

Glioblastoma Infiltration into Central Nervous System Tissue in Vitro: Involvement of a Metalloprotease

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Abstract. Differentiated oligodendrocytes and central nervous system (CNS) myelin are nonpermissive substrates for neurite growth and for cell attachment and spreading. This property is due to the presence of membrane-bound inhibitory proteins of 35 and 250 kD and is specifically neutralized by monoclonal antibody IN-1 (Caroni, P., and M. E. Schwab, 1988. *Neuron* 1:85-96). Using rat optic nerve explants, CNS frozen sections, cultured oligodendrocytes or CNS myelin, we show here that highly invasive CNS tumor line (C6 glioblastoma) was not inhibited by these myelin-associated inhibitory components. Lack of inhibition was due to a specific mechanism as the metalloenzyme blocker 1,10-phenanthroline and two synthetic dipeptides containing metalloprotease-blocking sequences (gly-phe, tyr-tyr) specifically impaired C6 cell spreading on CNS myelin. In the presence of these inhibitors, C6 cells were affected by the IN-1-sensitive inhibitors in the same manner as control cells, e.g., 3T3

fibroblasts or B16 melanomas. Specific blockers of the serine, cysteine, and aspartyl protease classes had no effect. C6 cell spreading on inhibitor-free substrates such as CNS gray matter, peripheral nervous system myelin, glass, or poly-D-lysine was not sensitive to 1,10-phenanthroline.

The nonpermissive substrate properties of CNS myelin were strongly reduced by incubation with a plasma membrane fraction prepared from C6 cells. This reduction was sensitive to the same inhibitors of metalloproteases. In our in vitro model for CNS white matter invasion, cell infiltration of optic nerve explants, which occurred with C6 cells but not with 3T3 fibroblasts or B16 melanomas, was impaired by the presence of the metalloprotease blockers. These results suggest that C6 cell infiltrative behavior in CNS white matter in vitro occurs by means of a metalloproteolytic activity, which probably acts on the myelin-associated inhibitory substrates.

IN the normal adult central nervous system (CNS)¹ cell mobility is extremely restricted and nerve fiber growth or regeneration over distances of >1 mm is almost absent. Several factors may contribute to this situation, which is so different from that in most peripheral organs: the blood-brain barrier restricting access of blood-borne cells to the CNS; the very dense nature of CNS tissue; and the presence of specific inhibitors for neurite growth and cell mobility. Such inhibitory constituents have been recently found as specific membrane proteins in the cell membranes of one type of CNS glial cells, the oligodendrocytes, and in their product, myelin (Caroni and Schwab, 1988a; Schwab and Caroni, 1988). They probably play an important role in the lack of regenerative neurite growth in the CNS of higher vertebrates.

In the case of malignant, invasive CNS tumors (mainly glioblastomas) infiltration of grey and white matter areas of the CNS is observed (Mørk et al., 1984). The specific

mechanisms allowing this infiltrative behavior in the CNS tissue have not been elucidated so far. Of particular interest is the interaction of the tumor cells with the myelin-associated inhibitory substrate components, which have been shown in vitro to inhibit not only nerve fiber growth but also fibroblast attachment and spreading (Caroni and Schwab, 1988a).

In vivo, detailed cell biological analysis is difficult because of the heterogeneity of most tumors and the complex local tissue, vascular, and inflammatory reactions that occur (Mørk et al., 1984). In vitro models successfully reproducing the inhibitory microenvironment of CNS white matter with regard to neurite growth and cell attachment have recently been described (Schwab and Thoenen, 1985; Schwab and Caroni, 1988; Savio and Schwab, 1988). In the present study we used as a model the rat C6 glioblastoma line (Benda et al., 1968), which is known to rapidly infiltrate CNS tissue in vivo (Auer et al., 1981; Yoshida et al., 1986). For the study of cell attachment and spreading, C6 cells were confronted with cultured oligodendrocytes, myelin, and purified neurite growth inhibitors. For the study of cell migration and infiltration, C6 cells were added to CNS frozen sections and optic nerve explants. Our results point to the crucial role of a

1. *Abbreviations used in this paper:* Cbz, benzyloxycarbonyl; cm, carboxymethyl; CNS, central nervous system; PLYS, poly-D-lysine; PNS, peripheral nervous system; TIMP, tissue inhibitor of metalloproteases.

membrane-associated metalloprotease for C6 motility in CNS white matter in all these experimental paradigms.

Materials and Methods

Cell Cultures

Rat C6, mouse NIH 3T3, and B16 cells were cultured in DME, supplemented with 10% FCS, usually to, at most, 70–80% confluency. Cells were harvested with a short trypsin treatment (0.1% in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's medium for 90 s), stopped by addition of FCS in excess, followed by centrifugation (1,000 rpm, 6 min). Cells were resuspended in either DME/FCS or defined serum-free medium (MEM α) and used for the experiments. Dissociated rat CNS glial cells were prepared starting from optic nerves of 6–7-d-old Lewis rats as described (Schwab and Caroni, 1988) and plated into poly-D-lysine (PLYS)-coated wells (100 mm², 100 μl medium) at a density of 20,000 cells per well. The culture medium was an enriched L15 medium with 5% rat serum, penicillin, and streptomycin. C6, 3T3, and B16 cells were added to 2-d-old cultures at a concentration of 30,000 cells per well, incubated for 2 h, and fixed with warm 4% formalin in 0.1 M phosphate buffer, pH 7.4, 5% sucrose. Inhibitory oligodendrocytes were identified by double labeling using the specific antibodies O1 and O4 (Sommer and Schachner, 1981; Schwab and Caroni, 1988).

Preparation of Nerve Explants for Infiltration Assay

Optic nerve and sciatic nerve explants were prepared as described (Schwab and Thoenen, 1985). Briefly, the nerves were rapidly dissected from ~8-wk-old male rats, cleaned from the meninges, frozen and thawed three times using liquid nitrogen, and placed under a teflon ring (13 mm diam, 1 mm thick) sealed to a culture dish with silicon grease. Two chambers connected only by the explants were obtained this way. 300,000 C6, 3T3, or B16 cells were plated in the inner chamber in DME/FCS and incubated for 5–20 d. The medium was changed every other day. Cultures were fixed overnight with 4% formalin. The nerve explants were mounted with Tissue-Tek (Reichert-Jung, Nussloch, Federal Republic of Germany); 10–15- μm sections were cut in a cryostat and collected on gelatine-coated coverslips. After drying at room temperature overnight the sections were stained in 0.75% cresyl violet, and evaluated. We counted the infiltrated cells starting from the tip of the nerves at which they were added. Each point represents the numbers of infiltrated cells per 0.7 μm . Only the most central part of the nerves was considered (0.25 mm), where the explants presented the best histology. Inhibition experiments were performed with nerve explants previously injected from both sides with 2 μl of 3 mM cbz-tyr-tyr or cbz-ala-phe solutions.

CNS Frozen Sections and Myelin as Substrates

Adult rat cerebellum frozen sections were prepared and dried on glass coverslips (Savio and Schwab, 1989). 70,000 C6, 3T3, or B16 cells in 100 μl were added to each well containing slices previously rinsed with cold DME/FCS. Cultures were incubated for 2 d at 37°C. Cultures were then fixed and stained with cresyl violet (Savio and Schwab, 1989). Three to four cerebellum slices were used per point per experiment, each experiment being repeated at least two times.

Myelin from rat spinal cord (CNS) or sciatic nerve (peripheral nervous system [PNS]) purified on a discontinuous sucrose gradient (Colman et al., 1982; Schwab and Caroni, 1988) was dried overnight onto PLYS-coated wells (20 μg protein per well of 100 mm² surface). Unbound membranes were removed by three washes with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's solution. Myelin-coated wells were immediately used in substrate testing assays by the addition of 9,000 cells (C6, 3T3, or B16) per cm². Alternatively, we used extracted CNS myelin protein, or SDS-PAGE-purified 35- and 250-kD inhibitory proteins reconstituted in liposomes (Caroni and Schwab, 1988a). Experiments were scored at different time points using a phase-contrast microscope equipped with a photcamera. Quantifications were done using a surface integration program; three arbitrary fields containing at least 25 cells were photographed for each well at 80 \times . Each point represents the mean of at least three wells \pm SEM. Results are expressed as μm^2 of projected cell surface, or as spreading, which was calculated by subtracting from the projected surface value of a spreading cell the surface value of a completely spheric cell.

