Proc. Natl. Acad. Sci. USA
Vol. 75, No. 3, pp. 1554–1558, March 1978
Medical Sciences

Degradation of basic protein in myelin by neutral proteases secreted by stimulated macrophages: A possible mechanism of inflammatory demyelination

(plasminogen activator/plasmin/cell-mediated immunity/urokinase/protease inhibitors)

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Communicated by Harry Eagle, December 27, 1977

ABSTRACT **In inflammatory demyelinating diseases such as multiple sclerosis and experimental allergic encephalomyelitis, myelin destruction occurs in the vicinity of infiltrating mononuclear cells. The observations that myelin can be altered prior to phagocytosis and in areas not contiguous with inflammatory cells suggests a common mechanism for the initial stages of demyelination. Because stimulated macrophages secrete several neutral proteases, including plasminogen activator, we have investigated the possibility that myelinolysis could be mediated directly or indirectly by these enzymes. Isolated myelin was incubated with conditioned media from cultures of thioglycollate-stimulated mouse peritoneal macrophages in the presence and absence of plasminogen. Myelin appeared to be vulnerable to attack by at least two proteolytic activities secreted by the macrophages, a plasminogen-dependent and a plasminogen-independent activity; of the major proteins in myelin, the basic protein was most susceptible. The direct myelinolytic activity of macrophage-conditioned media was abolished by EDTA, and the plasminogen-dependent hydrolysis was abolished by *p*-nitrophenylguanidinobenzoate, an inhibitor of plasminogen activator and plasmin. These results suggest that the plasminogen activator released by the stimulated macrophages generated plasmin which hydrolyzed basic protein in intact myelin. This interpretation was confirmed by the observation that urokinase, a plasminogen activator, in the presence of plasminogen brought about marked degradation of basic protein in myelin. We propose that the release of neutral proteases by stimulated macrophages involved in cell-mediated reactions, and its amplification by the plasminogen-plasmin system, may play a significant role in the demyelination observed in several inflammatory demyelinating diseases.**

The classification "demyelinating diseases" is now reserved by most neuropathologists and neurologists exclusively for the acquired inflammatory diseases of myelin in which there is loss of myelin with sparing of axons (1, 2). The most extensively studied disorders in this category are multiple sclerosis (MS) and the model disease, experimental allergic encephalomyelitis (EAE). These diseases are characterized by perivascular infiltration of hematogenous cells in association with areas of demyelination (1, 2).

Lesion formation in inflammatory demyelinating diseases is generally believed to be related to an immunological response, either autoimmune or the result of a viral infection. However, the mechanism by which the inflammatory cells, predominantly macrophages and lymphocytes in MS and EAE, participate in the process of demyelination has not yet been defined. Most attention has been directed toward acid proteases and lysosomal hydrolases which increase in and around MS plaques (3–9) and in lesions in animals with EAE (10–15). It has been established that mouse peritoneal macrophages stimulated by thioglycollate broth or by products of activated lymphocytes secrete several neutral proteases (16–19), including plasminogen (Plg) activator (20–24, 18). Such extracellular proteases generated from macrophages activated as a consequence of a reaction of sensitized T-lymphocytes with antigen, if capable of degrading myelin proteins, could initiate a process of myelin destruction common to inflammatory and cell-mediated immune reactions occurring in nervous tissue. We have investigated the feasibility of such a mechanism, by studying the proteolytic activity of products of activated macrophages on purified myelin.

MATERIALS AND METHODS

Reagents. Materials were obtained from the following suppliers: porcine Plg, Sigma Chemical Co., St. Louis, MO; glycine, Matheson, Coleman and Bell, Norwood, OH; urokinase (EC 3.4.99.26) (approximately 1.2 Ploug units/μg), Calbiochem, La Jolla, CA; thioglycollate broth and lactalbumin hydrolysate, Difco Laboratories, Detroit, MI; Dulbecco's medium and fetal calf serum, Grand Island Biological Co., Grand Island, NY. In most experiments, immediately before use the Plg was pretreated with Sepharose (Pharmacia, Piscataway, NJ) conjugated to lima bean trypsin inhibitor (Worthington Biochemical Corp., Freehold, NJ) (25) in order to remove traces of free plasmin. *p*-Nitrophenylguanidinobenzoate (NPGB) was a gift from D. Rifkin and can be purchased from ICN Nutritional Biochemicals, Cleveland, OH.

