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Biochemistry

Monoclonal antibody to a plasma membrane antigen of neurons

(lymphocyte hybridoma cells/ganglioside/retina/brain)

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ABSTRACT **Fusion of spleen cells from a mouse immunized with chicken embryo retina cells with clonal mouse myeloma cells yielded a lymphocyte hybrid cell line that produced antibody that bound to neural tissue such as retina, brain, spinal cord, and dorsal root ganglia but not to other tissues tested. The antigen was shown by indirect immunofluorescence to be associated with plasma membranes of most, or all, neuron cell bodies in chicken retina, but little or no antigen was detected on axons or dendrites, Müller cells, or retina pigment cells. The activity of antigen A2B5 is relatively stable at 100°C, is insensitive to trypsin, exhibits the solubility properties of a ganglioside, and is destroyed by neuraminidase. Antibody A2B5 cytotoxicity against retina cells is inhibited by a GQ ganglioside fraction from bovine brain (estimated half-maximal inhibition at 0.2 μM) or by *N*-acetylneuraminic acid (half-maximal inhibition at 5000 μM) but not by other purified gangliosides tested. These results suggest that the antigen is a complex ganglioside in plasma membranes of retina neuron cell bodies but not axons or dendrites.**

Chicken embryo retina cells have been used to study biological processes that require interactions between neurons, such as cell aggregation (1, 2), adhesiveness (3), and synapse formation (4–7). To define surface molecules of retina neurons, we have used the technique, introduced by Milstein and coworkers (8–10), of antibody production by hybrid cells formed by fusion of mouse myeloma cells with spleen cells from mice immunized with chicken embryo retina cells. This approach is useful because lymphocyte hybridomas can synthesize large quantities of monoclonal antibody with specificity for a single antigen determinant. Hybridoma cell lines have been reported which synthesize antibodies specific for cell surface antigens as diverse as the Forsmann antigen (11), HLA determinants (12), tumor-specific antigens (13), and an antigen detected on human neuroblastoma cells and fetal brain (14) (also see ref. 15).

Other techniques also have been used to produce antisera against nervous system antigens. These include xenogenic immunization followed by extensive absorption with non-neuronal tissues (16, 17) and immunization with neural cell lines (18–20), partially purified synaptosomes, plasma membranes, and protein fractions (21, 22).

In this report we describe the characterization of a monospecific antibody synthesized by lymphocyte hybrid A2B5 cells that recognizes a surface antigen restricted to cell bodies of most, or all, retina neurons.

METHODS AND MATERIALS

Immunization of Mice and Production of Hybrid Cells. Female BALB/c mice were immunized by intraperitoneal injections at zero time and 7 days with 1×10^7 8-day-chicken embryo retina cells [dissociated with 0.2 mM sodium ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate and fixed with 0.1% glutaraldehyde in Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (P_i/NaCl) for 5 min], suspended in 0.25 ml of complete Freund's adjuvant, followed at 37 days by intravenous and intraperitoneal injections of 5×10^6 cells (each) without adjuvant. On day 41, two spleens were removed, the cells were dissociated mechanically, and 2×10^8 spleen cells were fused with 2×10^7 P3X63 Ag8 mouse myeloma cells (8) [obtained from John Minna] in the presence of 0.8 ml of 50% polyethylene glycol 1000 (Baker) according to the methods of Galfre *et al.* (23). After fusion, cells were suspended in medium A [Dulbecco's modification of Eagle's minimal essential medium containing 100 μM hypoxanthine, 1 μM aminopterin, 16 μM thymidine, and 20% fetal bovine serum (heat inactivated at 56°C for 30 min)] and inoculated into 92 wells (2.2×10^6 cells per ml of medium per well per 2-cm² surface area) of Costar 3524 multiwell plates. A2B5 hybrid cells (also termed F12A2B5) also were grown as ascites tumors in BALB/c mice that had been injected intraperitoneally with 0.5 ml of pristane (Aldrich) 7 days prior to injection of $1–10 \times 10^6$ cells.