C6 Plasma Membranes and Conditioned Medium Preparation

C6 cells grown to 80% confluency were washed twice with Hank's medium, and harvested in 20 ml 8.5% sucrose, 50 mM NaCl, 10 mM Tris buffer, pH 7.4 using a rubber policeman. After mechanical homogenization through a series of needles of decreasing size, a low purity plasma membrane fraction was obtained by centrifugation (5 min at 3,000 g, 10 min at 8,000 g, and then 2 h at 100,000 g). A higher purity fraction was isolated by loading the material on a discontinuous sucrose gradient, containing 50 mM NaCl, 10 mM Tris, pH 7.4 (Quigley, 1976). 20–40% sucrose interphase (C6 plasma membranes fraction) and 40–60% sucrose interphase (C6 mitochondrial fraction) were collected, washed in Hank's medium, and resuspended in MEM α .

Conditioned media were obtained by cultivating 80% confluent C6 cell cultures for 1 d in MEM α . The medium was then collected and centrifuged for 10 min at 3,000 g. In some experiments the conditioned medium was concentrated 10 times using Centricon 10 microconcentrators (model Centricon 10, Amicon Corp., Danvers, MA).

Treatment of CNS Myelin with C6 Plasma Membranes

CNS myelin-coated PLYS wells were prepared as described in the previous section, but instead of being immediately tested as substrate, they were first incubated with 50 μl of C6 plasma membranes (containing 0.8 mg protein/ml MEM α) at 37°C for 30 min. Dishes were then rinsed twice with Hank's medium and immediately used as substrates for 3T3 cells. In some experiments protease blockers were added to the membranes using 10 \times solutions.

Results

C6 Glioblastomas but Not 3T3 Fibroblasts or B16 Melanomas Infiltrate Optic Nerve and CNS White Matter In Vitro

Frozen optic nerve and sciatic nerve explants were placed under a teflon ring and sealed with silicon grease (Schwab and Thoenen, 1985). C6 or 3T3 cells were plated into the ring, in contact with one end of the nerve explants. Culture medium was exchanged every other day, and after 5–20 d of incubation the nerves were fixed, and sectioned with a cryotome. Infiltrated cells were recognized by cresyl violet staining. PNS explants supported diffuse infiltration of both, C6 and 3T3 cells (Fig 1, *c* and *d*). C6 cells were present in the explants at higher density. In the optic nerve explants a different situation emerged (Fig. 1, *a* and *b*); 3T3 cells did not infiltrate the nerves, with the exception of very few cells which migrated along blood vessels (Fig. 1 *b*, *arrow*). On the other hand, C6 cells infiltrated deep into the optic nerves with a diffuse pattern, reaching a maximum distance of ~3 mm from the entry point in 14 d (migration rate: ~0.2 mm per day).

As an alternative model adult rat cerebellum frozen sections were used as a culture substrate for C6, B16, or 3T3 cells (Savio and Schwab, 1989). The highly metastatic B16 melanoma cells were found to clearly discriminate between the substrate qualities of the gray and white matter with regard to cell attachment, spreading, and migration. In fact, B16 cells (exclusively attached and spread on gray matter regions and, even if plated at high cell densities, they did not attach on or migrate into white matter areas of the sections (Fig. 2, *e* and *f*). The same picture emerged for 3T3 cells, which formed dense monolayers on gray matter, but not on white matter (Fig. 2, *c* and *d*). Much in contrast to B16 and 3T3 cells, C6 cells were found frequently on white matter, and extensive spreading of these cells was observed on gray as

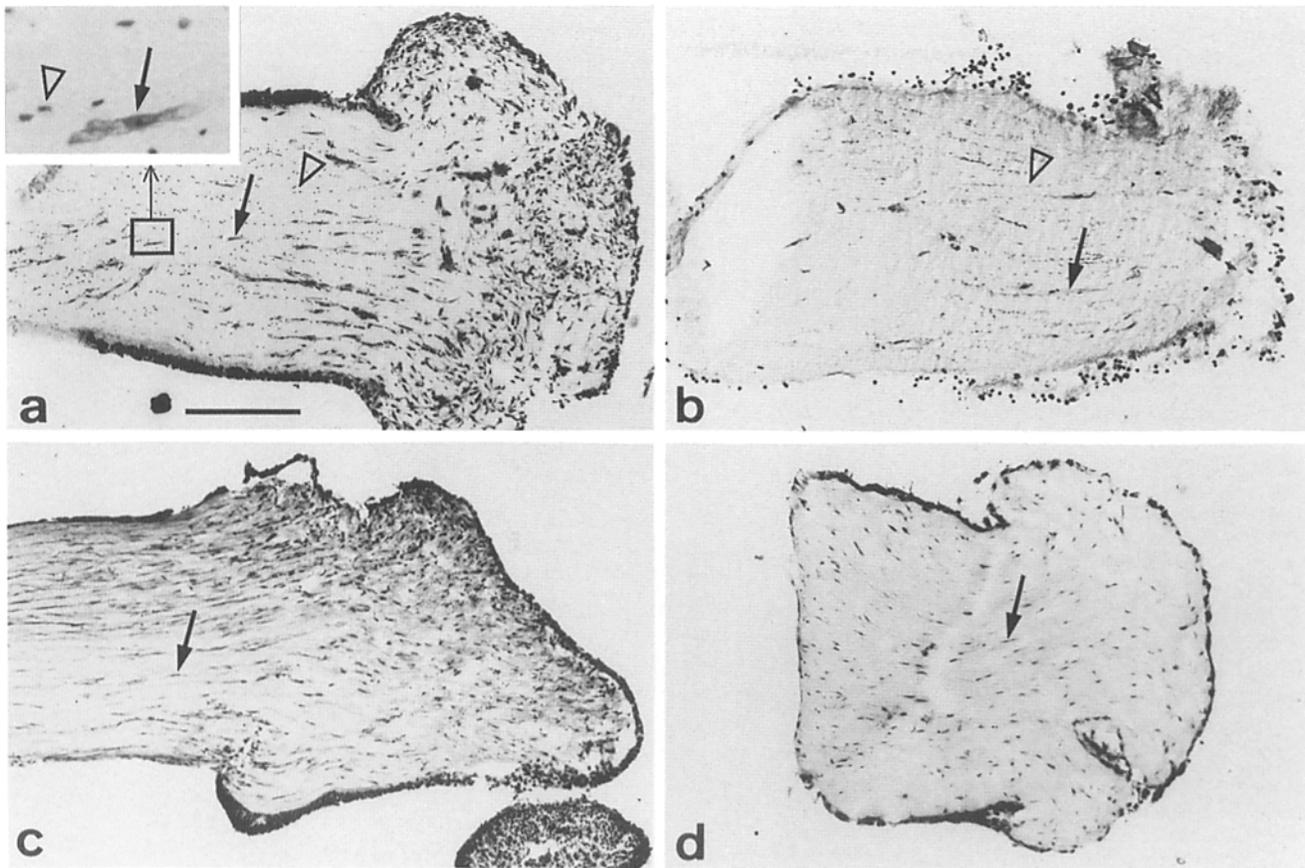


Figure 1. C6, but not 3T3 cells, infiltrate optic nerve explants. Phase-contrast microphotographs of 10- μ m frozen sections of rat optic (a and b) or sciatic (c and d) nerve explants, after 2-wk incubation with C6 (a and c) or 3T3 (b and d) cells. Cells were added to one tip of the explants. Infiltrated cells (arrow in a–d) can be seen after cresyl violet staining in both sciatic nerves (c and d), but only for C6 cells in the optic nerve (a). (Inset in a) An enlargement of the boxed area, showing the large size of the migrating C6 cells in comparison to the nuclei of optic nerve glial cells (arrowheads in a and b). The arrow in b points to few 3T3 cells adjacent to blood vessels. Bar, 0.2 mm.

well as on white matter (Fig. 2, a and b). In some cases we found that C6 cells were denser on the white matter than on the molecular layer of the gray matter, where they often formed little aggregates which spread with difficulty.