Preparation of Supernatants (Conditioned Media) from Macrophages. Unstimulated and thioglycollate-stimulated macrophages were obtained from Oxford Swiss mice and were cultivated at 1–2 × 10^7 cells per flask in Dulbecco's medium plus 5% acid-treated fetal bovine serum for 48 hr before the preparation of conditioned medium in Dulbecco's medium plus 0.1% lactalbumin hydrolysate (10 ml per flask) (18). Supernatants T_x and T_y (both stimulated) were collected after 3 days, centrifuged to remove cellular debris, and kept frozen (−20°). Other preparations of conditioned media were collected at different intervals, dialyzed against 10 mM Tris, pH 7.5/1 mM $CaCl_2$, and lyophilized. The supernatants were assayed for Plg activator by measuring Plg-dependent fibrinolysis on ^{125}I-labeled fibrin-coated plates (18). Samples were dissolved in $\frac{1}{10}$ volume of distilled water before use.

Incubation of Myelin with Macrophage-Conditioned Media. Purified myelin (26) was stored as a lyophilized powder

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Abbreviations: Plg, plasminogen; BP, myelin basic protein; MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; NaDodSO$_4$, sodium dodecyl sulfate; NPGB, *p*-nitrophenylguanidinobenzoate.

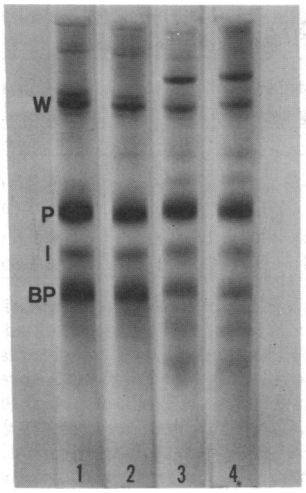

FIG. 1. Myelin proteins separated by polyacrylamide gel electrophoresis after incubation of myelin with a supernatant from stimulated macrophages in the presence and absence of Plg. Protein bands: W, Wolfgram protein; P, proteolipid protein; I, intermediate proteins; and BP, basic protein. Myelin (1 mg) was incubated for 45 min at 37° in a total volume of 220 μl containing 18 mM Tris·HCl (pH 7.5) plus the following amounts of supernatant and Plg: gel 1, none; gel 2, 5 μg of Plg; gel 3, 50 μl of supernatant T_2; gel 4, 50 μl of supernatant T_2 plus 5 μg of Plg.

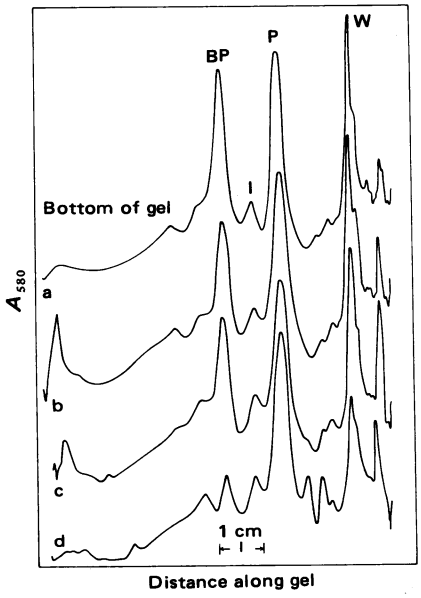

FIG. 2. Spectrophotometric scans of stained myelin proteins on polyacrylamide gels after incubation of myelin with a stimulated macrophage supernatant alone or plug Plg. The gels were scanned in the Gilford spectrophotometer at 580 nm. Curves: a, myelin alone; b, 50 μl of supernatant T_x; c, 5 μg of Plg; and d, 50 μl of supernatant T_x plus 5 μg of Plg. Myelin protein bands are labeled as in Fig. 1.

