytotoxic cell population, we cannot be certain that this is the case given the complexity of the mixed lymphocyte reaction. It is the object of our present investigations to clarify this point.

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