ANTIBODY-INDEPENDENT COMPLEMENT ACTIVATION BY MYELIN VIA THE CLASSICAL COMPLEMENT PATHWAY*

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The possible participation of the complement (C)[1] system in the pathogenesis of various nervous disorders has been suggested in experimental animals and in man (1). Abnormal concentrations of C components have been detected in cerebrospinal fluid (CSF) from patients with systemic lupus erythematosus (2, 3) or degenerative neurological diseases such as multiple sclerosis (4) and subacute sclerosing panencephalitis (5). These C abnormalities have not always paralleled changes of immunoglobulin (Ig) G levels in CSF. It has been shown that liver DNA (6), myocardial mitochondria (7), certain lymphocytes, thymocytes (8), or killed kidney cells (9) can activate the C system directly without the participation of antibody. These reactions may be important pathologically in the induction of secondary nonspecific inflammation after injury to these organs.

The present study was undertaken to examine whether nervous tissues can also activate the C system directly. Activation of C following contact of sera with nervous tissue would provide a mechanism for inflammatory changes in various neurological disorders.

Materials and Methods

Experimental Animals. Inbred BALB/c mice were obtained from Cumberland View Farms, Clinton, Tenn. Unless otherwise stated, 3–6-mo-old healthy adult mice were used. New Zealand white rabbits weighing 3 kg were obtained from Dutchland Co., Denver, Pa.

Sera. Normal human sera (NHS) was obtained from healthy laboratory personnel. The presence or absence of C-reactive protein (CRP) was determined by immunoprecipitation (10). Agammaglobulinemic serum obtained from a patient with X-linked infantile agammaglobulinemia used in our study contained <10 μg/ml IgG, no IgM, and no IgA. Rabbit blood was obtained by cardiac puncture; mouse blood was obtained by retro-orbital puncture. The blood was allowed to clot for 30 min at room temperature, and sera obtained by centrifugation and stored in aliquots at −70°C.

Brain Homogenates. Animals were killed by cervical dislocation (mice) or by exsanguination (rabbits), and their brains were immediately removed and weighed. After washing in phosphate-

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[1] *Abbreviations used in this paper:* C, complement; CRP, C-reactive protein; CSF, cerebrospinal fluid; DFDNB, difluorodinitrobenzene; EAE, experimental allergic encephalomyelitis; EAN, experimental allergic neuritis; $GGVB^{++}$, glucose-gelatin-veronal-buffered saline with Mg^{++} and Ca^{++}; GVB, gelatin-veronal-buffered saline; MBP, myelin basic protein; NHS, normal human serum; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; TCH50, total hemolytic complement.

buffered saline (PBS), pH 7.4, the brains were homogenized in ice with a Teflon-glass homogenizer (Talboys Engineering Corp., Emerson, N. J.) driven at 1500 rev/min.

Material for Complement Titrations. Sheep erythrocytes were purchased from the Colorado Serum Co., Denver, Colo.; sheep hemolysin was from BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.; guinea pig C was from Texas Biological Laboratories, Ft. Worth, Tex.

Determination of Total Hemolytic C and C Components. Glucose-gelatin-veronal-buffered saline (pH 7.2) containing 1.0 mM Mg^{++} and 150 μM Ca^{++} ($GGVB^{++}$) and gelatin-veronal-buffered saline (GVB) were prepared as described previously (11). Total hemolytic complement (TCH50) was determined by a modified method described previously (12) using IgM hemolysin-sensitized sheep erythrocytes (1×10^8 cells/ml). Titration of C components (C1, C4, and C2) was performed using intermediate cells $EAC1^{gp}$ for C4, $EAC1^{gp}4^{hu}$ for C2, and $EAC4^{hu}$ for C1. Functionally pure C1 and C2 were prepared according to methods previously described (13). Guinea pig C diluted 1:25 in 0.04 M EDTA was used as a source of C3–C9. The components C3–C7 were each titrated using $EAC1^{gp}4^{hu}$ and guinea pig or human C components obtained from Cordis Laboratories Inc., Miami, Fla. C8 and C9 were titrated using stable intermediate cells $EAC1^{gp}4\text{–}7^{hu}$ (Cordis Laboratories).

EDTA in GVB (EDTA-GVB) and 10 mM EGTA containing 5 μM MgCl (Mg^{++}-EGTA-GVB) were used to deplete magnesium and or calcium ions, respectively.