at −20° over $CaSO_4$. Immediately prior to its use in incubation mixtures, the myelin was suspended in cold distilled water in a Dounce homogenizer, to yield 10 mg of myelin per ml of suspension. Incubation mixtures contained 10–36 mM Tris·HCl (pH 7.5), macrophage-conditioned medium (15–75 μl) or urokinase with or without 2.5–5 μg of Plg (as indicated for each experiment), and 100 μl of the myelin suspension in a total volume of 220 μl. The incubations were for 30–60 min at 37°, and the reactions were stopped by addition of 2 mg of solid sodium dodecyl sulfate ($NaDodSO_4$) and boiling for 2 min. Two control incubation takes containing only myelin, Tris, and water and two control tubes containing myelin, Tris, Plg, and water were included in every experiment.

Gel Electrophoresis of Myelin and Analysis of Myelin Proteins. Polyacrylamide gels (15%) were prepared and electrophoresis was carried out according to the method of Greenfield *et al.* (27), modified by including 1% $NaDodSO_4$ instead of 0.1% in the gels. A 50-μl portion of each incubated sample in $NaDodSO_4$ was added to 50 μl of a sample solvent (27) containing 1% $NaDodSO_4$ and 2% 2-mercaptoethanol, and the 100-μl mixtures, containing 80–100 μg of protein (28), were electrophoresed for 18 hr at 45 V. The fast green-stained gels were scanned in a Gilford spectrophotometer, and the areas of the individual myelin protein bands were quantitated by triangulation (27).

Calculation of Protein Degradation. The areas of the four major myelin protein bands were summed, and the area of the myelin basic protein (BP) band was divided by the sum. The ratio of BP to total protein (approximately 0.40) in the control samples containing myelin incubated without supernatants or Plg was taken as 100% BP. In the other samples, the respective ratios of BP to total protein were subtracted from the control values (e.g., 0.40), and this difference divided by the control value was taken as percentage BP degraded.

Decreases in the total myelin protein recovered were primarily accounted for by the losses in BP. Qualitatively, the only other protein bands in which decreases could be perceived after incubation with supernatants and Plg were the Wolfgram protein and the small intermediate protein which was not always resolved from the large intermediate protein (29). Because the Wolfgram and small intermediate bands each account for only about 10% of the total myelin protein, changes in their magnitudes could not be quantitated with sufficient accuracy. Therefore, calculated values are presented only for the percentage BP degraded.

RESULTS

Degradation of BP by Macrophage Supernatants Plus Plg. The principal proteins of myelin are the Wolfgram protein (molecular weight, 55,000), the proteolipid protein (24,000), the intermediate proteins (~20,000), and the BP (18,000) (30). Fig. 1 gives a qualitative indication of the changes occurring in the protein pattern on $NaDodSO_4$/polyacrylamide gels of myelin that had been incubated alone or with conditioned medium from stimulated macrophages in the presence or absence of Plg. After incubation of the myelin with macrophage supernatant alone (gel 3), the band for BP diminished significantly compared to myelin incubated without enzymes (gel 1). Incubation with supernatant plus Plg gave greater breakdown of BP (gel 4) than occurred with the supernatant alone (gel 3). Incubation with purified Plg along (gel 2) had little effect. Faint stained bands of degradation products can be seen below the BP band in gels 3 and 4.