Cytotoxicity and Absorption Assays. Synthesis of anti-retina antibodies by hybridoma A2B5 cells was measured by complement-dependent cytoxicity. Retinas obtained from White Leghorn chicken 8-day embryos (Truslow Farms, Chestertown, MD) were dissociated by incubation with 0.5% trypsin (crystallized 3 times, Worthington) for 5 min and washed by centrifugation. Approximately $1.2–1.6 \times 10^8$ cells were suspended in 4 ml of Eagle's minimal essential medium supplemented with 10% fetal bovine serum containing 400 μCi of $Na^{51}CrO_4$ (4.8 μM; 1.5×10^7 becquerels) incubated for 40 min at 37°C in an atmosphere of 95% air/5% CO_2. Cells were washed twice with P_i/NaCl, incubated at 4°C for 30 min in P_i/NaCl, washed three times with P_i/NaCl, and then suspended in P_i/NaCl. ^{51}Cr-Labeled retina cells (1×10^6 cells, approximately 2000–10,000 cpm) were incubated in a mixture of 95 μl of P_i/NaCl, 5 μl of guinea pig serum as a source of complement, and 50 μl of culture medium or diluted antibody in microtiter U plates (catalog no. 76-205-042, Flow General, Hamden, CT). The cell suspension was incubated for 30 min at 37°C; cells then were sedimented at $1000 \times g$; the supernatant solution was harvested by using Titertek harvesting filters (Flow General, catalog no. 78-210-05) and the radioactivity in the supernatant solutions that was absorbed by the filters was determined. Saturating concentrations of antibody A2B5 in the presence of complement released 50–60% of radioactive material from cells compared to that released by 0.3% Triton X-100. In the absence of complement, <5% of the radioactive material was released.

Abbreviation: P_i/NaCl, Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+}.

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Cells used for absorption of antibody were prepared by mincing the embryonic tissues into 1-mm pieces, triturating the suspension with a 10-ml pipette, and forcing the cells through a Nitex 130 nylon filter. Various concentrations of cells suspended in 100 μl of P_i/NaCl were incubated with 0.4 μg of antibody [antibody concentration determined by assay with ^{125}I-labeled protein A (24)] for 30 min at room temperature. Cells were removed by centrifugation at 8000 × *g* for 2 min, and 50 μl of the supernatant solution (absorbed antibody) was added to each well to assay cytotoxicity against 8-day-embryo retina cells.

Antigen Characterization. White Leghorn chicken eggs fertilized 8 days previously were obtained from Truslow Farms. Ten 8-day-embryo retinas and three 8-day-embryo brains were homogenized separately in 3.3 ml of methanol/chloroform/water, 2:1:0.3, (vol/vol), in a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 1000 × *g* for 20 min at 4°C, the supernatant fraction was removed, and the pellet was extracted with 3.8 ml of methanol/chloroform/water, 2:1:0.8 (vol/vol). After centrifugation (1000 × *g* for 20 min at 4°C), the supernatant fractions were combined (fraction A). Two milliliters of chloroform and 2 ml of H_2O were added to the pooled supernatant fraction, the mixture was centrifuged at 1000 × *g* for 20 min, and the lower chloroform phase (fraction B) and upper methanol/H_2O phase were separated. Methanol was removed from the upper phase by flash evaporation (fraction C), and the residue was tested for ability to inhibit antibody A2B5-dependent cytotoxicity. In other experiments, the upper phase was dialyzed overnight at 4°C against 50 vol of H_2O (changed three times) and lyophilized, and the residue was dissolved in chloroform/methanol/H_2O, 10:5:1 (vol/vol) (fraction D). Solvents were removed from fractions by evaporation under a stream of nitrogen or air. The residues were dissolved in H_2O and assayed for inhibition of cytotoxicity.

GM_1, GD_1, and GT_1 were obtained from Supelco; GT_1 and GQ fractions were kindly provided by Peter Fishman (Laboratory of Neurochemistry, National Institutes of Health). The GQ fraction purified by DEAE column chromatography and silicic acid column chromatography (25) was approximately 1000-fold enriched in GQ compared to unfractioned bovine brain gangliosides.