Protein Determinations. Protein determinations were carried out according to the method of Lowry et al. (14).

Consumption of C by Brain Homogenates. Mouse or rabbit brain was dissected, homogenized at 4°C, and the protein concentration determined. Various dilutions in GVB^{++} of the brain homogenate were incubated with undiluted autologous serum or with normal human serum obtained from a healthy adult. The mixtures were incubated at 37°C for 30 min, centrifuged, and the residual C determined. The supernatants were also tested for C3 cleavage products by crossed immunoelectrophoresis according to the method of Weeke (15) using 1% agarose in barbital buffer at pH 8.6 containing 10 mM EDTA.

Preparation of Neuronal Cells, Glial Cells, and Myelin Fractions. Whole fresh mouse brains were finely chopped with a razor blade and transferred into ice-cold 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4. The minced tissue was then disrupted by passage through nylon mesh, and the suspension was sieved through mesh of descending pore size down to 50 μm. All steps were performed at 4°C. Neuronal cells, glial cells, and the myelin fraction were separated by the method of Blomstrand and Hamberger (16). The myelin enriched fraction was further purified by the procedure of Norton and Poduslo (17).

Isolation of Myelin Basic protein. Myelin basic protein (MBP) was extracted by either of the following methods. (*a*) This method was based on the procedure of Lim et al. (18) as modified by Golds and Braun (19). Briefly, isolated myelin fragments that contained ~10 mg of MBP were partially delipidated by two extractions with 10 ml of cold acetone. The pellet containing MBP was recovered by centrifugation for 10 min at 10,000 *g*. MBP was solubilized from the pellet by the addition of 0.1 N HCl and stirring for 1 h at 4°C. The acid-insoluble material was removed by centrifugation and MBP was precipitated from the supernatant by the addition of 10 ml of cold acetone. The MBP precipitate was collected by centrifugation at 10,000 *g* for 20 min at −20°C and residual acetone removed by aspiration. (*b*) MBP was also solubilized directly from isolated myelin fragments without prior delipidation by stirring with 10% acetic acid containing 0.4 M NaCl for 4 h at 4°C. After centrifugation at 10,000 *g* the supernatant was dialyzed for 48 h against three changes of PBS.

Cross-linking of Isolated MBP. The cross-linked form of MBP was obtained by two methods. One method was done according to Golds and Braun (20) and the other was a modified method of Golds (personal communication). In the method described by Golds and Braun (20), 1 mg/ml of MBP in PBS was incubated with 0.5 mg of difluorodinitrobenzene (DFDNB) (Sigma Chemical Co., St. Louis, Mo.) for 30 min at room temperature. The reaction was terminated by the addition of 3 vol of 1.0 M Tris-glycine, pH 9.0. After incubation for an additional 10 min, the cross-linked myelin fragments were recovered by centrifugation at 10,000 *g* for 10 min, washed three times with water, and resuspended in PBS. In the modified method 100 μg of purified MBP was reacted with 50 μg of DFDNB in 1 ml of acetone. The mixture was incubated

at room temperature for 30 min, and the reaction was terminated by the addition of 300 μl of 1 M glycine-Tris pH 9. After incubation for an additional 10 min, 1 ml of 50% trichloroacetic acid (TCA) was added and the mixture was left on ice for 15 min. After centrifugation the mixture was reacted with 1 ml of ether:ethanol (3:2) mixture at −20°C for 10 min and centrifuged. This latter procedure was repeated three times, and the residual protein, treated with ether (−20°C) for 10 min, was resuspended in PBS to its original concentration.

Results

Consumption of C by Brain Homogenates from Adult Rabbit, Adult Mouse, and Newborn Mouse. Fig. 1 presents the consumption of C after incubation of different concentrations of either mouse brain homogenate with NHS, or different concentration of rabbit brain homogenates with normal rabbit serum or NHS. As shown, the consumption of C by both homogenates was dose dependent. The degree of C depletion by rabbit brain homogenate was higher using rabbit serum. 6 mg protein/ml of mouse brain homogenate also reduced total hemolytic C by 37% in sera from a patient with agammaglobulinemia (<10 μg/ml IgG/ml) (data not shown).

To determine the time of appearance of the anticomplementary activity in mouse brain, homogenates of brain (6 mg/ml) of BALB/c mice at different ages were incubated with NHS. As shown in Fig. 2, brain homogenates of newborn mice did not consume C. The anticomplementary activity first appeared 7 d after birth and thereafter increased dramatically with age. Brain homogenates of newborn rabbits were not studied.