Quantitative differences among the protein bands on stained gels became more obvious in the densitometric scans of gels from another experiment (Fig. 2). Macrophage-conditioned medium had the ability to cause some degradation (39% in this experiment) of BP directly (curve b), and this observation was consistent with subsequent findings, suggesting that BP was vulnerable to attack by neutral proteases in the macrophage-conditioned media. In the presence of unpurified Plg alone (curve c), 23% of the BP was degraded. However, preferential loss of BP was most dramatic when both Plg and conditioned medium were present (curve d; 77% BP degraded), suggesting that the macrophages were secreting a Plg activator (20, 22, 18) capable of converting Plg to plasmin and that plasmin was

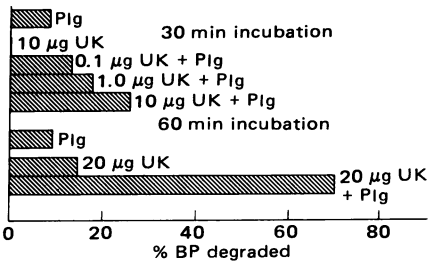

FIG. 3. Effect of plasmin on BP. Incubation tubes contained 34 mM Tris·HCl (pH 7.6) plus 5 μg of Plg and/or 0.1–20.0 μg of urokinase (UK) in a total volume of 120 μl. After a 15-min preincubation at 37°, 100 μl of a bovine myelin suspension (10 mg/ml) in water was added. The tubes were incubated at 37° for 30 or 60 min, as indicated.

highly effective at hydrolyzing BP. (In curve d, the height of the Wolfgram band also appeared to be diminished.)

Degradation of BP by Plasmin. To verify that this sequence of events was plausible, myelin was incubated with purified urokinase, the human Plg activator, together with Plg. As shown in Fig. 3, myelin BP was indeed highly vulnerable to proteolysis by plasmin. The amount of BP degraded was dependent upon the quantity of urokinase used and the incubation time; with 20 μg of urokinase, a maximum loss of 70% of the BP was obtained in a 1-hr incubation in the presence of 5 μg of Plg. The preincubation of urokinase, Plg, and buffer, prior to addition of myelin, was later found to be unnecessary for both urokinase and conditioned media, indicating that the activation of Plg occurred much more rapidly than breakdown of BP.

Comparison of Supernatants from Resting and Stimulated Macrophages. It had previously been demonstrated that thioglycollate-stimulated peritoneal macrophages, but not resting macrophages, from mice secrete various neutral proteinases (16–18), including Plg activator (18, 20, 22), and it was therefore of interest to compare the myelinolytic effects of conditioned media derived from stimulated and from resting peritoneal macrophages. The results of such experiments confirmed the existence of both Plg-dependent and Plg-independent activities capable of degrading BP in myelin, as illustrated for stimulated macrophages in Fig. 4. The proteolytic activities were dose-dependent, although under the conditions used it was not possible to decrease the BP band by greater than 70%.

A summary of the data from several preparations of conditioned media (Table 1) shows that both proteolytic activities were usually greater in supernatants from thioglycollate-stimulated macrophages than in those from resident macrophages, although supernatant U_2 had levels of both BP degradative activities similar to those of its counterpart, T_2, from stimulated cells. Comparisons of the myelinolytic activity of the conditioned media with the fibrinolytic data given in the legend to Table 1 indicate a greater susceptibility of myelin in

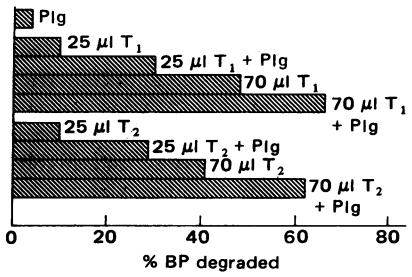

FIG. 4. Quantitation of BP degradation by macrophage supernatants in the absence and presence of Plg. Incubation tubes contained 10 mM Tris·HCl (pH 7.6) and 1.0 mg of myelin suspended in water plus 5 μg of Plg and/or macrophage supernatants in a total volume of 220 μl. The tubes were incubated at 37° for 30 min.