Glioblastoma Cell Spreading Is Not Inhibited by CNS Myelin

CNS myelin has been shown to be a highly nonpermissive substrate for neurons, neuroblastoma cells, astrocytes, and 3T3 cells (Schwab and Caroni, 1988). This property is due to the presence of two membrane proteins (35 and 250 kD), which inhibit cell attachment and growth cone and lamellipodia mobility (Caroni and Schwab, 1988a). The spreading behavior of C6 glioblastomas on CNS myelin adsorbed to PLYS-coated wells was compared with that of B16 melanomas and 3T3 fibroblasts. B16 melanoma reaction to a CNS myelin substrate strongly resembled that of 3T3 fibroblasts: spreading of both cell types was inhibited by CNS myelin (Figs. 3 and 4). The differences between cells on CNS myelin or on PLYS persisted also with prolonged incubation times (up to 1 d). On PLYS or PNS myelin 3T3 cells attached to the sub-

strate in the first 30 min, then immediately started to increase in size and flatten out. Numerous processes could be seen forming in all directions (Fig. 3 b). About 3 h after plating, 3T3 cells started to produce polarized lamellipodia, typical for migrating cells (Fig. 3 h). Interestingly, on CNS myelin 3T3 cells initially attempted to spread in the usual way (Fig. 3 e), but in a short time the typical sequence of spreading steps was retarded, the main processes retracted, and further spreading was strongly inhibited (Fig. 3 l). B16 cell spreading on purified CNS myelin was also strongly impaired in a way similar to 3T3 cells (Fig. 3, c, f, i, and m). C6 glioblastomas on the other hand were always slightly retarded at the beginning by CNS myelin, but in <2 h no difference was observed between cells on CNS myelin or on PLYS (Fig. 3, a, d, g, and k). Quantification of cell spreading confirmed these observations (Fig. 4): 3T3 or B16 cells spreading on CNS myelin was strongly impaired, whereas C6 cell spreading was slightly reduced at the beginning (90 min), but no further appreciable differences were detected at later time points.

As the nonpermissive substrate nature of white matter and CNS myelin have been shown to be due to two membrane

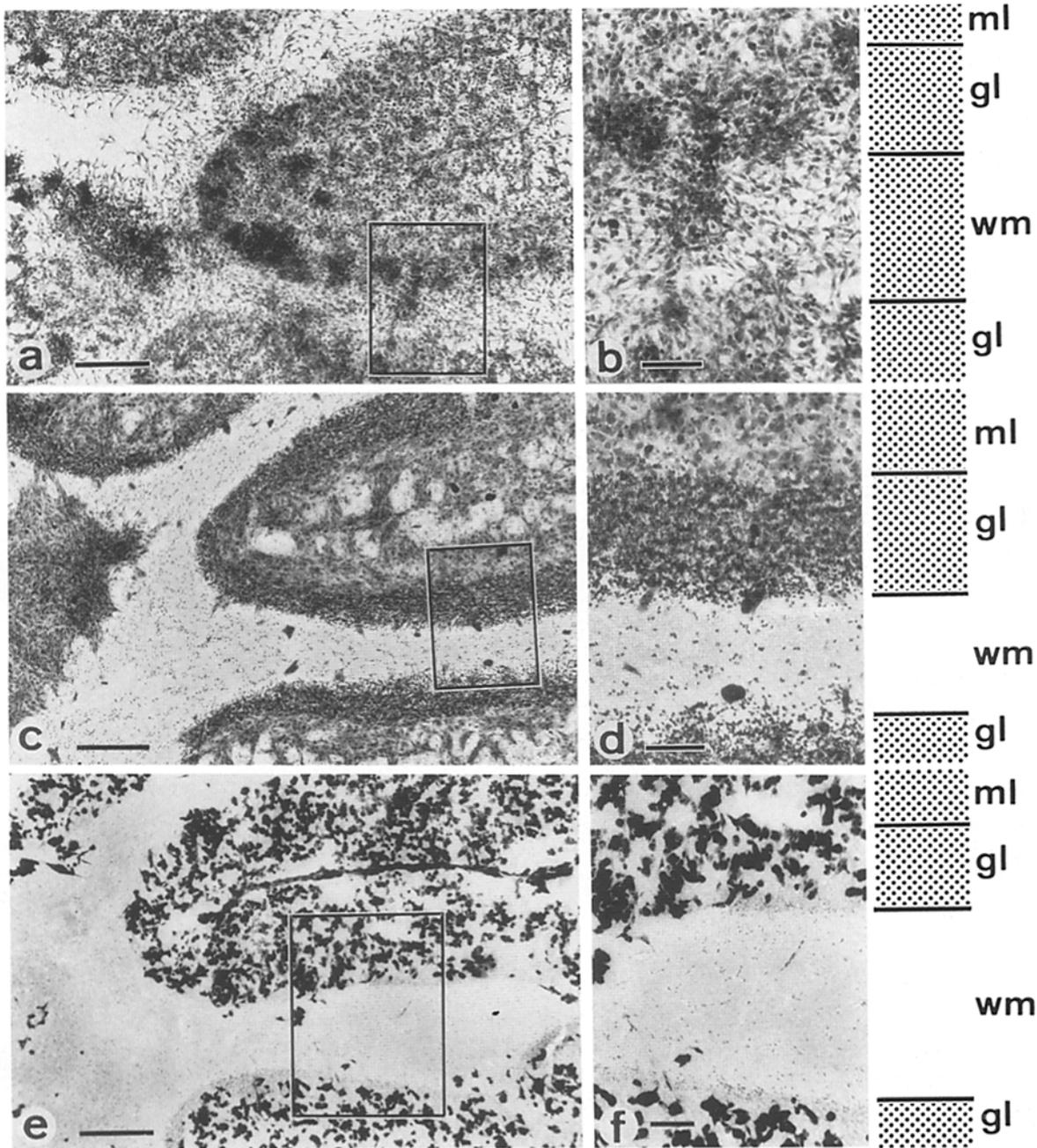


Figure 2. C6, but not 3T3 or B16 cells attach and spread on CNS white matter of rat cerebellar frozen sections. Phase-contrast micrographs of rat cerebellar frozen sections (25 μ m) on which C6 (*a* and *b*), 3T3 (*c* and *d*), or B16 (*e* and *f*) cells were cultured for 2 d. *b*, *d*, and *f* are enlargements of the boxed areas in *a*, *c*, and *e*, respectively. A clear difference on white matter (*wm*) emerges for 3T3 and B16 cells compared with C6 cells. The extent of the different cell monolayers is represented by the dotted areas. *gl*, granular layer; *ml*, molecular layer. Gray matter is composed of granular and molecular layer. Bars: (*a*, *c*, and *e*) 0.3 mm; (*b*, *d*, and *f*) 0.1 mm.

proteins of oligodendrocytes and myelin (Caroni and Schwab, 1988*a*), C6 cells were confronted with the SDS-PAGE-purified inhibitors (35 and 250 kD) reconstituted in liposomes, and also with living, cultured oligodendrocytes. Again 35- and 250-kD liposomes strongly inhibited 3T3 cell spreading, but they did not impair C6 cell spreading; C6 cells adhered and rapidly assumed the well-spread characteristic "fried egg" appearance also on these reconstituted CNS myelin fractions (not shown).