Indirect Immunofluorescence. Chicken neural retina was fixed with 3% formaldehyde in P_i/NaCl for 30 min, washed with P_i/NaCl, immersed in liquid nitrogen, and then sectioned (16 μm). Sections were incubated with 50–100 μl of A2B5 ascites fluid diluted 1:100 (2–4 μg of antibody) for 45 min at room temperature and then washed with P_i/NaCl for 45 min. For immunofluorescence of monolayer cultures, cells were dissociated from 8-day-embryo retina (0.5% trypsin for 5 min at 37°C) and cultured for 24 hr in 35-mm petri dishes (1×10^6 cells per dish) in 90% Eagle's minimal essential medium (GIBCO)/10% fetal bovine serum. The cells were fixed as described above, washed, and incubated with 4 μg of antibody in 100 μl of P_i/NaCl for 45 min. Cultured retina cells or sections were incubated with fluorescein-labeled rabbit antibody directed against mouse IgG for 30 min at room temperature and then were washed with P_i/NaCl.

RESULTS

Spleen cells from a mouse immunized with chicken retina cells from 8-day embryos and P3X63 Ag8 myeloma cells which lack hypoxanthine phosphoribosyltransferase activity (EC 2.4.2.8) (8) were fused in the presence of polyethylene glycol. Two weeks after fusion, colonies were present in all wells. Part of the medium was removed and assayed (*i*) for antibody directed against retina cells by assaying binding of ^{125}I-labeled protein A to 8-day embryo retina cells with bound immunoglobulin (26) and (*ii*) for complement-dependent cytotoxicity by using 8-day-embryo retina cells labeled with ^{51}Cr as targets. Media harvested from 45 of the 92 wells assayed contained antibodies that bound to retina cells and to protein A. Cytotoxic antibodies were detected in three wells and the properties of one, A2B5, is described in this communication. Antibody was obtained by harvesting the medium from cultured cells or ascites fluid from BALB/c mice with A2B5 ascites tumors. The ascites fluid was cytotoxic, at a dilution of 1:25,000, to 8-day-embryo retina cells and was used for most of the studies described. A2B5 cells were cloned six times by dilution (0.8 cell/well) in multiwell plates. After 2 weeks of incubation for each cloning cycle, approximately 30% of the wells contained colonies. Initially, both positive and negative sublines of A2B5 were obtained with respect to the synthesis of antibody directed against chick retina; however, cycle 5 of cloning resulted in a subline (A2B5 clone 105) that, when cloned again, gave rise only to positive colonies. Ascites fluid from mice bearing subline A2B5 clone 105 was cytotoxic, at a dilution of 1:300,000, to 8-day-embryo retina cells.

Different tissues were assayed for antigen A2B5 by incubating the antibody with the cells and, after absorption, determining the remaining cytotoxic activity against 8-day-embryo retina cells. The cytotoxic activity of antibody A2B5 was removed by 8- and 16-day-embryo retina or brain cells but not by muscle, heart, kidney, or liver cells or erythrocytes (Fig. 1). The antigen also was detected in human and bovine brain and chicken dorsal root ganglion (data not shown).

The neural retina of the chicken 16-day embryo consists of three layers of cell bodies separated by two layers of axons and dendrites connected by synapses. The distribution of antigen A2B5 in sections of 16- and 8-day-embryo retina and adult