Table 1. Rate of degration of BP (nmol/hr per 100 μl of supernatant)

	Supernatants from resting cells			Supernatants from stimulated cells	
	Plg-indep.	Plg-dep.		Plg-indep.	Plg-dep.
U_A	0.25 ± 0.35 (2)	0.8 ± 1.1 (2)	T_A	2.1 ± 0.5 (4)	4.1 ± 1.8 (2)
U_B	0 ± 0 (2)	1.8 ± 2.5 (2)	T_B	2.1 ± 0.1 (4)	5.2 ± 0.28 (2)
U_1	2.1 ± 1.2 (5)	3.4 ± 1.9 (2)	T_1	6.3 ± 1.4 (5)	5.6 ± 3.5 (2)
U_2	5.2 ± 1.3 (4)	3.4 ± 1.2 (2)	T_2	5.4 ± 1.0 (3)	3.6 ± 2.5 (4)

Plg-dependent and Plg-independent degradation of BP in myelin by supernatants from resting and thioglycollate-stimulated peritoneal macrophages. Unstimulated (U) and thioglycollate-stimulated (T) macrophages were cultivated for 2 days before preparation of conditioned medium in Dulbecco's medium plus 0.1% lactalbumin hydrolysate. U_A, U_B, T_A, and T_B were collected at 2 days; U_1 and T_1 and U_2 and T_2 were consecutive 3- and 4-day collections. One hundred microliters of reconstituted conditioned medium represented the product of 2×10^6 macrophages and contained fibrinolytic activity as follows: U_1 and U_2, 2.3–4.2 units for Plg-dependent and -independent activity; T_1 and T_2, 176 and 85 units for Plg-dependent and 14 and 6.4 units for Plg-independent activity, respectively. The data from various experiments, similar to those illustrated in Fig. 4, with different aliquots and incubation times were used to calculate the values in the table. Percentages of BP degraded were converted to nmol on the basis of: bovine brain myelin is 25% protein (20); the molecular weight of BP is 18,000 (30); and BP constitutes 40% of the protein in myelin. The Plg-dependent rates were obtained by subtracting the rates of BP degradation by supernatant alone and by Plg alone from the rate of BP degradation during incubation with the supernatant plus Plg. The data are recorded as means ± SD with numbers of experiments in parentheses.

suspension than of fibrin monolayers to both Plg-dependent and Plg-independent proteolytic activities: whereas the fibrinolytic data show a large difference between supernatants from stimulated and unstimulated cells, the differences in myelinolysis (comparing U_1 and U_2 to T_1 and T_2) are less striking.

Use of Inhibitors to Distinguish between Plg Activator and Other Neutral Proteases. The observation of neutral protease activity in macrophage-conditioned media capable of degrading BP in the absence as well as the presence of Plg suggested that the degradation of myelin was effected by more than one proteolytic enzyme. NPGB, a specific inhibitor of both Plg activator and plasmin (18), and EDTA, which inhibits other macrophage-secreted neutral proteases (18), were used to assess the relative contributions of the Plg-dependent and -independent mechanisms. The Plg-independent breakdown of BP in myelin was markedly inhibited by the presence of EDTA (Fig. 5). However, when Plg was added to conditioned media in the presence of EDTA, the Plg-dependent degradation of BP was found to be unaffected, remaining comparable to the Plg-dependent increment produced in the absence of inhibitors. Conversely, NPGB substantially decreased the Plg-dependent increment in BP degradation, whereas the direct proteolytic activity of the macrophage-conditioned medium alone was not affected. The simultaneous addition of both NPGB and EDTA virtually abolished BP degradation by macrophage-conditioned medium in the presence or absence of Plg. The degradation of BP in myelin by Plg-dependent as well as EDTA-sensitive neutral proteases secreted by stimulated macrophages was consistently found in experiment with nine preparations of conditioned media prepared by two separate laboratories. Furthermore, macrophage supernatants plus Plg were effective in degrading BP in brain myelin prepared from the three species tested—cow, cat, and rabbit. In additional experiments, purified BP isolated from myelin was found also to be sensitive to proteolytic degradation by macrophage-conditioned medi-