Living oligodendrocytes from dissociated optic nerves extend large radial process networks over two days in culture (Fig. 5, *a* and *c*). These cells and processes are strictly avoided by growing neurites (Schwab and Caroni, 1988) or 3T3 cells (Fig. 5 *b*), also at high 3T3 plating density. After plating C6 glioblastomas into such cultures, however, the oligodendrocytes were rapidly and extensively covered by the spreading cells, so that in the phase-contrast picture the oligodendrocytes were difficult to recognize. Overgrowth of

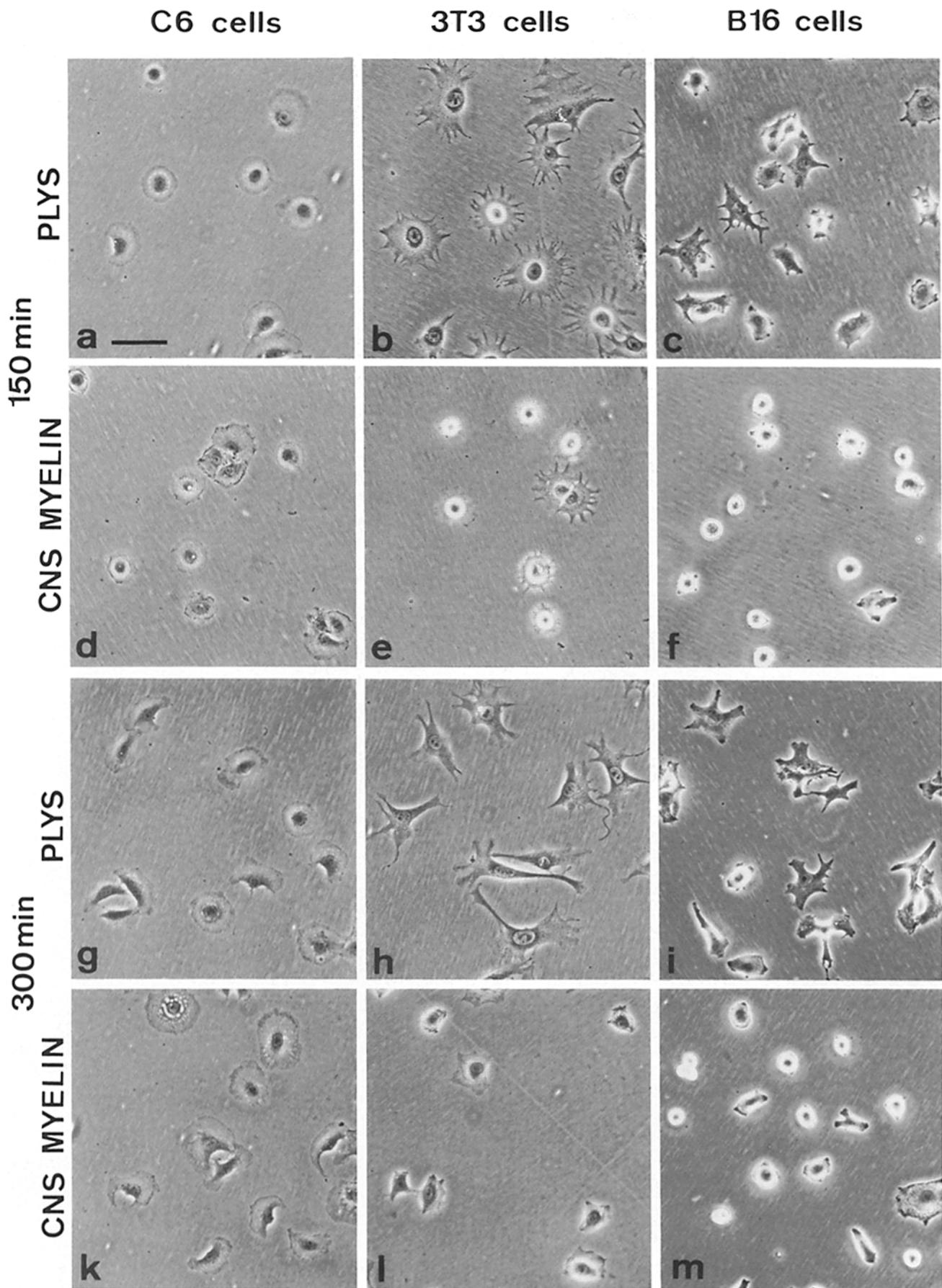


Figure 3. 3T3 and B16 cells are inhibited in their spreading on CNS myelin, but C6 cells are not impaired. Morphology of C6, 3T3, and B16 cells cultivated on either PLYS or on CNS myelin. Micrographs were taken at two different time points: 150 or 300 min after plating. Bar, 50 μ m.

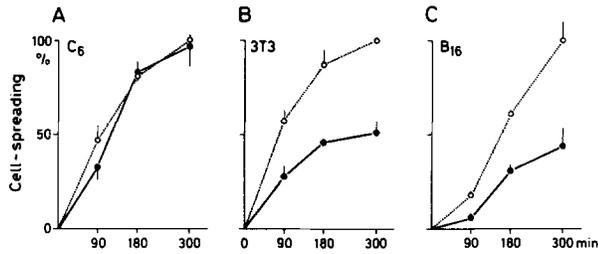


Figure 4. C6 cells overcome the inhibitory substrate property of CNS myelin. Spreading of C6 (a), 3T3 (b), and B16 (c) cells on PLYS or CNS myelin. Spreading is calculated as described in Materials and Methods using micrographs similar to those of Fig. 3. 0% spreading, round cells; 100% spreading, average value at 300 min. The experiment was repeated three times. (○) PLYS; (●) CNS.

oligodendrocytes was also observed at low densities of C6 cell plating (Fig. 5 d).

Specific Blockers of Metalloproteases Inhibit C6 Cell Spreading on CNS Myelin

In the past years different proteolytic activities were shown to be increased in tumorigenic cell lines (Matrisian et al.,

1986; Mignatti et al., 1986), in primary tumor explants (Mullins and Rohlich, 1983), or in transformed cells (Quigley, 1976; Mehdevi and Hynes, 1978; Chen et al., 1984; Wilhelm et al., 1987). In many cases it was also shown that the proteases were membrane associated. Protease treatment was shown to be able to destroy the inhibitory substrate property of myelin and white matter (Caroni and Schwab, 1988a). To test the possible involvement of proteases in C6 behavior, we investigated the effect of inhibitors of proteases on C6 cell spreading on either CNS myelin or PLYS. Cysteine-, serine-, and aspartyl protease blockers at the adequate concentrations had no discernible effect on C6 spreading on CNS myelin (Table I). The specific metalloprotease blocker 1,10-phenanthroline on the other hand, resulted in a strong inhibition of C6 spreading specifically on CNS myelin: 1,10-phenanthroline inhibited C6 spreading on myelin up to 67% after 150 min in culture (Table I). Not one of the blockers tested showed a significant effect on C6 cell spreading on PLYS. 1,10-phenanthroline is a general metalloprotease inhibitor due to its property of metal ion chelator. However, inhibition by this substance is not sufficient to define a proteolytic activity, since other metallo-dependent enzymes are also inhibited. Many other inhibitors of metalloproteases have been found, but they usually turned out not to be as general as 1,10-phenanthroline. Phosphoramidon (Komiya et al., 1975), bestatine (Umezawa et al., 1976),