Kinetics and Temperature Dependence of C Activation by Brain Homogenates. Brain homogenates (6 mg protein/ml) of adult rabbits or mice were incubated with NHS and aliquots were removed at timed intervals and assayed for residual TCH50. As shown in Fig. 3, a maximum decrease of TCH50 occurred 30 min after incubation with either the mouse or rabbit brain homogenate. The consumption of TCH50 by 6 mg/ml of either mouse or rabbit brain homogenate was also temperature dependent

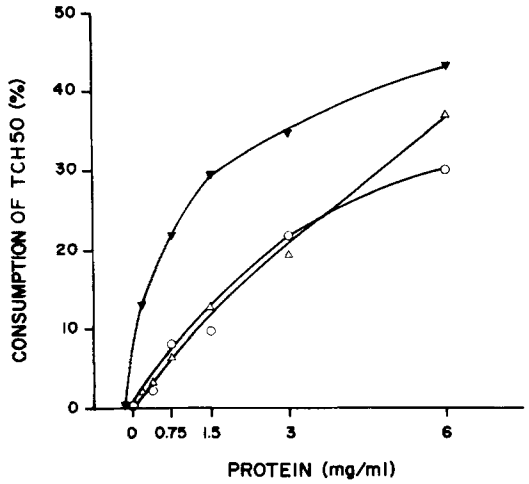

FIG. 1. Concentration-dependent depletion of TCH50 by brain homogenates. Different concentrations of BALB/c mouse brain homogenates (○) were incubated with NHS, or rabbit brain homogenates were incubated with NHS (△) or autologous serum (▼) at 32°C for 30 min. Residual TCH50 was then determined.

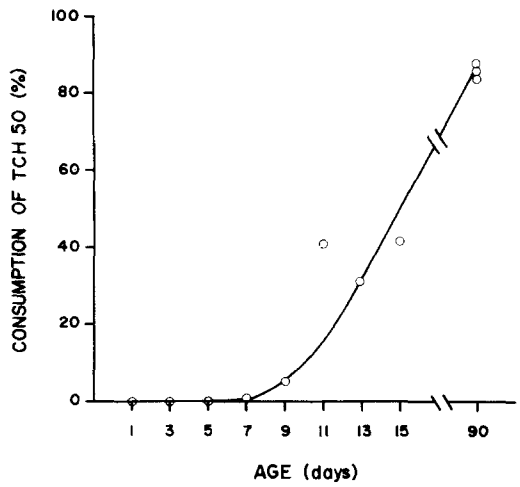

FIG. 2. Age-related appearance of anticomplementary activity in murine brains. Brain homogenates from BALB/c mice at various ages (12 mg/ml) were incubated with equal volumes of NHS at 37°C for 30 min, and residual TCH50 was determined.

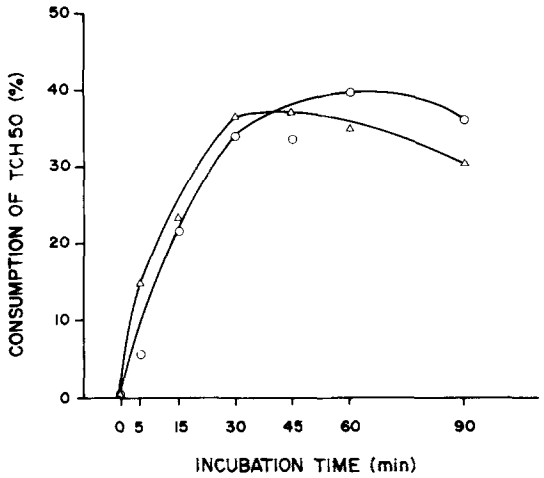

FIG. 3. Time-dependent depletion of TCH50 by brain homogenates. Brain homogenates (6 mg/ml in GVB^{++}) from BALB/c mice (○) and rabbit (△) were incubated with equal volumes of NHS at 37°C for various times, and residual TCH50 was determined.

(Fig. 4), and no significant differences were observed in the reactivity between the two homogenates at any temperature.

Physical Properties of Brain Homogenates. Mouse or rabbit brain homogenates were incubated at 50°C or 56°C for 30 min and then reacted with NHS. Controls consisted of NHS incubated with GVB^{++}. In another experiment, brain homogenates were subjected to freezing and thawing three or five times before testing for anticomplementary activity. Table I shows that the anticomplementary activity was heat labile at 56°C for 30 min, and that freezing and thawing 5 times reduced the anticomplementary activity of mouse and rabbit brain homogenate by 26 and 9%, respectively, of the control.

