BRAIN AND THYMUS LIPID INHIBITION OF ANTIBRAIN-ASSOCIATED θ-CYTOTOXICITY*

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Reif and Allen (1–3) demonstrated that θ-antigen was present on the surface of mouse thymocytes and a number of murine leukemia cells, in brain tissue and, to a lesser extent, in lymph nodes and spleen. They later showed that θ-antigen was controlled by a single locus with two alleles: θ-AKR found in AKR mice, and θ-C3H found in most inbred strains of mice, including CBA.

Golub (4) has employed the fact that θ-antigen is present in brain to produce an antibrain-associated θ-antiserum (BAθ)[1] by injecting mouse brain into rabbits. The activity of BAθ antiserum parallels that of AKR anti-θ C3H and C3H anti-θ AKR. The relationship between θ-C3H, θ-AKR, and BAθ has recently been shown by Clagett et al. (5) and Peter et al. (6) to consist of a complex system of shared and unshared antigens between mouse θ-C3H, θ-AKR, and rat Lewis (Le) thymocytes.

The immunochemical reactions of lipids have been the subject of a review (7). Among the most studied of these antigens of haptenic determinants are the Forssman hapten (8, 9), cytolipin R, K, and H (7), and the ABH and Le^b isoantigens (10). Antisera can be prepared toward gangliosides with extended immunization schedules using pure gangliosides or brain (11, 12).

We now describe the partial characterization of a lipid which has been isolated from mouse thymocytes and brain, and which is capable of inhibiting the cytotoxicity of anti-BAθ antiserum. The data presented here are consistent with the proposal that BAθ antigen is G_{D1b} ganglioside.

Materials and Methods

Animals.—CBA/J female mice 4–12-wk old were used as donors of thymocytes and brains. Young Dutch Belt rabbits (3 kg) were used for preparation of antisera.

Thymocytes.—Care was exercised upon removal of mouse thymus to avoid contamination with surrounding mediastinal lymph nodes. Thymuses were placed in Eagle's minimal essen-

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[1] *Abbreviations used in this paper*: BAθ, brain-associated θ-antigen; Gal, galactosyl; Glc, glucosyl; GalNAc, *N*-acetylgalactosaminyl; NANA, *N*-acetylneuraminyl; cer, ceramide (2-*N*-acylsphingosine); G_{M3}, NANA-Gal-Glc-cer; G_{M2}, GalNAc-Gal(NANA)-Glc-cer; G_{M1}, Gal-GalNAc-Gal(NANA)-Glc-cer; G_{D1a}, Gal(NANA)-GalNAc-Gal(NANA)-Glc-cer; G_{D1b}, Gal-GalNAc-Gal(NANA-NANA)-Glc-cer; G_{T1}, Gal(NANA)-GalNAc-Gal(NANA-NANA)-Glc-cer; G_{D2}, GalNAc-Gal(NANA-NANA)-Glc-cer. Gangliosides are abbreviated according to the nomenclature of Svennerholm (19).

tial medium (MEM) and dispersed by mincing the organs with iris scissors. This step was followed by successive aspiration with syringe and needles progressively from a 19 gauge needle to a 23 gauge and finally a 27 gauge. Cells were suspended in culture medium after a single wash.

Anti-CBA/J Brain Antiserum.—Brains of CBA/J were removed, homogenized, and injected into Dutch Belt rabbits for production of anti-BAθ according to protocol used by Golub (13). The cytotoxic titer of the antiserum used for all the experiments reported herein was 1:128.

Absorption of Antisera and Complement.—The antisera and normal rabbit serum (C) were absorbed with CBA/J mouse erythrocytes, liver, and agarose (14).

Cytotoxic Tests.—0.05 ml of absorbed rabbit anti-CBA brain diluted 1:10 was added to 0.1 ml of cells at a density of 2×10^7/ml. 0.05 ml of absorbed C was added next and the mixture was incubated 1 h at 37°C. Cytotoxic potency of the antisera was determined by trypan blue exclusion. The cytotoxic index (C.I.) was calculated from the following formula:

$$\text{C.I.} = \frac{\%\text{ dead with antiserum} - \%\text{ dead with normal rabbit serum}}{100 - \%\text{ dead with normal rabbit serum}}.$$

A killing of 100% of the cells would give a C.I. of 1.0. Absorptions of antibrain serum with lipid fractions (prepared as discussed below) were incubated for 1 h before incubation with thymocytes and complement.

Lipid Extraction.—CBA/J brain or thymocyte gangliosides were prepared by homogenization of 8.3 g of CBA/J brains or 2.4×10^9 CBA/J thymocytes with chloroform-methanol 2:1 (vol/vol) in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) as described previously (15). The extract was submitted to a Folch partition (16), the ganglioside-rich upper layer was dialyzed and the nondialyzable material was evaporated to dryness in vacuo and further purified by thin-layer chromatography. All solvents were redistilled before use. Further details of isolation and separation procedures are given in another report (15).