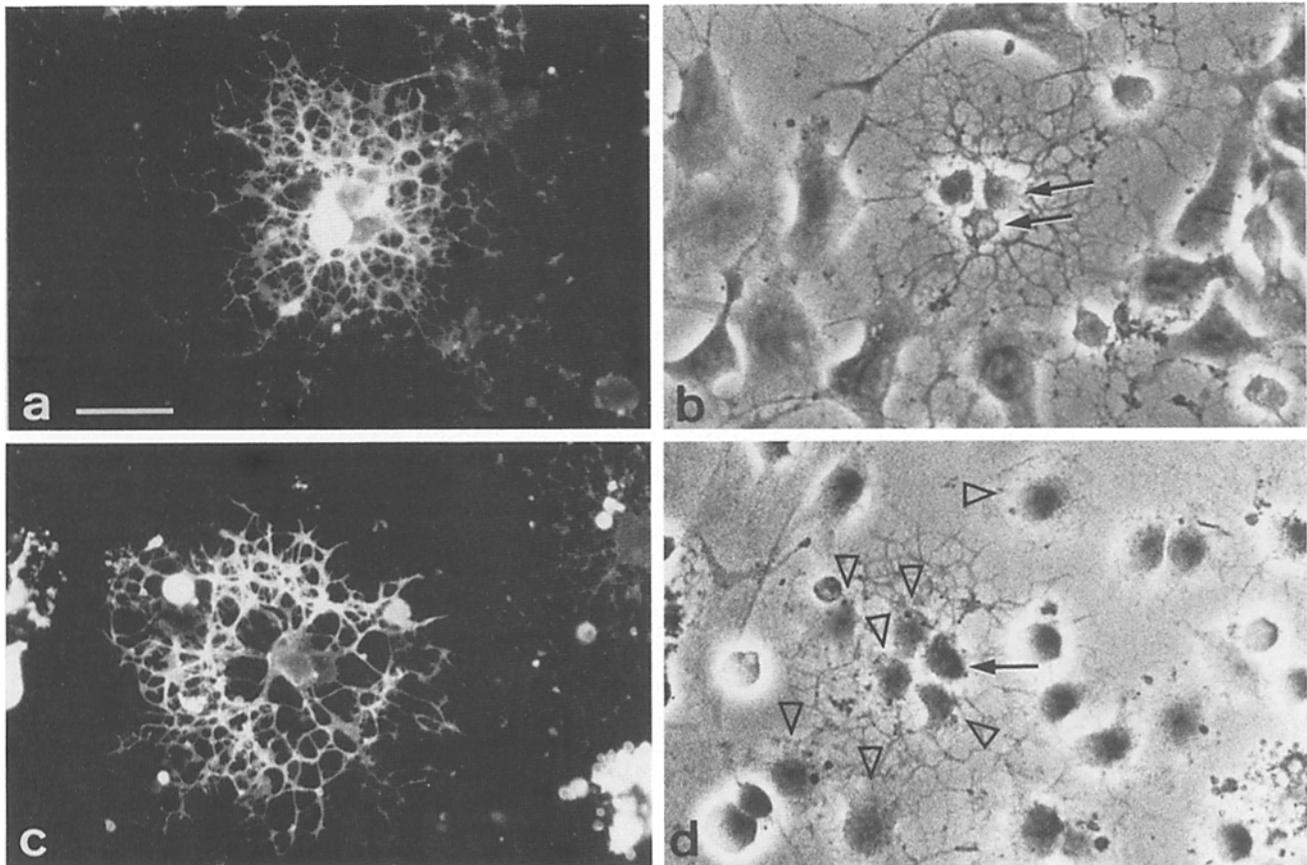


Figure 5. Highly branched oligodendrocytes are avoided by 3T3 cells, but not by C6 cells. 3T3 (a and b) or C6 cells (c and d) were added to 2-d-old rat optic nerve cell cultures. Arrows point to the cell bodies of highly branched, inhibitory oligodendrocytes (HBO). C6 cells (arrowheads in d) spread on the processes of an HBO. These processes were completely avoided by 3T3 cells (b). Oligodendrocytes were identified by immunofluorescence staining with antibody O1 (a and c). Bar, 30 μ m.

and the tissue inhibitor of metalloprotease (TIMP; Cawston et al., 1981) did not impair C6 cell spreading (Table I). Carboxymethyl-phe-leu (cm-phe-leu), a modified peptide with high affinity for enkephalinase (Almenoff and Orłowski, 1983; Fournie-Zaluski et al., 1983), did not inhibit C6 cell spreading (Table I). On the other hand, we found that the dipeptides benzyloxycarbonyl-gly-phe-NH₂ (cbz-gly-phe-NH₂) and cbz-tyr-tyr lead to 55% inhibition of C6 cell spreading on CNS myelin, but not on PLYS, PNS myelin, or glass. These peptides are substrate peptides with metalloprotease specificity (Almenoff and Orłowski, 1983; Baxter et al., 1983; Couch and Strittmatter, 1983; Chen and Chen, 1987; Lelkes and Pollard, 1987).

The previous results gave no information about the nature and localization of the substrates for the metalloprotease activity. To exclude a possible general enhancement of C6 cell spreading on unfavorable substrates, we tested metalloprotease-dependent C6 cell spreading on two other substrates in addition to PLYS and CNS myelin (Fig. 6): PNS myelin and glass. PNS myelin was chosen as a control for the general properties of a myelin membrane fraction (e.g., high content of lipids), and glass because of its well-known unfavorable substrate qualities. Half-maximal inhibition of spreading on CNS myelin was obtained with 0.2 mM 1,10-phenanthroline. On PLYS, glass and PNS myelin 1,10-phenanthroline did not impair C6 cell spreading at concentrations up to 0.5 mM (Fig. 6).

Absorption of CNS myelin with a monoclonal antibody (IN-1) raised against CNS myelin inhibitory components (Caroni and Schwab, 1988b) largely reversed 1,10-phenanthroline-dependent inhibition of C6 cell spreading on CNS myelin liposomes (Table II). IN-1 also almost completely neutralized the inhibitory substrate property of CNS myelin protein liposomes for 3T3 cells (Table II). These results indicate that the putative metalloprotease(s) plays an important role for overcoming of CNS myelin inhibitory substrates by neutralization of IN-1-sensitive inhibitory properties.

A C6 Plasma Membrane-associated Activity Neutralizes the Inhibitory Substrate Property of CNS Myelin

CNS myelin-coated culture wells were incubated with C6 conditioned medium or C6 plasma membranes, and subsequently tested for their inhibitory substrate property by spreading of 3T3 cells. We found that C6 plasma membranes contained an activity which strongly reduced CNS myelin inhibitory activity (Fig. 7; Table III). The same treatment also decreased the inhibitory effect of CNS myelin protein liposomes or SDS-PAGE-purified, reconstituted 35- and 250-kD inhibitory components (results not shown). The decrease in CNS myelin inhibitory activity for 3T3 cell adhesion and spreading was quantified by measuring spreading values and DNA synthesis (Table III). 1,10-phenanthroline, EDTA, and the dipeptide cbz-gly-phe-NH₂ completely blocked the C6

Table I. Effect of Different Protease Inhibitors on C6 Cell Spreading on PLYS or CNS Myelin

Protease class	Inhibitor		Spreading on		Percent inhibition on CNS (% of control on PLYS)
			PLYS (% of control on PLYS)	CNS	
	None, control		100	95	5
Serine	6-amino-capronate	3.0 mM	93	100	0
	Hirudine	1.0 nM	nq	nq	0
	PMSF	4.0 mM	100	94	6
	Trasyolol	200 U/ml	98	93	5
Cysteine	Leupeptine	0.3 mM	91	83	8
Aspartyl	Pepstatine	0.3 mM	98	95	3
Metallo	1,10-phenanthroline	0.3 mM	97	30	67
	Bestatine	0.1 mM	nq	104	0
	Phosphoramidon	0.3 mM	nq	91	9
	TIMP	10 µg/ml	102	93	9
	cm-phe-leu	0.5 mM	95	92	3
	cbz-gly-gly-NH ₂	1.0 mM	nq	99	1
	cbz-gly-phe-NH ₂	1.0 mM	100	45	55
	cbz-ala-phe	0.3 mM	98	90	8
	cbz-tyr-tyr	0.3 mM	101	56	45
General	α2-macroglobuline	3.0 µM	70	52	18
	Cocktail -		nq	nq	0
	Cocktail +		nq	nq	++

Cells were plated on PLYS or CNS myelin-coated culture dishes. Spreading was determined after 150 min as described in Materials and Methods. Inhibition values are calculated by subtracting spreading values on CNS myelin from the values on PLYS. Each value is the mean of at least three experiments. SEM <11%. PMSF, phenylmethylsulfonyl fluoride. TIMP, tissue inhibitor of metalloproteases. Cocktail -, 200 u/ml trasyolol, 0.3 mM leupeptine, 0.3 mM pepstatine. Cocktail +, same as cocktail -, but with 0.3 mM 1,10-phenanthroline. nq, not quantified, only qualitative.

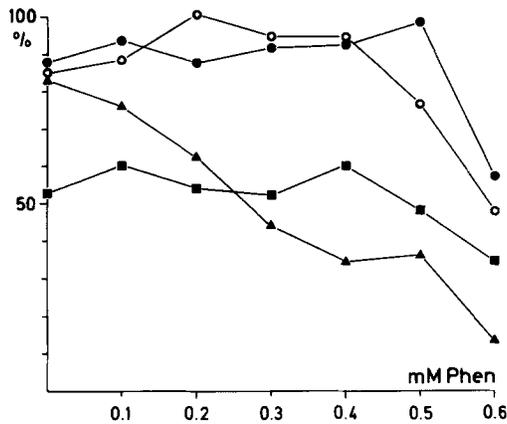


Figure 6. 1,10-phenanthroline inhibits C6 cell spreading specifically on CNS myelin. C6 cells were cultured for 3 h on the indicated substrates in presence of increasing doses of 1,10-phenanthroline. Spreading is inhibited by low doses exclusively on CNS myelin. 1,10-phenanthroline concentrations above 0.5 mM exert a general toxic effect on all substrates. Spreading was quantified as indicated in Fig. 4. Experiment was repeated three times. SEM <10%. (●) Plys; (○) PNS; (▲) CNS; (■) Glass.

plasma membrane effect. Trasylol, leupeptine, and pepstatine did not inhibit this effect (not shown).