Consumption of Human C Components and Cleavage of Human C3 by Mouse Brain Homoge-

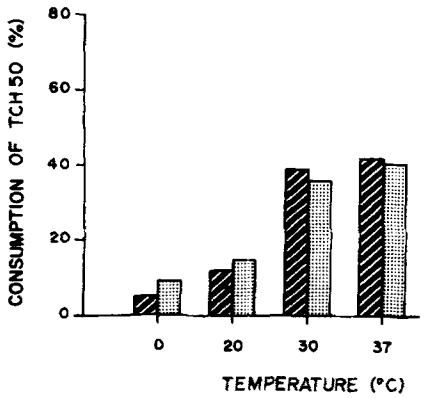

FIG. 4. Temperature-dependent depletion of TCH50 by brain homogenates. BALB/c (▨) and rabbit (▩) brain homogenates (6 mg/ml) were incubated with equal volumes of NHS for 30 min at various temperatures, and residual TCH50 was determined.

TABLE I

Effect of Temperature and Freezing and Thawing on Complement Depletion by Mouse and Rabbit Brain Homogenates

Pretreatment*	Decrease in anticomplementary activity‡	
	Mouse	Rabbit
	%	%
50°C 30 min	69	19
56°C 30 min	96	80
Freezing and thawing three times	5	0
five times	26	9

* Mouse and rabbit brain homogenate were treated as described, NHS added, and the mixtures incubated at 37°C for 30 min.

‡ Activities were compared with the TCH50 of NHS incubated with GVB^{++} under identical conditions.

nates. Fig. 5 presents levels of residual C components in human serum following incubation with mouse brain homogenates (12 mg/ml). As shown, each of the components from C1–C7 was markedly decreased. The later components C8 and C9 did not change as much as the earlier components.

C3 tested by crossed-immunoelectrophoresis showed a dramatic cleavage of C3 to C3d following incubation of NHS with 12 mg/ml brain homogenate (Fig. 6, panel A) when compared with C3 of NHS incubated with GVB^{++} for the same period of time (panel C). When the concentration of mouse brain homogenate was reduced to 6 mg/ml and incubated with NHS, native C3 as well as a C3b peak was observed (panel B).

Effect of Ca^{++} and Mg^{++} on Activation of Human C3 by Mouse and Rabbit Brain Homogenates. The requirements of Ca^{++} and Mg^{++} for activation of human C3 were studied next because C activation by the classical pathway requires Ca^{++} and Mg^{++}, and the alternative pathway is strongly influenced by Mg^{++} alone. Serum was chelated with 10 mM EGTA to bind Ca^{++} and to prevent classical C pathway activation, and then supplemented with 1 mM Mg^{++} to allow for alternative pathway activation. Brain homogenates incubated with unchelated serum (Table II) strongly

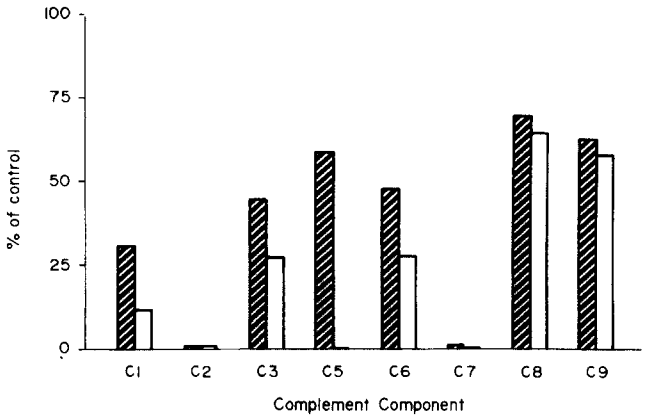

FIG. 5. Hemolytic complement component titers after incubation with BALB/c mice brain homogenates. Brain homogenates (12 mg/ml in GVB^{++}) were incubated with equal volumes of NHS at 37°C for 30 min (▨) or for 60 min (□) and functional C assays were performed.

activated C3 (89% using mouse brain and 100% using rabbit brain). No C3 activation occurred, however, when the same homogenates were incubated with NHS containing either Mg^{++}-EGTA or EDTA (Table II). This result indicates that the activation of C3 by brain homogenate occurs via the classical pathway.