Thin-Layer Chromatography.—Dialyzed upper phases were evaporated to dryness in vacuo and applied to a thin-layer plate of 0.25 mm of Silica Gel G (Analtech, Inc., Wilmington, Del.). The plates were developed with chloroform-methanol-2.5 N ammonium hydroxide 60:40:9 (vol/vol/vol) in unlined thin-layer tanks. Ganglioside-containing areas were visualized by spraying with resorcinol solution (17). Areas to be used for immunological or chemical tests were visualized with iodine vapors, and bands were scraped into disposable pipettes with glass wool plugs which were eluted with 10–15 ml of chloroform-methanol-water 10:10:3 (vol/vol/vol). Blueprints were made of resorcinol- or iodine-stained plates using diazo paper (18). Sialic acid was determined by the modified method (19) of Svennerholm.

Auxiliary Lipids.—Mixed upper-phase (ganglioside-rich) lipids and purified lipids were formulated with cholesterol-lecithin (auxiliary lipid) mixtures. The glycolipid (1–4 μg) and the auxiliary lipids (5 μg cholesterol and 5 μg lecithin [Supelco, Inc., Bellefonte, Pa.]) were mixed in a small volume of chloroform-methanol 1:1. The solvents were evaporated under nitrogen and 50 μl of water was added, followed by sonication in an ultrasonic cleaner (Mettler Electronics Corp., Anaheim, Calif.) for 30 s, and heating at 100°C for 1 min. The emulsion was made isotonic with 50 μl of 1.7% NaCl and mixed on a vortex mixer.

RESULTS

Total lipid extracts prepared from CBA/J mouse thymocytes were partitioned between chloroform-methanol-water two-phase systems. The lower phase (neutral lipids) and the upper phase (ganglioside-rich) were tested for inhibitory activity (Table I). Lower-phase lipids demonstrated little or no

TABLE I

Inhibition of Cytotoxicity with Thymocyte Lipid Extracts

Lipid	Cells extracted	Cholesterol: Lecithin	Cytotoxicity index
		μg:μg	
Lower phase*	2.4×10^5	0:0‡	0.86§
	2.4×10^6	0:0	0.84
	2.4×10^7	0:0	0.74
Upper phase	4.8×10^5	0.05:0.05	0.81
	4.8×10^6	0.5:0.5	0.35
	4.8×10^7	5:5	0.01

* Upper and lower phase refer to the Folch partition (see Materials and Methods).

‡ Cholesterol and lecithin were not used with lower phase lipid mixtures.

§ Unabsorbed controls gave a C.I. of 0.84–0.89.

ability to inhibit the cytotoxicity of anti-BAθ antiserum. This fraction was not mixed with auxiliary lipids because cholesterol and lecithin are present in the lower-phase lipid mixture. Upper-phase thymocyte lipids extracted from 4.8×10^7 cells completely inhibited cytotoxity (C.I. = 0.01). The antigen preparation from 4.8×10^5 cells gave no inhibition (C.I. = 0.81). The upper phases derived from lipid extracts of CBA/J brain (θ-positive) and CBA/J kidney (θ-negative) (2) were also tested for activity. The upper phase from 0.45 mg of brain (cholesterol:lecithin; 0.5 μg:0.5 μg) significantly inhibited cytotoxicity (C.I. = 0.35). On the other hand, little or no inhibition of cytotoxicity was observed with the extract of 10 mg of kidney (cholesterol:lecithin, 5 μg:5 μg) (C.I. = 0.71).

Upper-phase lipids were separated by thin-layer chromatography (Fig. 1) and isolated as described in the Materials and Methods. Individual mouse brain (MB) gangliosides were identified by comparison of their R_f and pattern to human brain gangliosides (HB) of known structure (20). Thymocyte upper-phase lipids or gangliosides were compared to mouse and human brain gangliosides by thin-layer R_f. Strong resorcinol reactions were obtained with the brain gangliosides, but the thymocyte lipids gave only weak reactions because of the small amount of material present.

The isolated lipids were formulated with auxiliary lipids and tested for ability to inhibit cytotoxicity of anti-BAθ antiserum (Table II). Area E (Fig. 1) of the thymocyte lipids (TC) was capable of complete inhibition (C.I. = 0.01). The only other thymocyte lipid demonstrating slight activity was the adjacent area F (C.I. = 0.70), and this probably represents overlap with area E. Comparable inhibition was obtained from upper-phase lipids extracted from 4.8×10^7 thymocytes, C.I. = 0.01 (Table I) and isolated lipid from 5.9×10^7 thymocytes, C.I. = 0.01 (E, Table II). These results indicate a high yield of active BAθ antigen during the purification steps.

