Immunochemical Properties of Glycolipids

ROSCOE O. BRADY, Laboratory of Neurochemistry, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland

Abstract

Lipids of the nervous system and other tissues which contain at least one molecule of a carbohydrate have been shown to exhibit haptenic properties. The specificity of the antibodies produced by the administration of these materials is directed by specific portions of the carbohydrate moiety. Examples of antibodies induced by the polyhexoside and N-acetyl neuraminic acid portions of gangliosides will be shown. The potential usefulness of such antigen-antibody systems for structural studies will be illustrated. Some consideration will be given to the possible role of glycolipid haptens in pathological conditions such as experimental allergic encephalomyelitis and multiple sclerosis.

STUDIES OF THE PROPERTIES and reactions of lipid haptens are of considerable interest for investigators concerned with immunological phenomena in at least four areas. The first deals with immune reactions which occur with an incompatible blood type. The second is the phenomenon of transplantation immunity. The third, perhaps related to the second, is the immunopathology of neoplasms. The fourth arises from attempts to discover some insight into the pathogenesis of demyelinating diseases such as multiple sclerosis. All of these fields seem to have two characteristics in common. The haptens or antigens responsible for these immune reactions arise from components of cellular membranes or are contiguous with them as in the case of the endoplasmic reticulum of cells (1,2). Secondly, the haptenic material is generally not a simple chemical entity, but frequently is a covalently-bound complex of lipid, carbohydrate and protein. We are primarily concerned in this presentation with immunochemical studies of haptens containing the first two of these substances.

Sphingolipid Haptens

The simplest sphingoglycolipid is a cerebroside which is comprised of 1 molecule each of sphingosine, long chain fatty acid, and hexose. The carbohydrate may be either galactose or glucose. Cerebrosides account for approximately 4% of the fresh (wet) weight of brain tissue. The largest portion is in the white matter, and galactocerebrosides, which constitute 95% of the cerebrosides of nervous tissue, are a characteristic lipid of the myelin sheath.

It has been known for nearly 40 years that alcoholic extracts of brain tissue contain what appears to be an organ-specific lipid hapten (3,4). Recent investigations in two laboratories have rather conclusively shown that this hapten is galactocerebroside. The first identification of the haptenic property of this material was accomplished by Niedieck (5) in studies with sera of rabbits sensitized by the intradermal injection of peripheral nerve, brain or spinal cord, along with an adjuvant in the manner characteristically employed to produce experimental allergic encephalomyelitis (EAE). Anticerebroside antibodies were found in the serum and some correlation was claimed between the onset of the signs of EAE and the appearance of anti-cerebroside antibodies. The other study was carried out by Joffe et al. (6) by intraperitoneal injections of brain particulate material. Antigalactocerebroside antibodies were found in the serum from these animals. Neither glucocerebroside nor ceramide-lactose cross-reacted with this antiserum.

It therefore seems quite likely that galactocerebroside was the alcohol-soluble hapten with which the earlier investigators were dealing. These studies indicated that the presence of a single hexose molecule conferred haptenic properties to an otherwise nonhaptenic material, in this case ceramide (N-acyl sphingosine). Furthermore, in keeping with the known high degree of specificity of immunological reactions, epimerization at C-4 of the hexose molecule completely alters the immunological specificity of the cerebroside molecule.

In spite of the evidence for the production of anticerebroside antibodies, most investigators agree that induction of antibodies by injection of cerebrosides is not responsible for the neurological lesions which occur in EAE (7,8). Furthermore, it appears that the neurological manifestations are actually due to an antibody directed against a basic protein component of the myelin sheath (9). The role of galactocerebroside as an inducer of an autoimmune phenomenon in multiple sclerosis seems even more remote. This concept is supported by the findings of Sherwin et al. (10) and Somers (11) who clearly demonstrated that antimyelin antibodies are present in sera from animals with EAE, but absent in patients with multiple sclerosis. Hence the etiological similarity between these conditions seems evanescent. However, the reservation must be made that anticerebroside antibodies may not have been detected because of improper testing procedures. This may be due in part to difficulties in examining the hapten, in this case cerebroside, in a properly solubilized form. In addition, studies in which the explicit status of the patient is carefully controlled have yet to be done. It is conceivable that antimyelin (cerebroside, basic protein) antibodies may have been completely adsorbed from the serum by components of the nervous system during periods of remission of the pathological process.