Location of Anticomplementary Activity in Brain Tissue. Whole brain was separated into myelin, glial cell (astrocytes, oligodendrocytes, and microglia), neuronal cell, and cell nuclei fractions. After homogenization and adjustment to equal protein concentrations (1 mg/ml), the ability of each fraction to activate C in NHS was compared. As shown in Fig. 7, the summarized data of eight independent experiments, >70% of the anticomplementary activity was recovered in the myelin fraction; no significant anticomplementary activity was observed with the other fractions.

Anticomplementary Activity of MBP. The myelin fraction described above still contained several components. Murine myelin was further purified by the method of Norton and Poduslo (17). Approximately a third of the anticomplementary activity was still retained. The isolated murine myelin was then purified to obtain myelin basic proteins by two procedures. One procedure eliminates lipid components during isolation (19). The other technique does not involve the use of any organic solvent during isolation (19). The fractions of purified myelin, MBP, free myelin, and cross-linked MBP were brought up to 1 mg protein. MBP extracted by either of the two methods showed almost no anticomplementary activity (<5% reduction in TCH50). However, the myelin residues remaining after MBP extraction also possessed no anticomplementary activity (<3% reduction in TCH50). These results are shown in Fig. 8.

MBP, however, exists in nervous tissue as a dimer while extracted MBP is a monomer (19). Purified MBP was therefore dimerized with DFDNB and reassayed for anticomplementary activity. After polymerization, MBP was now anticomplementary (Fig. 8) and the specific activity per mg protein was higher than in purified myelin. Polymerized MBP-free myelin and DFDNB used as controls were not anticomplementary (data not shown). Fractionated 3-d-old mouse brain (myelin and cellular fractions) also exhibited no ability to activate C (data not shown).

In addition to the above experiments we used murine (C57Bl/6) MBP purified by

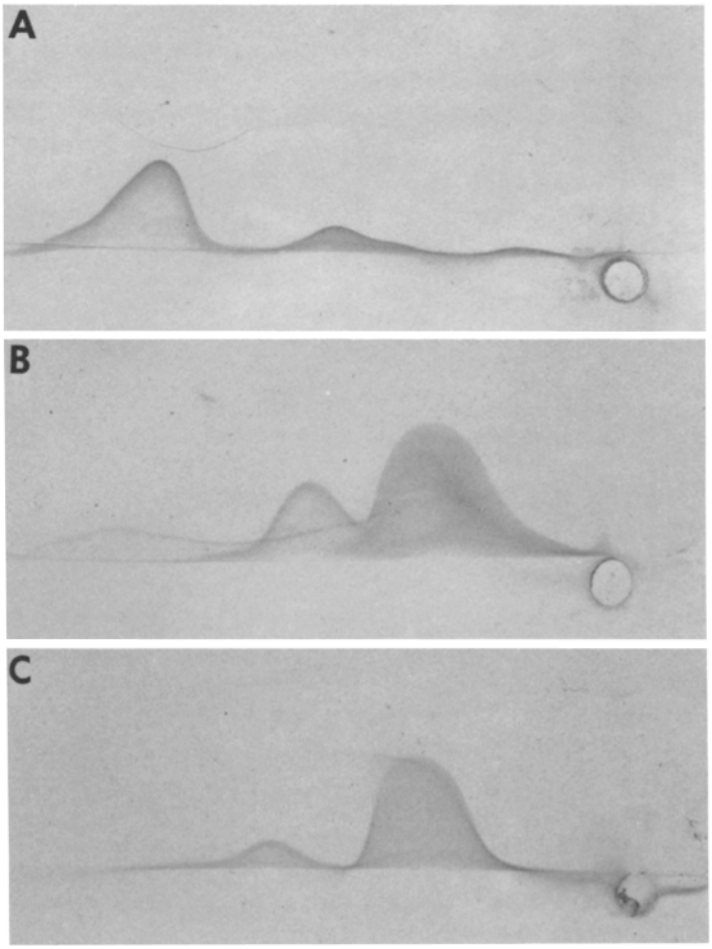

FIG. 6. C3 activation by BALB/c mice brain homogenate. NHS was incubated with an equal volume of (A) 12 mg/ml or (B) 6 mg/ml brain homogenates or with GVB^{++} (C) for 30 min at 37°C. The sera were then subjected to cross immunoelectrophoresis to locate C3 cleavage products. The anode is to the left.

