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-antisera (BA0) by injecting mouse brain into rabbits. The activity of BA0 antiserum parallels that of AKR anti-θ C3H and C3H anti-θ AKR. The relationship between θ-C3H, θ-AKR, and BA0 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 (L) 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 Leb 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-BA0 antiserum. The data presented here are consistent with the proposal that BA0 antigen is GDlb 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-
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-BA0 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 \times 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:

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

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 \times 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-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 inhibitory activity (Table I).

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 |
|----------------|----------------|-----------------------|--------------------|
| Lower phase*   |                |                       |                    |
| 2.4 × 10⁵      | 0:0‡           | 0.86§                 |                    |
| 2.4 × 10⁶      | 0:0            | 0.84                  |                    |
| 2.4 × 10⁷      | 0:0            | 0.74                  |                    |
| Upper phase    |                |                       |                    |
| 4.8 × 10⁵      | 0.05:0.05      | 0.81                  |                    |
| 4.8 × 10⁶      | 0.5:0.5        | 0.35                  |                    |
| 4.8 × 10⁷      | 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-BA0 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 × 10⁷ cells completely inhibited cytotoxicity (C.I. = 0.01). The antigen preparation from 4.8 × 10⁴ 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 Rᵢ 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 Rᵢ. 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 × 10⁷ thymocytes, C.I. = 0.01 (Table I) and isolated lipid from 5.9 × 10⁷ 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 Rᵢ
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 GM1 and GM2 are unknown lipids and GM4 and GM7 were not visualized because of their low concentration.

| Thymocyte lipid* | Cytotoxicity index | Brain ganglioside | µg‡ | Cytotoxicity index |
|-----------------|-------------------|------------------|-----|-------------------|
| A‡              | 0.93              | GM1, GM2         | 0.40| 0.84              |
| B               | 0.94              | GM1             | 22.20| 0.82              |
| C               | 0.88              | GD1a            | 6.05| 0.83              |
| D               | 0.80              | GD2             | 1.80| 0.71              |
| E               | 0.01              | GD1b            | 2.19| 0.07              |
| F               | 0.70              | GT1             | 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 X 10⁷ 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 BAα antigen was the
finding that auxiliary lipids were essential for activity. No activity was ob-
served when 1.19 μg of G_{D_{12}} 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 in-
hibiting the cytotoxicity of anti-BA0 antisera. 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_{D_{12}} ganglioside.

These data are consistent with the proposal that BA0 antigen is G_{D_{12}} ganglioside.

Reif and Allen (3) reported that 0-C3HeB/Fe isoantigen of thymus was non-
dialyzable and could not be recovered in active form after treatment with lipid sol-
vents, 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_{D_{12}} and G_{M_{1}} (11, 12). From this information we conclude that there are anti-G_{D_{12}}
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-BA0 antisera.

Atwell et al. (21) have reported that 0-antigen is a protein with a mol wt of
about 60,000 daltons. Using a similar system of immunoprecipitation of radio-
iodinated cell-surface proteins, we were unable to precipitate significant protein
material (assessed by SDS-gel electrophoresis) using rabbit anti-CBA brain
antisera. The antigenic activity specified by AKR anti-0 C3H antisera, on
the other hand, may be different from the antigen activity specified by rabbit
anti-CBA brain antisera. The difference, if any, remains to be determined by
examining the specificity of AKR anti-0 C3H antisera.

*Addendum:* Vitetta et al. (22) have recently presented evidence that the 0-antigen
(or Thy 1) of murine thymocytes is a glycolipid.

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