Higher Ceramide Oligosaccharides

Ceramide-Lactose

The next higher naturally occurring sphingolipid homologue is ceramide-lactose. This substance was actually the first sphingoglycolipid hapten to be definitively characterized and was conclusively identified by Rapport et al. (12). It was called cytolipin H by these investigators. It was shown to be a hapten and was obtained from methanolic extracts of human epidermoid carcinoma explants grown in rats. The components were shown to be equimolar proportions of sphingosine, fatty acid, glucose and galactose. Later this material was identified in serum from normal human beings (13). These two observations afforded a reconciliation of the findings of Goodman and Brady (14) that serum samples from normal

TABLE I

Hapten Inhibition of Agglutination of Cells Coated with Asialoganglioside (22)

Inhibitor	Concentration
Asialoganglioside	< 1 mg/ml
Ceramide-glucose-galactose-N-acetylgalactosamine	1 mg/ml
Lactose	0.0015 M
4-O-β-D-galactosyl-N-acetylgalactosamine	0.020 M
Globoside	Ineffective
Ganglioside	Ineffective

Serum from rabbits immunized with asialoganglioside was used as source of antibody (24).

humans as well as patients with multiple sclerosis appeared to contain an antibody which reacted with chicken erythrocytes coated with ceramide-lactose.

Ceramide-Trisaccharides

One of the next higher homologues, a ceramide-trisaccharide (glucose-galactose-N-acetylgalactosamine) has also been demonstrated in normal human serum. To date, no immunization studies with this substance have been reported. One wonders about the possible relationship of this material to the Forsmann hapten (15) which as yet is not well characterized. The latter substance is said to contain ceramide plus *at least* 1 molecule each of galactose and N-acetylgalactosamine (16).

Another ceramide-trisaccharide which contains glucose and galactose in the ratio of 1:2 has been isolated from the kidney tissue of a patient with Fabry's disease (17). We have confirmed the accumulation of ceramide-trihexoside in Fabry's disease by examining the lipid composition of an enlarged lymph node obtained from a patient with this condition (18). It is not yet known if an auto-immune response to this material plays a role in the pathogenesis of the renal failure which often occurs in these patients although it would appear to be a likely possibility.

Ceramide-Tetrasaccharides

At the present time two fairly well-defined ceramide-tetrasaccharides have been demonstrated. The first, called globoside by Yamakawa and his associates, consists of ceramide-glucose-galactose-galactose-N-acetylgalactosamine (19). It is said to be the main glycolipid of erythrocyte stroma. The production of antibodies with the use of this material as hapten has not been reported as yet. Recent experiments with this material indicate the immunological identity of globoside and another sphingoglycolipid isolated from kidney tissue, termed cytolipin K (20). It was known that the latter material contained the same type and number of carbohydrate residues as globoside, but the arrangement of the hexoses had not been clarified. The immunochemical studies by Rapport and Graf indicate that they are most likely identical materials. Furthermore, the haptenic properties of cytolipin K have been recently established (21).

Another ceramide-tetrasaccharide, generally called asialoganglioside, has been produced by mild acid hydrolysis of gangliosides. In this process, N-acetylneuraminic acid molecules are cleaved from the naturally-occurring parent gangliosides. The arrangement of hexose molecules in asialoganglioside is believed to be ceramide-glucose-galactose-N-acetylgalactosamine-galactose. The haptenic properties of this material have been investigated by Somers et al. (22). An antiserum was prepared by injecting rabbits with red cells coated with the glycolipid. The specificity of the antibody produced by the injections of asialoganglioside was examined by the hapten inhibition technique (23). The asialoganglioside antiserum was incubated with the various sugars or glycolipids for 15 min at 23C before examining the hemagglutination of red cells coated with asialoganglioside. With this technique, inhibition was observed by prior incubation with asialoganglioside and aminoglycolipid (ceramide-glucose-galactose-N-acetylgalactosamine) (Table I). No inhibition was observed with globoside in spite of the fact that the same carbohydrate residues are present as in asialoganglioside. These observations indicate that the arrangement of the carbohydrate portion confers immunological specificity to the molecule and provides confirmatory evidence that the sequence is indeed different in globoside and asialoganglioside. Of considerable interest was the additional finding that gangliosides did not appear to be an inhibitor in the anti-asialoganglioside test system.

These studies were extended to immunization experiments using red cells coated with gangliosides as hapten (24). Antiganglioside antibodies were produced with this technique. The antiganglioside antibody was inhibited by preincubating the antiserum with ganglioside, N-acetylneuraminic acid and colominic acid, a polymer of N-acetylneuraminic acid (Table II). It was not inhibited by asialoganglioside. These findings again indicate the specificity of the antibody, and in this instance, it appears to be conferred by the N-acetylneuraminic acid portion of the molecule.