Dr. E. Barbarese. Dr. Barbarese has identified four proteins that are antigenically related to the myelin basic protein designated prelarge (mol wt 21,500), large (18,500), presmall (17,000), and small (14,000) (21). We tested a mixture of 14K and 18.5K MBP. The purity of the two proteins we used is shown in Fig. 9. As presented in Table III, the purified MBP (14K and 18.5K) was not anticomplementary. After dimerization according to the modified method of Golds (personal communication), complement was activated by >95%.

Discussion

Myelination of the postnatal murine central nervous system occurs in at least two stages (22). The first stage, at 8–15 d of age, involves the proliferation of oligodendroglial cells. During the second stage, at 16–25 d of age, histologically recognizable

TABLE II

Effect of Calcium and Magnesium Ion on C3 Depletion by Mouse and Rabbit Brain Homogenates

Brain source	Buffer	Mode of action		C3 titer after incubation	Percent reduction of C3 activity
		Classical	Alternative		
BALB/c	Diluted GVB^{++}	+	+	260	88
	Mg-EGTA	−	+	3812	5
	EDTA	−	−	3742	6
Rabbit	GVB^{++}	+	+	0	100
	Mg EGTA	−	+	3659	5
	EDTA	−	−	4583	5
Buffer alone	GVB^{++}	+	+	2173	—
	Mg-EGTA	−	+	3600	—
	EDTA	−	−	3936	—

NHS diluted 1:3 by GVB^{++}, Mg^{++}-EGTA, or EDTA was incubated with 6 mg protein/ml brain homogenate at 37°C for 30 min.

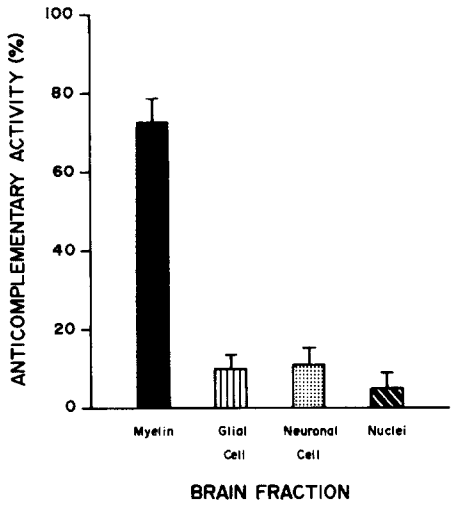

FIG. 7. Localization of anticomplementary activity in murine brain tissue. Whole BALB/c mice brain homogenates were fractionated on a sucrose-ficoll discontinuous gradient, and each fraction was assayed for anticomplementary activity. The percentage of total anticomplementary activity in each fraction is plotted on the graph.

myelin can be observed (17). Our data clearly indicate that the ability of the murine brain to activate complement very closely parallels the pattern of myelination.

Brain homogenates from both mouse and rabbit were shown to possess anticomplementary activity. Following brain fractionation, the activity was localized in myelin. It was further demonstrated that polymerized MBP but not monomeric MBP was anticomplementary. Dimerization of MBP by the DNDFB method has been extensively studied by Golds and Braun (20). The reaction depends on the concentration of both the reagent and protein. In addition to reaction with amino groups on the protein, the reagent can react with sulfhydryl imidizole tyrosine-hydroxyl groups, but only the amino group modification is stable. By the above method, 20% of the MBP

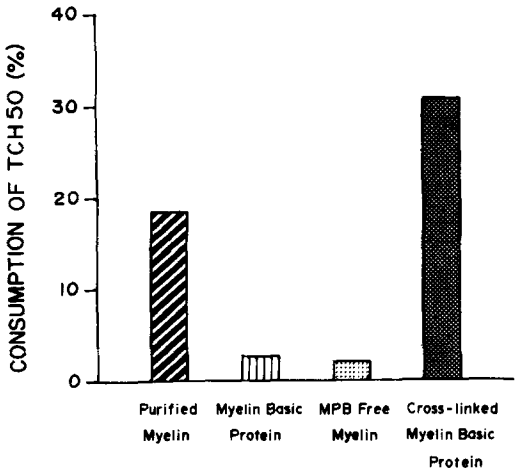

FIG. 8. Anticomplementary activity of myelin and MBP. The ability of purified myelin, purified myelin basic protein, residues of myelin after extraction of MBP, and cross-linked myelin basic protein to reduce the TCH50 of NHS was determined.