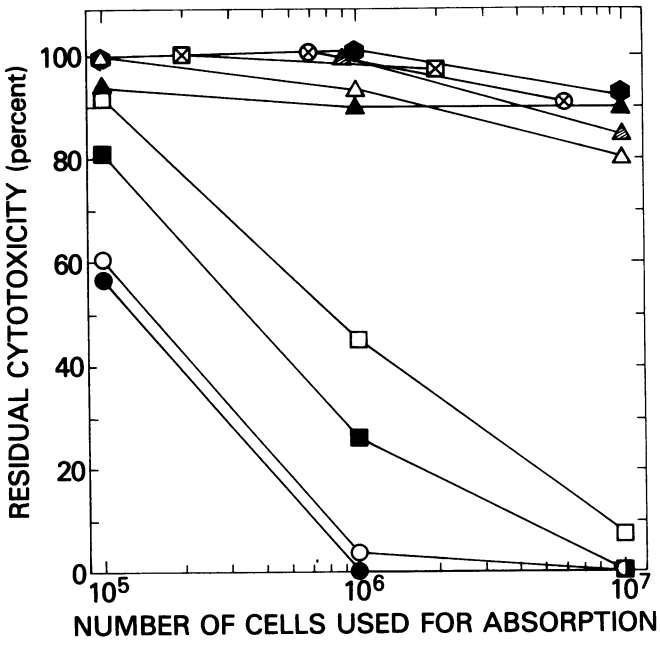

FIG. 1. Residual cytotoxicity of antibody A2B5 after absorption with different concentrations of cells from 8- or 16-day-embryo tissues: ●, 8-day, brain; ○, 16-day, brain; ■, 8-day, retina; □, 16-day, retina; ▲, 8-day, liver; △, 16-day, liver; ▲, 16-day, muscle; ⊗, 16-day, heart; ⊠, 16-day, kidney; ●, 16-day, erythrocytes. Residual cytotoxicity = (cpm released due to antibody A2B5 after absorption/cpm released due to unabsorbed antibody A2B5) × 100. One hundred percent residual cytotoxicity corresponds to the release of 785 cpm from 8-day-embryo cells labeled with ^{51}Cr, in response to 0.4 μg of antibody A2B5.

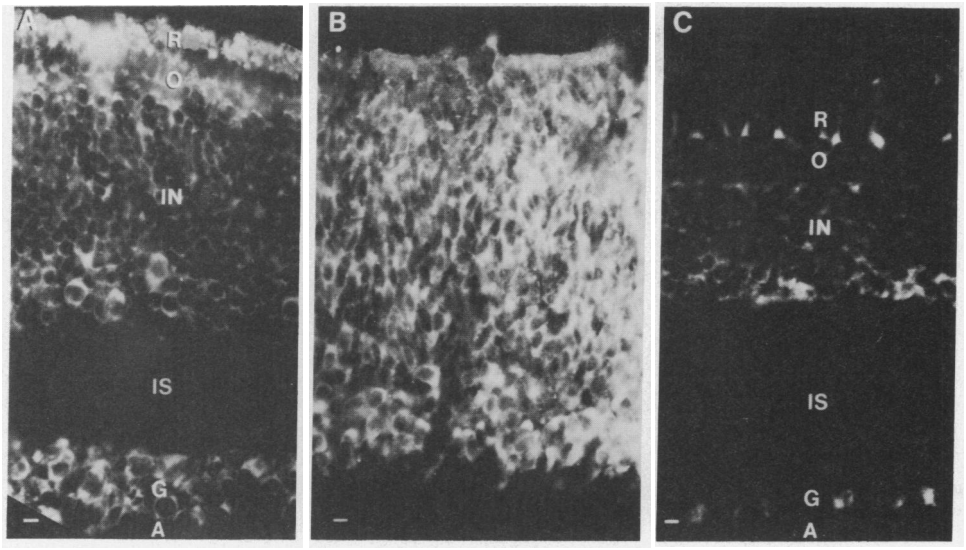

FIG. 2. Distribution of antigen A2B5 in sections of chicken retina from 16-day embryo (*A*), 8-day embryo (*B*), and adult (*C*). Letters refer to retina layers: R, photoreceptor cell layer; O, outer synaptic layer; IN, inner nuclear layer containing cell bodies of horizontal, bipolar, and amacrine neurons and Müller cells; IS, inner synaptic layer comprised of processes and synapses of amacrine, bipolar, and ganglion neurons; G, ganglion neuron cell bodies; A, ganglion neuron axons. (Bar, 4 μm.)