It is apparent that such specificity studies have been helpful in substantiating structural differences between various glycolipids, and in the case of cytolipin K, provide a rather conclusive method for identifying this compound. Studies of this nature have been used to help elucidate the structure of M and N substances present in erythrocyte membranes as well as the

TABLE III

Relationship Between Chemical Structure and Blood Group Activity in Hemagglutination Inhibition Tests (24)

Inhibitor	Concentration in mg/ml required to inhibit the respective antigen-antibody test systems				
	Anti-A	Anti-B	Anti-M	Anti-N	Anti-Rho(D)
Ganglioside	5.0	[a]	2.5	5.0	2.5
Purified ganglioside 4-G[b]	....	....	....	....	0.0013
Asialoganglioside	[a]	[a]	[a]	[a]	[a]
N-acetylneuraminic acid	[a]	[a]	[a]	[a]	[a]

[a] Denotes lack of blood group activity at highest level tested (10 mg/ml).
[b] Dodd, M. C. et al., Nature *204*, 549 (1964).

TABLE II

Hapten Inhibition of Agglutination of Cells Coated with Ganglioside (22)

Inhibitor	Concentration
Ganglioside	< 1 mg/ml
N-acetylneuraminic acid	0.05 M
Colominic acid	2 mg/ml
Asialoganglioside	Ineffective
Globoside	Ineffective
Lactose	Ineffective

Serum from rabbits immunized with gangliosides [Yokoyama et al., J. Immunol. *90*, 372 (1963)] was used as source of antibody.

TABLE IV

Demonstration of Anti-Ganglioside Antibodies in Human Sera in Various Pathological Conditions (26,27)

Condition	Sera examined	Anti-ganglioside antibody	
		No.	Titre
Normal	42	0	
Multiple sclerosis	42	8	1:1–1:8
Amyotrophic lateral sclerosis	3	1	1:8
Schizophrenia	14	1	1:8
Tay-Sachs' disease	14	0	

Rho(D) factor (24,25) (Table III). However, investigations along these lines have not been pursued so far as those described in the earlier portions of this communication.

Finally, some attention has been directed towards the possibility that anti-ganglioside antibodies might occur in the serum of patients with various lesions of the central nervous system, particularly with regard to multiple sclerosis (26). Antiganglioside antibodies are not normally present in human serum. A low but demonstrable antiganglioside antibody titre does occur in about 20% of patients with multiple sclerosis, and amyotrophic lateral sclerosis (Table IV). The antibody in the sera from patients with multiple sclerosis appears to be specific for monosialoganglioside (27). However, the role of antiganglioside antibodies in the etiology of pathological conditions such as multiple sclerosis and amyotrophic lateral sclerosis remains obscure. As in the case of animals in which anticerebroside antibodies were induced, no signs of neurological disability could be attributed to the presence of circulating antiganglioside antibody (28).

In retrospect, one might conclude that serum anticerebroside or antiganglioside antibodies would not necessarily be found in patients with multiple sclerosis even if an autoimmune reaction plays a role in the pathogenesis of this disease. This concept is supported by the widely accepted view that delayed hypersensitivity is a cellularly-mediated phenomenon and that circulating antibodies seem relatively unimportant in reactions of this nature (29).