C6-conditioned medium used as such or concentrated 10 times did not contain any degradative activity able to neutralize CNS myelin inhibitory substrate properties.

Inhibitors of Metalloproteases Impair C6 Cell Spreading on CNS White Matter and C6 Infiltration of CNS Explants

To investigate the relevance of the C6 plasma membrane metalloprotease activity not only for C6 cell attachment and spreading, but also for C6 cell migration and infiltration, C6 cells were plated on cerebellar frozen sections or added to optic nerve explants in the presence of two metalloprotease inhibitors (1,10-phenanthroline and cbz-tyr-tyr). Parallel

cultures contained inhibitors for the three other classes of proteases (leupeptine, pepstatine, or trasylol), or a control dipeptide (cbz-ala-phe).

On cerebellar frozen sections as culture substrates the presence of 1,10-phenanthroline at different concentrations (50, 100, 200, and 300 μ M), or of the dipeptide cbz-tyr-tyr (100 μ M) dramatically changed the distribution and behavior of C6 cells on the white matter areas (Fig. 8). On the gray matter the situation (for both molecular and granular layer) remained the same in all cases: C6 cells adhered in large numbers and spread extensively (Fig. 8).

Rat optic nerves were injected with 4 μ l of 3 mM solutions of either cbz-ala-phe or cbz-tyr-tyr. Cells were incubated with medium containing 0.5 mM peptide. In the outer chamber, where no cells were present, the peptide concentration was 1 mM. After 14 d the immigration of C6 cells into the explants differed greatly (Fig. 9). cbz-ala-phe-injected nerves contained more cells, and C6 cell infiltration was not affected, as compared with explants injected with culture medium only (not shown). On the other hand, cbz-tyr-tyr inhibited C6 cell infiltration in all the eight nerves examined (two experiments). C6 cells were found mainly at the cut end of these nerve explants, and deep infiltration, which occurred massively in control explants, was strongly reduced by cbz-tyr-tyr.

Discussion

Adult CNS tissue, in particular white matter, is strongly non-permissive for regenerative neurite growth, a property for which specific membrane proteins of oligodendrocytes and myelin are crucially involved (Caroni and Schwab, 1988a). The same constituents inhibit *in vitro* attachment, spreading, and migration of neurons, astrocytes, and fibroblasts (Caroni and Schwab, 1988a, b). The present results demonstrate that C6 glioblastoma cells, in contrast to neurons, fibroblasts, and B16 melanoma cells, were not impaired in their migration into optic nerve explants or in attachment and spreading on CNS white matter, isolated CNS myelin, or living oligodendrocytes. The fact that the behavior of C6 cells differed characteristically from that of several cell types in all the assay systems studied here suggests common underlying cell

Table II. Inhibition of C6 Cell Spreading by 1,10-phenanthroline on CNS Myelin Is Neutralized by Antibody IN-1

Cells	Antibody	1,10-phenanthroline mM	Spreading value on		Percent inhibition on CNS liposomes
			CNS liposomes	PLYS	
3T3	—	0	1.11	2.00	45
3T3	IN-1	0	2.03	2.26	10
3T3	Mouse IgM	0	1.16	2.18	47
C6	—	0	2.48	2.52	2
C6	—	0.3	1.35	2.49	46
C6	IN-1	0	2.46	2.48	1
C6	IN-1	0.3	2.25	2.54	11
C6	Mouse IgM	0	2.36	2.42	2
C6	Mouse IgM	0.3	1.41	2.39	41

CNS myelin protein liposomes were used as substrates, and were preadsorbed with monoclonal antibody IN-1 against the myelin inhibitory substrate constituents (Caroni and Schwab, 1988b), or with mouse IgM. Spreading was calculated after 150 min, is the mean of three experiments, and is expressed as $\mu\text{m}^2 \cdot 10^3$ (SEM <6%). Percent inhibition relates to PLYS.

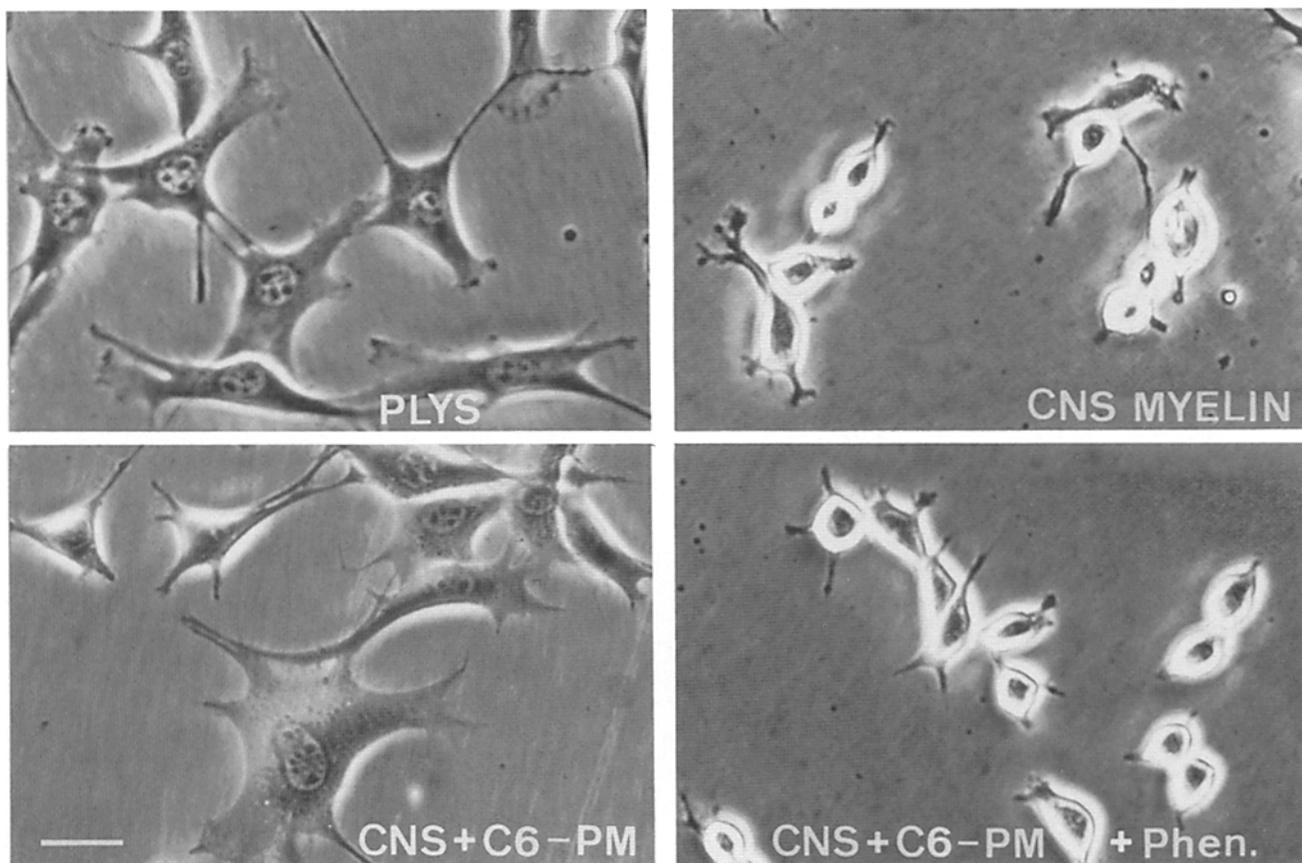


Figure 7. C6 plasma membranes degradation of CNS inhibitory substrate is 1,10-phenanthroline sensitive. Spreading of 3T3 cells on CNS myelin is induced by pretreatment of myelin with C6 plasma membranes. 1,10-phenanthroline abolishes this effect. Bar, 30 μ m.

biological mechanisms, both for C6 spreading on an inhibitory substrate as well as for C6 mobility in an environment (optic nerve), which does neither allow fibroblasts, Schwann cell, or melanoma cell migration, nor ingrowth of regenerating nerve fibers. This behavior of C6 cells was not due to "insensitivity" to the inhibitory components, since C6 cell motility was drastically inhibited on CNS myelin or white matter in the presence of specific metalloenzyme blockers, and this effect was reversed by selective neutralization of myelin-associated inhibitory proteins with a monoclonal antibody (IN-1).