retina, determined by indirect immunofluorescent staining, is shown in Fig. 2. All regions of the 16-day-embryo retina occupied by cell bodies bound antibody A2B5, but the antigen was not detected on axons or dendrites of neurons in the inner or outer synaptic layers of the retina or on axons of ganglion neurons (Fig. 2*A*). Layers of cell bodies separated from processes are not present in 8-day-embryo retina, and antibody A2B5 bound to cells in all regions of 8-day-embryo retina (Fig. 2*B*). In adult chicken retina (Fig. 2*C*), antibody A2B5 bound to cell bodies of photoreceptor cells, amacrine neurons, horizontal neurons, bipolar neurons, and ganglion neurons but not to cell processes in the inner and outer synaptic layers of the retina. In this and other retina sections, the inner segments of photoreceptor cells were intensely fluorescent and fluorescence was detected both within the cells and on the surface membrane. Thus, the antibody may be reacting with both plasma membrane and cytoplasmic antigens. Less antibody A2B5 bound to the central part of the inner nuclear layer which contains Müller cell bodies (retina glial cells) than to other areas of the retina with cell bodies. Antibody A2B5 also did not bind to processes of Müller cells which traverse the inner and outer synaptic layers. In contrast to the pattern of fluorescence with antibody A2B5, antibodies produced by other hybrid cell lines and rabbit anti-retina antibodies bound to all layers of the retina (data not shown).

The distribution of antigen A2B5 on retina cells dissociated from 8-day-embryo and cultured for 24 hr also was determined. These retina cells adhered to the substratum, extended pro-

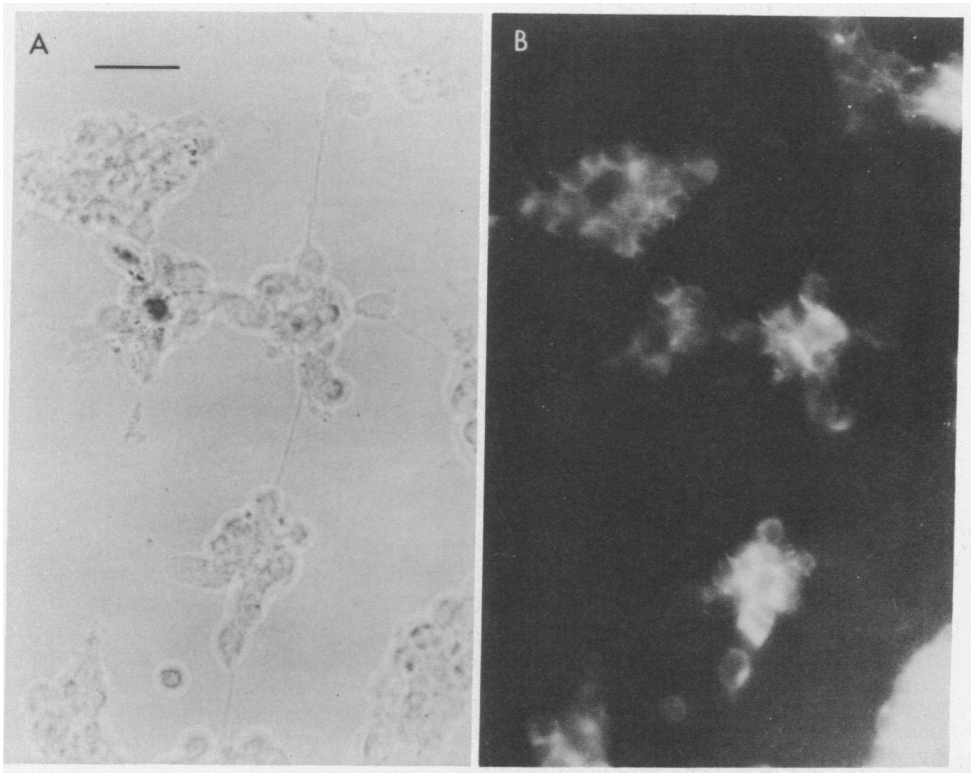

FIG. 3. Distribution of antigen A2B5 on chicken retina cells dissociated from 8-day-embryo and cultured for 1 day, detected by indirect immunofluorescence. (*A*) Bright-field view of the cultured cells showing cell bodies and processes (denoted by arrows). (*B*) Same field showing fluorescent cell bodies and cell processes without fluorescence. (Bar, 20 μm.)