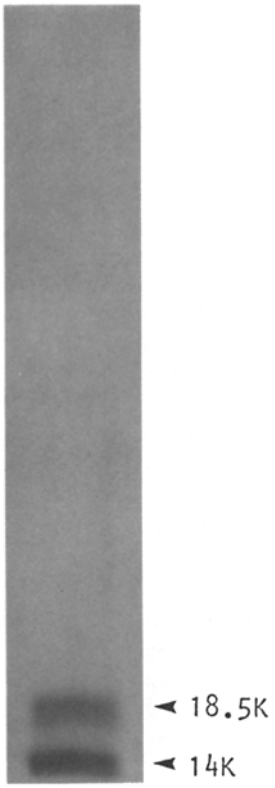

FIG. 9. Sodium dodecyl sulfate-polyacrylamide gel of murine MBP (14K and 18.5K).

TABLE III

Consumption of Total Hemolytic Complement Using Purified Murine MBP

	Total hemolytic C
	CH50 U/ml
NHS	43
MBP monomer (18.5K and 14K)	46
MBP cross linked	5

MBP (50 μg in 50 μl PBS) was reacted with 50 μl of 1:5 dilution of NHS at 37°C for 30 min, and the residual TCH50 was then tested. Control NHS treated under identical conditions contained 50 μl of PBS.

is dimerized and small amounts of higher heteropolymers are also produced (20). Whether it is the MBP dimer per se or other alterations of the MBP produced by DNDFB that account for the activation of the C system by treated MBP will have to be answered by subsequent experiments.

Siegel et al. (23) have reported that isolated, naturally occurring polycations including MBP activate C only in the presence of CRP. The sera we tested for activation of C did not have demonstrable CRP by the method used. However, because small amounts of CRP are present in normal blood, participation by CRP in the activation of C by myelin cannot be absolutely excluded. It seems possible that CRP may polymerize MBP or change its conformation so that it can readily activate the C system.

Current hypotheses concerning the pathogenesis of postinfectious and postvaccinal disorders of the nervous system focus on the role of the cellular immune system (24). Such hypotheses have developed largely as a result of the clinical and histopathological similarities between these human disorders and experimental allergic neuritis (EAN) and experimental allergic encephalomyelitis (EAE) in laboratory animals. Both EAN and EAE appear to result from delayed hypersensitivity to MBP. In addition, both EAE and EAN follow sensitization with nervous system antigen in the presence of Freund's complete adjuvant but not in the presence of incomplete adjuvant. The mechanism whereby a wide variety of infections, immunizations, and vaccinations induce similar sensitization to MBP has not, to date, been satisfactorily explained.

The histopathological features of postinfectious diseases of the nervous system have been recently reviewed (24). It was postulated that perivascular demyelination may also result from vascular injury alone, in the absence of delayed hypersensitivity reactions. The recent detection of antigen-antibody complexes circulating in the sera of patients with a variety of neurological disorders (25, 26) suggests a pathologic mechanism whereby the complexes induce a primary vascular lesion that, in turn, leads to demyelination. The present study suggests that neuronal tissue necrosis resulting in the exposure and/or release of MBP can lead to activation of the C system in the absence of autoantibodies. Since MBP is detected in sera and cerebral spinal fluids after brain injury (27) and in patients with multiple sclerosis (28), such C activation may be responsible, at least in part, for the development of acute inflammation, magnification of the damaged area, and the occurrence of vascular spasms following brain hemorrhage.

Summary

Murine or rabbit whole brain homogenates were shown to activate human complement via the classical pathway by an antibody-independent reaction. This activity

required Ca^{++} ions. Anticomplementary activity in fractionated murine brain was found to reside in the myelin fraction and in purified myelin. It was absent, however, both from highly purified myelin basic protein (MBP) and from the MBP-free residue. Because purified MBP is a monomer and this protein exists in brain tissue largely as a dimer, the ability of the cross-linked form of MBP to activate complement was investigated. MBP, dimerized with difluorodinitrobenzene, was highly anticomplementary. The murine brain, inactive when taken from the newborn mouse, was shown to first acquire the capacity to activate complement at 7 d after birth. This finding is consistent with the report that the synthesis of myelin protein has been shown to be initiated in murine brain 8 d after birth. Complement activation by MBP could play an important role in the pathological changes observed in neurological disorders.

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