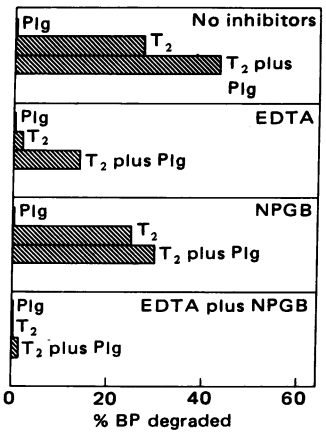

FIG. 5. Effects of EDTA and NPGB on myelin incubations with macrophage supernatants and Plg. Each tube contained 1 mg of bovine myelin, 19 mM Tris·HCl (pH 7.6), and 50 μl of macrophage supernatants and/or 5 μg of Plg, as indicated, in a total volume of 210 μl. The presence of 10 mM EDTA or 2 μg of NPGB in the incubation mixtures is shown at the right. Tubes were incubated for 45 min at 37°.

um, and the rate of proteolytic digestion was significantly increased by the inclusion of Plg in the reaction mixture.

DISCUSSION

The hypothesis underlying these experiments is that myelin may be disrupted, either directly or indirectly, by enzymes secreted by inflammatory cells, particularly macrophages. The data presented demonstrate that the BP in myelin is susceptible to at least two proteolytic activities secreted by macrophages, a Plg-independent activity that can be inhibited by EDTA and a Plg-dependent activity that can be inhibited by NPGB. Plasmin, produced by the action of urokinase, a purified Plg activator, on Plg, was also highly effective at degrading myelin BP.

Stimulated mouse macrophages secrete various neutral proteases, including Plg activator (20–23), collagenase (16, 19, 31), and elastase (17). Although the Plg-independent myelinolysis may have been produced directly by one of these proteases, the possible sequential activation of latent enzymes by macrophage-secreted enzymes, as has been demonstrated for a latent collagenase (32), precludes certain identification of the Plg-independent protease activity. There was a general parallel between Plg-dependent fibrinolytic and myelinolytic activities, but there were occasional quantitative discrepancies in the levels of the two activities that might be related to time of collection of the conditioned media and differences in sensitivity of the two assays.

In previous studies the secretion of macrophage neutral proteases has been related to the state of activation of the cells. Stimulation of macrophages by thioglycollate (16, 17, 20), priming with endotoxin and challenge with latex particles (21), or immunologic sensitization and challenge (33–35) result in macrophages that secrete greater levels of neutral proteases. These findings have been confirmed as well in the myelinolytic system, where thioglycollate-induced macrophages produced 3–6 times more proteolytic activity than did unstimulated cells. However, the myelinolytic assay is so sensitive that even uninduced macrophage-conditioned media contain easily demonstrable protease activity.

Because of the predominance of macrophages in lesions of inflammatory demyelinating disease such as MS and EAE, it has been suspected that such cells may be involved in degradation of myelin as well as in the final clearance of myelin debris by phagocytosis. Many studies have shown that acid proteases and other lysosomal enzymes are increased around active MS plaques (3–9, 36, 37) and in the central nervous system in EAE (10–15). The increase in acid proteases in plaques correlates with the decrease in BP (5, 6) which is known to be readily digested by proteases (13, 38, 39). However, neutral and acid protease activities were elevated to a similar degree in the spinal cord of rats with EAE (40).