Table 1. Properties of retina antigen A2B5 in 100,000 × *g* pellet fraction

Antibody A2B5 plus:	Residual cytotoxicity, %
No addition	100 ± 5
Pellet	4 ± 5
Pellet, 3 min at 100°C	23 ± 5
Pellet, 30 min at 100°C	40 ± 4
Pellet, 5 min with trypsin	6 ± 3
Pellet, 30 min with trypsin	3 ± 6
Pellet extracted with chloroform/methanol, 2:1	87 ± 13
Material extracted from 100,000 × *g* pellet into chloroform/methanol, 2:1	38 ± 9

Pellet fraction corresponded to 44 μg of unfractionated retina protein; antibody A2B5 was 0.4 μg per well. Release of ^{51}Cr from 8-day-embryo retina cells was determined. Where specified, the pellet fraction was incubated with 0.8 mg of trypsin in 400 μl at 37°C for 3 or 30 min; then 0.8 mg of soybean trypsin inhibitor was added and the effect on cytotoxicity was determined. Residual cytotoxicity is defined in the legend to Fig. 1. One hundred percent residual cytotoxicity corresponded to 1515 cpm released from 8-day-embryo retina cells labeled with ^{51}Cr. Each value is the mean ± SEM of three determinations.

Table 2. Effect of neuraminidase on antigen A2B5

Additions	Residual cytotoxicity, %
None	0
Antibody A2B5	100 ± 2
Antibody A2B5 + antigen A2B5	53 ± 4
Antibody A2B5 + neuraminidase	92 ± 6
Antibody A2B5 + antigen A2B5 + neuraminidase	86 ± 3
Antibody A2B5 + antigen A2B5 + boiled neuraminidase	43 ± 2

Antigen A2B5 extracted from 7 mg (wet weight) of chicken 8-day-embryo brain (fraction D) was incubated at 37°C for 60 min in a reaction mixture (final volume 320 μl) containing 30 mM sodium acetate (pH 5.5), 0.43 μg of neuraminidase protein from *Clostridium perfringens* purified by affinity chromatography (47 units/mg protein, Sigma), or heat-inactivated neuraminidase (100°C, 10 min). Then, 20 μl of the reaction mixture containing antigen A2B5 extracted from 440 μg (wet weight) of brain and 0.027 μg of neuraminidase or heat-inactivated neuraminidase protein was added to each well assayed for cytotoxicity (0.4 μg of antibody A2B5 per well). Residual cytotoxicity is defined in the legend of Fig. 1. One hundred percent residual cytotoxicity corresponded to the release of 196 cpm. Each value is the mean ± SEM of three determinations.

cesses, and formed aggregates (Fig. 3). Antibody A2B5 bound to >60% of the cell bodies but little or no antigen was detected on cell processes. The antibody did not bind to retina pigment cells or to cells with the morphology of fibroblasts or epithelial cells (these comprise <20% of the cell population). The remaining cells that lack antigen A2B5 may be Müller cells. Antigen A2B5 was detected on the soma and on the processes of cultured chicken embryo dorsal root ganglion cells; thus, localization to the cell soma is not an invariant property of the antigen.