The most active fraction among the brain gangliosides had a thin-layer R_f

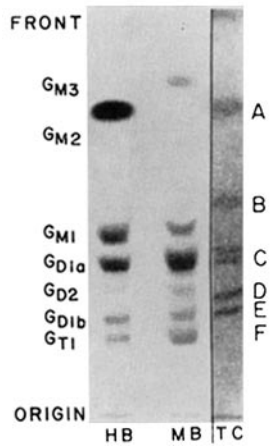

FIG. 1. Thin-layer chromatography of mouse brain and thymocyte upper-phase lipids. Human brain (HB), mouse brain (MB), and thymocyte (TC) upper-phase lipids were chromatographed and visualized with iodine vapors. Letters on the right refer to TC lipids and the ganglioside nomenclature on the left refers to HB and MB gangliosides. Spots appearing near G_{M3} and G_{M2} are unknown lipids and G_{M3} and G_{M2} were not visualized because of their low concentration.

TABLE II

Inhibition of Cytotoxicity with Isolated Thymocyte and Brain Lipids

Thymocyte lipid*	Cytotoxicity index	Brain ganglioside	μg§	Cytotoxicity index
A‡	0.93	G_{M3}, G_{M2}	0.40	0.84
B	0.94	G_{M1}	22.20	0.82
C	0.88	G_{D1a}	6.05	0.83
D	0.80	G_{D2}	1.80	0.71
E	0.01	G_{D1b}	2.19	0.07
F	0.70	G_{T1}	2.10	0.46

* Each thymocyte lipid and brain ganglioside was formulated with 5 μg of cholesterol and 5 μg of lecithin.

‡ Letters refer to thymocyte lipids in Fig. 1 (extracted 5.9×10^7 cells).

§ Micrograms of ganglioside added is based on the number of micrograms of sialic acid in the isolated lipid. The amounts reflect the relative proportions of the gangliosides isolated.

identical to G_{D1b} ganglioside and with the active thymocyte lipid. Inhibitory activity associated with G_{T1} and to a much lesser extent with G_{D2} was probably due to overlap with the active G_{D1b} fraction. When the gangliosides were further purified by thin-layer chromatography only the G_{D1b} fraction was inhibitory. Furthermore, the amount of G_{D1b} used in this assay was much more than the amount necessary to give complete inhibition, as little as 0.39 μg of G_{D1b} gave a C.I. of 0.04.

Further support for the glycosphingolipid nature of BAθ antigen was the

finding that auxiliary lipids were essential for activity. No activity was observed when 1.19 μg of G_{D1b} ganglioside was formulated without auxiliary lipids (C.I. = 0.84). When as little as 1 μg each of cholesterol and lecithin was added to the preparation complete inhibition was observed (C.I. = 0.07).

The possibility that the glycolipid antigen was interacting with complement was eliminated by absorbing the antiserum with the antigen, followed by a period of incubation with thymocytes. The thymocytes were then washed to remove glycolipid antigen and complement was added. The same degree of lysis was observed in this case as in experiments where lysis was performed as described in the Materials and Methods.

DISCUSSION

This report describes the partial characterization of an antigen which has been isolated from mouse thymocytes and brain and which is capable of inhibiting the cytotoxicity of anti-BAθ antiserum. The antigen is extractable into lipid solvents and is located in the upper-aqueous phase of a Folch partition (16). The antigen is water "soluble," requires auxiliary lipids for immunological activity, and migrates, by thin-layer chromatography with G_{D1b} ganglioside. These data are consistent with the proposal that BAθ antigen is G_{D1b} ganglioside.

Reif and Allen (3) reported that θ-C3HeB/Fe isoantigen of thymus was nondialyzable and could not be recovered in active form after treatment with lipid solvents, such as chloroform-methanol 2:1 (vol/vol). The active ganglioside reported here is nondialyzable in spite of its low mol wt (about 2,000) because it exists in solution in large aggregates or micelles (7).

Antisera to bovine brain have been found to have antibodies specific for gangliosides (12) and antisera to bovine brain gangliosides were found to contain antibodies for G_{D1b} and G_{M1} (11, 12). From this information we conclude that there are anti-G_{D1b} antibodies in the rabbit antimouse brain antisera and that this ganglioside, exposed on the surface of thymocytes and T lymphocytes, is responsible for the cytotoxicity of the anti-BAθ antisera.

Atwell et al. (21) have reported that θ-antigen is a protein with a mol wt of about 60,000 daltons. Using a similar system of immunoprecipitation of radioiodinated cell-surface proteins, we were unable to precipitate significant protein material (assessed by SDS-gel electrophoresis) using rabbit anti-CBA brain antiserum. The antigenic activity specified by AKR anti-θ C3H antiserum, on the other hand, may be different from the antigen activity specified by rabbit anti-CBA brain antiserum. The difference, if any, remains to be determined by examining the specificity of AKR anti-θ C3H antiserum.

Addendum: Vitetta et al. (22) have recently presented evidence that the θ-antigen (or Thy 1) of murine thymocytes is a glycolipid.

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