Transformed invasive cells are known to often express high

levels of proteolytic activities (Quigley, 1976; Mahdavi and Hynes, 1978; Mullins and Rohrich, 1983; Chen et al., 1984; Mignatti et al., 1986; Wilhelm et al., 1987). In fact, inactivation of myelin-associated inhibitory constituents occurred by living C6 cells as well as by C6 plasma membranes. Our experiments with a number of protease blockers with different known specificities showed that this C6-associated activity belongs to the metalloprotease family. The close parallelism observed between prevention of C6 cell spreading on CNS myelin and prevention of inactivation of myelin-associated inhibitory proteins strongly suggest that modification

Table III. C6 Plasma Membranes Reduce CNS Myelin Inhibitory Substrate Property for 3T3 Cells

Substrates	3T3 cell spreading	^3H Thymidine incorporation
	%	%
PLYS	100	100
CNS myelin	15	30
CNS myelin, C6 PM, 10-	52	83
CNS myelin, C6 PM, phenanthroline treated	17	50
CNS myelin, C6 PM, EDTA treated	13	ND

3T3 cells were plated on PLYS or CNS myelin. Spreading was assessed after 150 min and is the mean of three experiments, SEM <12%. CNS myelin was preincubated with a C6 cell plasma membrane fraction (C6 PM) in the absence or presence of metalloprotease inhibitors as indicated. 1,10-phenanthroline concentration was 0.5 mM, and EDTA concentration was 3 mM. ^3H Thymidine was added when 3T3 cells were plated, and incorporation was determined after 20 h. Values are the mean of two experiments, SEM <14%.

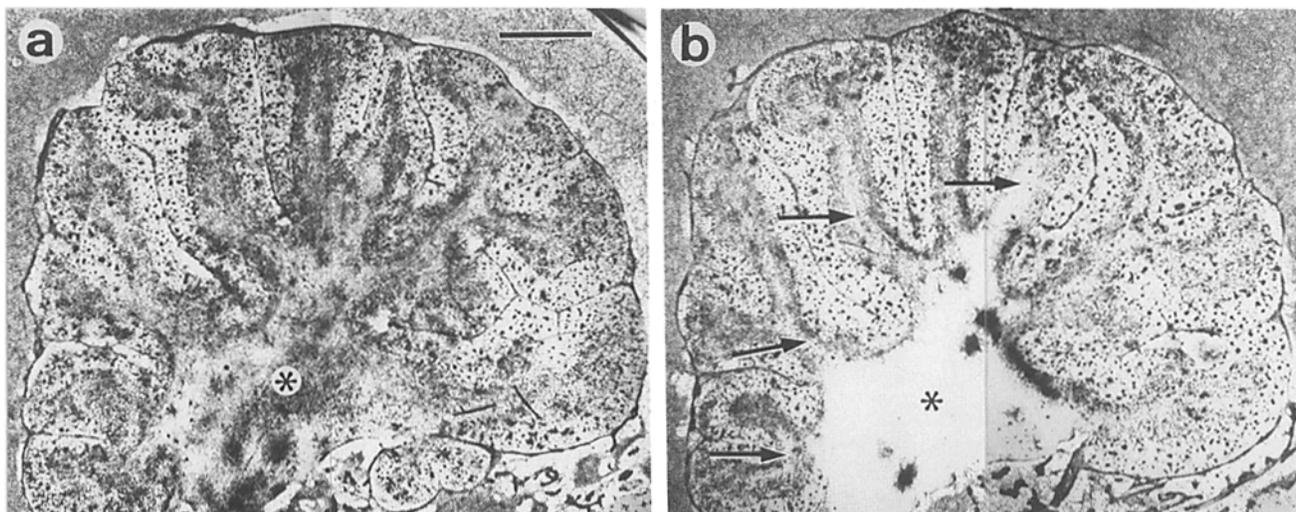


Figure 8. C6 cell attachment and spreading on CNS white matter of rat cerebellar frozen sections is impaired by metalloprotease blockers. Phase-contrast micrographs of C6 cells on rat cerebellar frozen sections cultivated in the presence of either 0.1 mM cbz-ala-phe (*a*) or 0.1 mM cbz-tyr-tyr (*b*). Inhibition of attachment and spreading is particularly evident in the center of the white matter (*asterisks*), but is also visible in the main white matter branches (*arrows*). On the gray matter there is no relevant difference between *a* or *b*. Bar, 1.0 mm.

of the inhibitory substrate components by a metalloprotease could be the mechanism which enables C6 cells to spread on myelin, on white matter, and to infiltrate optic nerve explants.

Metalloproteases form an increasingly numerous group, the members of which differ in their sensitivity to various blockers. The most general blocker is 1,10-phenanthroline which impaired C6 cell spreading on CNS myelin up to 67%, whereas most inhibitors of the other classes of proteases had no detectable effects. In the early (90 min) but not the later (300 min) phases of C6 cell spreading on myelin an effect of trypsin-like serine-protease inhibitors was also observed (not shown). Whether these enzymes act in parallel with the metalloproteases or could even activate them, as it has been reported (e.g., for the plasminogen activator-plasmin-collagenase system) (Mignatti et al., 1986), is an open question. The effect of 1,10-phenanthroline was dose dependent, with an IC_{50} of 0.2 mM. This effect was specific for CNS myelin as a substrate, since normal, rapid spreading of C6 cells was observed on other substrates such as CNS gray matter, PNS myelin, glass, or PLYS in the presence of 1,10-phenanthroline. Other known metalloprotease blockers like bestatine (inhibitor of aminopeptidases; Umezawa et al., 1976), phosphoramidone (inhibitor of thermolysin-like metalloproteases; Komiyama et al., 1975), and TIMP (inhibitor of extracellular matrix-degrading metalloproteases; Cawston et al., 1981) did not lead to inhibition of C6 cell spreading on CNS myelin. Since metalloproteases generally hydrolyze peptide bonds followed by large aliphatic or neutral aromatic amino acids, we tested the effect of dipeptide substrate analogues containing such residues. cbz-gly-phe-NH₂ (1 mM) or cbz-tyr-tyr (0.3 mM) inhibited C6 cell spreading specifically on CNS myelin. cbz-gly-phe-NH₂ was found to inhibit other 1,10-phenanthroline-sensitive enzyme activities with relative high specificity (Almenoff and Orlowski, 1983; Baxter et al., 1983; Couch and Strittmatter, 1983; Chen and Chen, 1987; Lelkes and Pollard, 1987).

Inactivity of C6-conditioned medium and cell fractionation experiments demonstrated that the myelin-directed proteolytic activity is associated with C6 plasma membranes. Isolation and characterization of a plasma membrane-bound metalloprotease (endopeptidase 24.11, enkephalinase), which is also blocked by 1,10-phenanthroline but not by TIMP, was reported by Almenoff and Orlowski (1983). However, the metalloprotease described here is probably not an enkephalinase, since cm-phe-leu, a peptide with high affinity for enkephalinase (Fournie-Zaluski et al., 1983), did not affect C6 spreading on myelin. A metalloprotease expressed by Rous sarcoma virus-transformed chick embryo fibroblasts and localized at adhesion sites and on "invadopodia" was described by Chen and Chen (1987). This enzyme is also inhibited by 1,10-phenanthroline and cbz-gly-phe-NH₂, but

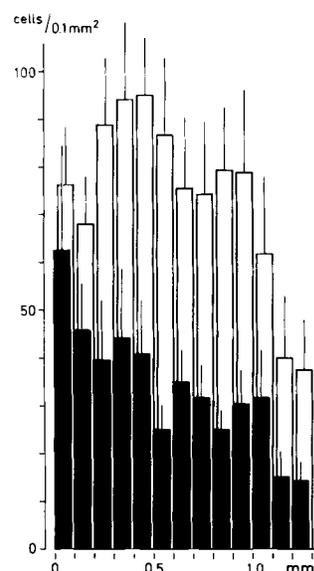


Figure 9. C6 cell infiltration into CNS explants is impaired by cbz-tyr-tyr. Cells were added to one tip of optic nerve explants (chamber cultures) in the presence of the metalloprotease inhibitor cbz-tyr-tyr (■), or of the control peptide cbz-ala-phe (□), as described in Materials and Methods. 14-d-old cultures were quantified. Infiltrated cells were counted in the first 1.3 mm of the explants. Each column represents the number of infiltrated cells per 0.1 mm. Only the most central part of the explants was considered (0.25 mm). Values represent means \pm SEM of two sets of experiments for a total of eight explants.

not by phosphoramidon, as the metalloprotease described here. However, we could not detect any fibronectin degradative activity on C6 cells so far. Indeed, the substrate specificity of the C6 metalloprotease is not yet known. Our observation of an inactivation of the myelin-associated inhibitory substrate molecules indicates, that the substrates relevant for the effects observed here may not be on the surface of the C6 cells. However, a more complex sequence of events resulting in the release of an inhibitor-blocking receptor fragment from C6 cell membrane can not be excluded.