Antigen A2B5 was assayed by inhibition of antibody A2B5-dependent cytotoxicity (Table 1). The antigen was inactivated only 36% on incubation at 100°C for 30 min, was insensitive to trypsin, was sedimented by centrifugation at 100,000 × *g* for 30 min, and was extracted from the pellet into chloroform/methanol, 2:1 (vol/vol). Antigen A2B5 was solubilized by extraction of 8-day-embryo chicken brain or retina with methanol/chloroform/H_2O, 2:1:0.3 (vol/vol). Two additional volumes of chloroform and 2 vol of H_2O were added. The lower chloroform phase and the upper methanol/H_2O phase were separated, taken to dryness, and assayed for inhibition of antibody A2B5-dependent cytotoxicity (Fig. 4). All of the antigen extracted from brain or retina partitioned into the methanol/H_2O phase and thus exhibited the solubility of a ganglioside. A2B5 antibody-dependent cytotoxicity was inhibited by compounds in the methanol/H_2O phase (Fig. 4*B*) but not by material in the chloroform phase (Fig. 4*A*). When material in the methanol/H_2O phase was fractionated by thin-layer chromatography [silica gel plates; propanol/H_2O, 70:30 (vol/vol)], antigenic activity remained close to the origin (R_F = 0.02), with GQ gangliosides.

Because the antigenic material exhibited the properties of a ganglioside, the effect of neuraminidase (purified by affinity

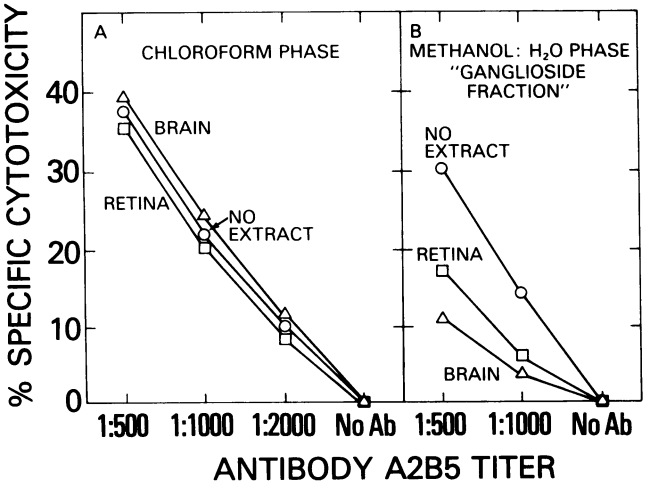

FIG. 4. Inhibition of antibody A2B5-dependent cytotoxicity by molecules extracted from 8-day-embryo chicken brain or retina into chloroform/methanol/H_2O, 3:1:0:3. Antigenic activities (corresponding to antigen extracted from 3.9 mg of brain or 1.0 mg of retina, wet weight) in the chloroform phase (fraction B) and in the methanol/H_2O phase (fraction C) are shown in *A* and *B*, respectively.

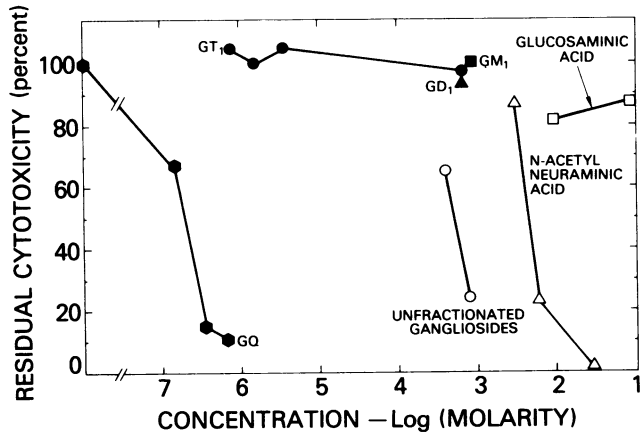

FIG. 5. Inhibition of antibody A2B5-mediated cytotoxicity by purified ganglioside preparations and saccharides. Each point represents the mean of three determinations. The effects of gangliosides GQ (●), GM_1 (■), GD_1 (▲), and GT_1 (●), bovine gangliosides (○), *N*-acetylneuraminic acid (△), and glucosaminic acid (□) were studied at the concentrations shown.

column chromatography) on the antigenic activity was determined (Table 2). Incubation with neuraminidase almost completely blocked the inhibitory effect of antigen A2B5 on antibody-dependent cytotoxicity.