We believe that secreted neutral proteases are likely to be a more significant factor than lysosomal enzymes in early stages of demyelination for several reasons. In morphologic studies of MS and EAE, disruption of myelin is seen to occur prior to identifiable phagocytosis. In EAE, macrophages can be seen penetrating and stripping the myelin lamellae (41, 42), and extensive vesicular disruptions of the sheath are commonly seen in the vicinity of mononuclear cells (41, 43, 44). A similar morphological picture is seen in the early stages of demyelination occurring as a "bystander effect" to a delayed hypersensitivity reaction to nonbrain antigens occurring in the vicinity of myelinated fibers (45, 46). In MS, macrophages are not seen engaged in active stripping of myelin from axons; rather, the early changes are disintegration of the sheath into lamellar and granular debris (47, 48). These observations support the view that the proteases act extracellularly. The level of activity of lysosomal hydrolases, if released extracellularly, is likely to be low because they are relatively inactive at physiologic pH.

A mechanism for extracellular degradation of myelin would predict the finding of myelin deficient in BP in lesions and the occurrence of myelin fragments in the cerebrospinal fluid of patients undergoing inflammatory demyelination. Both phenomena have been reported in MS. A decreased content of BP in MS myelin has been reported (49, 50) and was more pronounced in myelin isolated from plaques than from normal-appearing white matter (48). Recent studies show that there is serologically detectable BP-like material in the cerebrospinal fluid of MS patients and that the amount of BP or its fragments assayed by radioimmunoassay correlates with the clinical stage of the disease (51, 52). Of particular significance for the present studies are the data that show that the antigen in cerebrospinal fluid may not be intact BP but rather a fragment of it (52).

A model for inflammatory demyelination consistent with the present and previous observations would propose that sensitized lymphocytes, with specificity either for exogenous antigens (such as viruses) or for brain antigens in the case of EAE are activated by those antigens in the vicinity of myelinated fibers. As a consequence, products of activated lymphocytes are secreted which both increase chemotaxis for mononuclear cells and activate the macrophages attracted to the site to secrete neutral proteases. Some macrophage-secreted proteases might act to degrade myelin directly where there is close contact. A second aspect of the mechanism would be the amplification of the macrophage-produced proteolytic activities by interaction with zymogens, such as Plg, present in serum and tissue spaces. Plasminogen serves as an enormous reservoir in serum of potential proteolytic activity which could be activated locally to plasmin by Plg activator secreted by activated macrophages. Although serum proteins are normally excluded from the nervous system, Plg would be freely available to the brain parenchyma in any situation in which the blood/brain barrier is damaged, which is known to occur in inflammatory reactions. The amount of protease generated from Plg could be sufficient to overcome the high levels of natural protease inhibitors. The relative specificity of these reactions for causing demyelination

would lie in the remarkable vulnerability of myelin BP to proteases, which has been amply documented (38, 53–56) and which we have further demonstrated here. Although plasmin has been shown here to be capable of degrading BP in myelin, it is a key enzyme in a number of cascade systems including the coagulation and complement systems and thus could be involved in further amplifying both the inflammatory and the proteolytic components of such reactions.

The mechanism of demyelination initiated by macrophage-secreted proteases proposed here suggests several strategies for intervention to decrease the degree of demyelination. If demyelination is caused by one or more extracellular proteases, then inhibitors of these enzyme activities, currently available or to be developed, offer the possibility of limiting the extent of demyelination. In addition, secretion of Plg activator by activated macrophages is known to be markedly inhibited by dexamethasone and inducers of cyclic AMP (23). Thus, the extent of demyelination might be restricted by pharmacologic agents that control enzyme secretion by these cells. Further studies of inhibitors of macrophage protease production and activity will be of importance both for more clear definition of the mechanisms underlying demyelination *in vivo* and to provide new ways to control these processes.

We thank Mrs. Lesley Bieler, Miss Renee Sasso, and Miss Pat Clarke for excellent assistance in this study, Dr. Daniel Rifkin for a gift of NPGB, Dr. John Whitaker for kindly furnishing us with a preprint of his work, and Dr. Zena Werb for her helpful criticisms. This work was supported by National Multiple Sclerosis Society Grants 1089 and 1006, by Grants NS-02476, NS-03356, AI-07118, and AI-10702 from the U.S. Public Health Service, and by a grant from the Sloan Foundation.

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