An increase of proteolytic activities associated with transformation or tumorigenicity is well described (see above). Such proteases have been implicated in the degradation of extracellular matrix (Fairbairn et al., 1985) or basement membrane components (Liotta et al., 1986), facilitating migration of tumor cells through tissue barriers, and allowing extravasion from blood vessels and metastatic tissue invasion. We, therefore, tested the highly metastatic B16 mouse melanoma cells in all the assays used with C6 cells. Interestingly, B16 cells did not migrate into optic nerve explants, but responded to the myelin-associated inhibitors in a way very similar to 3T3 cells or neurons. In line with this in vitro behavior, B16 cells upon intracisternal injection, mainly showed meningeal tumor spread with occasional migration along blood vessels into the Virchow-Robin spaces (Schabet et al., 1987). Obviously, the mechanisms providing metastatic behavior to B16 cells in the periphery are different from those conferring high mobility to C6 cells in the CNS tissue.

Inhibition of C6-associated metalloproteases not only inhibited C6 spreading on CNS myelin, but also abolished C6 cell attachment, spreading, and migration on CNS white matter, and the dipeptide cbz-tyr-tyr strongly impaired the migration of C6 cells into optic nerve explants. This metalloprotease activity(ies) may, therefore, be crucially involved in the infiltrative behavior of C6 glioblastoma cells in CNS tissue, also in vivo.

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References

Almenoff, J., and M. Orlowski. 1983. Membrane-bound kidney neutral metalloendopeptidase: interaction with synthetic substrate, natural peptides, and inhibitors. *Biochemistry*. 22:590-599.

Auer, R. N., R. F. Del Maestro, and R. Anderson. 1981. A simple and reproducible experimental *in vivo* glioma model. *Le Journal Canadien des Sciences Neurologiques*. 8:325-331.

Baxter, D. A., D. Johnston, and W. J. Strittmatter. 1983. Protease inhibitors implicate metalloendopeptidase in synaptic transmission at the mammalian neuromuscular junction. *Proc. Natl. Acad. Sci. USA*. 80:4174-4178.

Benda, P., J. Lightbody, G. Sato, L. Levine, and W. Sweet. 1968. Differentiated rat glial cell strain in tissue culture. *Science (Wash. DC)*. 161:370-371.

Caroni, P., and M. E. Schwab. 1988a. Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *J. Cell Biol.* 106:1281-1288.

Caroni, P., and M. E. Schwab. 1988b. Antibody against myelin-associated inhibitor of neurite growth neutralizes non-permissive substrate properties of CNS white matter. *Neuron*. 1:85-96.

Cawston, T. E., A. Galloway, E. Mercer, G. Murphy, and J. J. Reynolds. 1981. Purification of rabbit bone inhibitor of collagenase. *Biochem. J.* 195:159-165.

Chen, J. M., and W. T. Chen. 1987. Fibronectin-degrading proteases from the membranes of transformed cells. *Cell*. 48:193-203.

Chen, W. T., K. Olden, B. A. Bernard, and F. F. Chu. 1984. Expression of transformation-associated protease(s) that degrade fibronectin at cell contact sites. *J. Cell Biol.* 98:1546-1555.

Colman, D. R., G. Kreibich, A. B. Frei, and D. D. Sabatini. 1982. Synthesis and incorporation of myelin polypeptides into CNS myelin. *J. Cell Biol.* 95:598-608.

Couch, C. B., and W. J. Strittmatter. 1983. Rat myoblast fusion requires metalloendopeptidase activity. *Cell*. 32:257-265.

Fairbairn, S., R. Gilbert, G. Ojakian, R. Schwimmer, and J. P. Quigley. 1985. The extracellular matrix of normal chick embryo fibroblasts: its effect on transformed chick fibroblasts and its proteolytic degradation by the transformants. *J. Cell Biol.* 101:1790-1798.

Fournie-Zaluski, M. C., P. Chaillet, E. Soroca-Lucas, H. Marçais-Collado, J. Costentin, and B. P. Roques. 1983. New carboxylalkyl inhibitors of brain enkephalinase: synthesis, biological activity, and analgesic properties. *J. Med. Chem.* 26:60-65.

Komiyama, T., T. Aoyagi, T. Takeuchi, and H. Umezawa. 1975. Inhibiting effects of phosphoramidon on neutral metalloendopeptidase and its application on affinity chromatography. *Biochem. Biophys. Res. Commun.* 65:352-357.

Lelkes, P. I., and H. B. Pollard. 1987. Oligopeptide inhibitors of metalloproteases activity inhibit catecholamine secretion from bovine adrenal chromaffin cells by modulating intracellular calcium homeostasis. *J. Biol. Chem.* 262:15496-15505.

Liotta, L. A., C. N. Rao, and U. M. Wewer. 1986. Biochemical interactions of tumor cells with the basement membrane. *Annu. Rev. Biochem.* 55:1037-1057.

Mahdavi, V., and R. O. Hynes. 1978. Proteolytic enzymes in normal and transformed cells. *Biochem. Biophys. Acta*. 583:167-178.

Matrisian, L. M., G. T. Bowden, P. Krieg, G. Fuerstenberger, J. P. Briand, P. Leroy, and R. Breathnach. 1986. The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. *Proc. Natl. Acad. Sci. USA*. 83:9413-9417.

Mignatti, P., E. Robbins, and D. B. Rifkin. 1986. Tumor invasion through human amniotic membrane: requirement for a protein cascade. *Cell*. 47:487-498.

Mørk, S., O. D. Laerum, and L. deRitter. 1984. Invasiveness of tumours of the central nervous system. In *Tumor Invasion*. M. M. Mareel, and K. Calman, editors. Oxford University Press, NY. 79-125.

Mullins, D. E., and S. T. Rohrllich. 1983. The role of proteinases in cellular invasiveness. *Biochem. Biophys. Acta*. 695:177-214.

Quigley, J. P. 1976. Association of a protease (plasminogen activator) with a specific membrane fraction isolated from transformed cells. *J. Cell Biol.* 71:472-486.

Savio, T., and R. E. Schwab. 1989. Rat CNS white matter, but not gray matter, is nonpermissive for neuronal cell adhesion and fiber outgrowth. *J. Neurosci.* In press.

Schabet, M., H. Wiethölter, and D. Meier. 1987. Experimental meningeal neoplasia in nude rats. *Neurology*. 37(Suppl. 1):311.

Schwab, M. E., and P. Caroni. 1988. Oligodendrocytes and CNS myelin are non-permissive substrate for neurite growth and fibroblasts spreading *in vitro*. *J. Neurosci.* 8:2381-2393.

Schwab, M. E., and H. Thoenen. 1985. Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. *J. Neurosci.* 5:2415-2423.

Sommer, I., and M. Schachner. 1981. Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Dev. Biol.* 83:311-327.

Umezawa, H., M. Ishizuka, T. Aoyagi, and T. Takeuchi. 1976. Enhancement of delayed-type hypersensitivity by bestatin, an inhibitor of aminopeptidase B and leucine aminopeptidase. *J. Antibiot.* 29:857-859.

Wilhelm, S. M., I. E. Collier, A. Kronberger, A. Z. Eisen, B. L. Marmor, G. A. Grant, E. A. Bauer, and G. I. Goldberg. 1987. Human skin fibroblast stromelysin: structure, glycosylation, substrate specificity, and differential expression in normal and tumorigenic cells. *Proc. Natl. Acad. Sci. USA*. 84:6725-6729.

Yoshida, T., K. Shimizu, Y. Ushio, T. Hayakawa, N. Arita, and H. Mogami. 1986. Development of experimental meningeal gliomatosis models in rats. *J. Neurosurg.* 65:503-507.