The effects of partially purified ganglioside fractions and several monosaccharides on release of ^{51}Cr from 8-day-embryo retina cells due to antibody A2B5 cytotoxicity are shown in Fig. 5. A GQ ganglioside fraction (ganglioside molecules with four *N*-acetylneuraminic acid residues) purified approximately 1000-fold inhibited cytotoxicity 50% at an estimated concentration of 0.2 μM. A crude bovine ganglioside fraction and *N*-acetylneuraminic acid also inhibited the cytotoxic effect of antibody A2B5 with half-maximal inhibitions estimated to be 600 and 5000 μM, respectively. Gangliosides GT_1, GD_1, and GM_1 and glucosaminic acid had little or no effect on cytotoxicity. These results suggest that antibody A2B5 recognizes an antigen with the properties of a complex ganglioside such as a GQ ganglioside. Further work is needed to define the structure of the antigen.

In a preliminary study, antibody A2B5 was used to explore the possible role of the antigen in synapse formation. Saturating concentrations of antibody A2B5 did not inhibit the formation of synapses between dissociated 8-day-embryo chicken retina cells and rat myotubes or the transmission across synapses.

DISCUSSION

A hybrid cell line that synthesizes a cytotoxic antibody directed against chicken embryo retina neurons was obtained by fusion of mouse myeloma cells with spleen cells of mice immunized with retina cells. The antigen also was found in chicken embryo brain, spinal cord, and dorsal root ganglion neurons and in bovine and human brain but was not detected in heart, muscle, liver, kidney, or erythrocytes of chicken embryos. The antigen of chicken retina was shown by indirect immunofluorescence to be associated with plasma membranes of most, or all, neuron cell bodies including photoreceptor cells, horizontal neurons, amacrine neurons, bipolar neurons, ganglion neurons, and neuroblasts. The antigen was not detected on axons or dendrites in the synaptic layers of the retina, even though the synaptic layers contain more plasma membrane than equivalent areas of the retina composed of cell bodies. Bipolar neurons may contain less antigen A2B5 because they fluoresced less intensely than did other retina neurons. In addition, inner segments of photoreceptor cells fluoresced more intensely than outer segments. Antigen A2B5 was not detected on retina Müller cells, pigment cells, or cells with the morphology of fibroblasts or epithelial cells. Molecules in chicken retina with a distribution complementary to that of antigen A2B5 (i.e., preferentially localized to axons or dendrite plasma membranes) have been reported, such as cell adhesion molecules (27), acetylcholinesterase (EC 3.1.1.7) (28), nicotinic acetylcholine receptors (29), and muscarinic acetylcholine receptors (30).

A2B5 antigenicity was not destroyed by trypsin or by incubation at 100°C but was inactivated during incubation with neuraminidase. The antigen sedimented at 100,000 × *g* and was soluble in chloroform/methanol (2:1). A GQ ganglioside fraction from bovine brain purified 1000-fold inhibited the cytotoxic effect of antibody A2B5 on retina cells. The concentration for half-maximal inhibition was estimated to be 0.2 μM. In contrast gangliosides with three or less *N*-acetylneuraminic acid residues that were tested had no effect on cytotoxicity at concentrations of 1 mM. Antibody A2B5 may not interact with all molecules in the GQ ganglioside fraction; thus, the observed half-maximal inhibition of cytotoxicity may be a minimal estimate of the affinity of the antigen for the antibody.

The localization of antigen A2B5 on the exterior surface of cell bodies of neurons raises questions concerning the function of the antigen. Whether the antigen is involved in the sorting out of neurites from cell bodies or in determining the relative positions of neuronal soma in the retina is unknown. However, the availability of large quantities of monoclonal antibody should be useful for further purification of the antigen, for separating populations of neurons that possess the antigen from other cell types, and for studies on the synthesis, localization, and function of the antigen.

We thank Dr. Peter Fishman for generously supplying us with a purified GQ ganglioside fraction and Dr. Roscoe Brady for helpful suggestions concerning fractionation of chicken brain gangliosides.

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