REFERENCES

1. Herzenberg, L. A., and L. A. Herzenberg, Proc. Nat. Acad. Sci. (U.S.) *47*, 762 (1961).
2. Davies, D. A. L., Biochem J. *84*, 307 (1962).
3. Brandt, R., H. Guth and R. Müller, Klin. Wochschr. *5*, 655 (1928).
4. Witebsky, E., and J. Steinfeld, Z. Immunitätetsforsch. *58*, 271 (1928).
5. Niedieck, B., Z. Immunitäts und Allergieforschung *126*, 40 (1964).
6. Joffe, S., M. M. Rapport and L. Graf, *Nature 197*, 60 (1963).
7. Kuvert, E., and B. Niedieck, personal communications.
8. Kies, M., personal communications.
9. Kies, M., R. H. Laatsch, O. L. Silva and E. C. Alvord, Jr., Z. Immunitäts and Allergieforschung, *126*, 25 (1964).
10. Sherwin, A. L., M. Richter, J. B. Cosgrove and B. Rose, Science *134*, 1370 (1961).
11. Somers, J. E., Neurology *14*, 232 (1964).
12. Rapport, M. M., L. Graf, V. P. Skipski and N. F. Alonzo, Cancer *12*, 438 (1959).
13. Svennerholm, E., and L. Svennerholm, Nature *198*, 688 (1963).
14. Brady, R. O., in "Metabolism and Physiological Significance of Lipids," edited by R. M. C. Dawson and D. N. Rhodes, John Wiley and Sons, Ltd., London, 1964, p 95.
15. Forsmann, J., Biochem Z. *37*, 78 (1911).
16. Papirmeister, B., and M. F. Mallette Arch. Biochem. Biophys. *57*, 94 (1955).
17. Sweeley, C. C., and B. Klionsky, J. Biol. Chem. *238*, PC 3148 (1963).
18. Brady, R. O., and L. Laster, unpublished observations.
19. Yamakawa, T., S. Yokoyama and N. Handa, J. Biochem. (Tokyo) *53*, 28 (1963).
20. Rapport, M. M., and L. Graf, Federation Proc. *24*, 176 (1965).
21. Rapport, M. M., L. Graf and H. Schneider, Arch. Biochem. Biophys. *105*, 431 (1964).
22. Somers, J. E., J. N. Kanfer and R. O. Brady, Biochemistry *3*, 251 (1964).
23. Landsteiner, K., "The Specificity of Serological Reactions," revised ed., Harvard University Press, Cambridge, Mass., 1945.
24. Yokoyama, M., E. G. Trams and R. O. Brady, J. Immunol. *90*, 372 (1963).
25. Dodd, M. C., N. J. Bigley and V. B. Geyer, Science, *132*, 1398 (1960).
26. Yokoyama, M., E. G. Trams and R. O. Brady, Proc. Soc. Expl. Biol. Med. *111*, 350 (1962).
27. Somers, J. E., J. N. Kanfer and R. O. Brady, Proc. Soc. Expl. Biol. Med. *114*, 350 (1963).
28. Sherwin, A. L., J. A. Louden, and L. S. Wolfe, Canad. J. Biochem. *42*, 1640 (1964).
29. Kabat, E. A., and M. M. Mayer, "Experimental Immunochemistry," second edition, Charles C Thomas, Publisher, Springfield, Ill., 1961.

The Structure and Chemistry of Sulfatides

PIERRE J. STOFFYN, Harvard Medical School and McLean Hospital, Belmont, Massachusetts

Abstract

Recent advances in the chemistry of cerebroside sulfates which have led to a precise formulation of their structure and to a detailed knowledge of their composition are reviewed.

Qualitative and quantitative analyses of the constituent fatty acids and sphingosine-type bases of cerebrosides and sulfatides have shown a close relationship between the cerebrosides and their sulfate esters.

Sulfatides were converted into cerebrosides in high yield by mild desulfation with methanolic HCl at room temperature. The products of partial degradation of cerebrosides—ceramide and psychosine—were found to be identical to the products obtained under similar conditions from desulfated sulfatides.

By methylation and periodic acid oxydation, the sulfate group has been located conclusively and apparently only at C-3 of the galactose moiety in the sulfatide molecule.

A β-configuration of the galactosidic linkage in cerebrosides and in sulfatides has been demonstrated by direct comparison of the products of periodate oxidation of dihydropsychosine obtained from cerebrosides and from sulfatides, with the products of oxidation of 1-D-glyceryl β-D-galactoside of known configuration.

Investigations of the composition and structure of sulfatides abnormally accumulated in the brain and kidneys in cases of metachromatic leukodystrophy, a demyelinating disorder, are reported.

Introduction

SEVEN YEARS AGO Jatzkewitz (1) and Austin (2) identified as sulfatides the metachromatic substance (3) which accumulates abnormally in the central nervous system (4) and the kidneys (5) of patients afflicted with metachromatic leukodystrophy, a diffuse demyelinating disorder. This finding, confirmed many times since (6–9), has undoubtedly been largely responsible for the growing interest shown recently in the chemistry and biochemistry of the cerebrosides sulfuric esters and their relationship to other complex lipids with which they are associated, notably as constituents of the myelin membrane.

The present article is intended to summarize the advances which have been made lately in the chemistry of sulfatides, and to discuss some of the experimental data which are leading to a more accurate and detailed knowledge of the composition, the structure and the chemical properties of these sulfolipids.

A general review entitled "The Sulfolipids" and covering the different aspects of earlier work in the field has been published by Goldberg in 1961 (10).