Retro-Klempnerization: Look for the unpublished data. :)

Matrix Metalloproteinases in the Cerebrospinal Fluid of Patients with Lyme Neuroborreliosis

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Neurologic manifestations of Lyme disease include meningitis, encephalopathy, and cranial and peripheral neuropathy. There are no sensitive markers for neuroborreliosis, and diagnosis is often based on clinical presentation and cerebrospinal fluid (CSF) abnormalities, including intrathecal antibody production. Matrix metalloproteinase (MMP) activity in CSF was compared in patients with neuroborreliosis, patients with diverse neurologic disorders, and healthy controls. The CSF of 17 of 18 healthy subjects and 33 of 37 patients with neurologic symptoms and normal CSF and imaging studies contained only MMP2. The CSF of several patients with neurologic disorders contained MMP2, MMP9, and gelatinolytic activity at 130 and 250 kDa. The 130-kDa MMP was found without the 92-kDa MMP9 in the CSF of 11 (79%) of 14 patients with neuroborreliosis and only 7 (6%) of 118 control patients ($P < .001$). This pattern of CSF gelatinase activity may be a useful marker for neuroborreliosis.

Lyme disease or Lyme borreliosis is caused by the tickborne spirochete *Borrelia burgdorferi* and is the most common vectorborne infection in the United States and Europe [1]. The illness usually begins in late spring to early fall with a characteristic skin lesion, erythema migrans, which occurs at the site of the tick bite. Days to weeks later, the spirochete often spreads hematogenously and may affect the joints, heart, or nervous system [2–4]. Acute neurologic manifestations include meningitis, cranial neuropathy, or radiculoneuritis [5]. Months to years later, chronic neurologic abnormalities may develop, including a subtle encephalopathy, polyneuropathy, or, rarely, encephalomyelitis [6–8].

Patients with neuroborreliosis and encephalopathy often have a subtle neuropsychiatric illness, with memory deficit, irritability, or somnolence, occurring months to years after classic manifestations of Lyme disease [9, 10]. Such patients usually have cerebrospinal fluid (CSF) abnormalities, including elevated total protein, intrathecal antibody production to *B. burgdorferi* [11], or a positive polymerase chain reaction (PCR) test for spirochetal DNA [6, 11]. In addition, increased amounts of neurofilaments and glial fibrillary acidic protein degradation products have been noted in CSF [12]. The polyneuropathy is usually accompanied by spinal radicular pain or peripheral dysesthesias, and electromyography often shows a diffuse axonal polyneuropathy.

Matrix metalloproteinases (MMPs) are enzymes that contain zinc in their active center and require Ca^{2+} for proteolytic activity. Several MMPs have been identified in the human brain, including gelatinase A (MMP2), gelatinase B (MMP9), stromelysin (MMP3), and collagenase (MMP1) [13–17]. Two additional bands with gelatinolytic activity and electrophoretic mobility corresponding to 130 and 250 kDa have been identified but not fully characterized. It has been suggested that the 130-kDa band is a complex of gelatinase B and tissue inhibitor of metalloproteinase-1 [14, 18, 19] or a dimer of the active 68-kDa form of gelatinase B [20]. Increased concentrations of MMPs have been identified in the central nervous systems (CNS) of patients with Alzheimer's disease, brain injury, and brain tumors [13, 16, 21]. MMPs have also been detected in the CSF of patients with inflammatory diseases, amyotrophic lateral sclerosis, and multiple sclerosis [18, 19, 22]. We recently reported the presence of 130-kDa gelatinolytic activity in the CSF of a patient with documented Lyme neuroborreliosis [23]. Here we analyze the pattern of MMPs in the CSF of patients with Lyme neuroborreliosis compared with healthy persons and patients with other neurologic diseases.

Materials and Methods

American patients with chronic Lyme neuroborreliosis. CSF samples were available from 8 patients who were evaluated for

Received 27 March 1997; revised 13 August 1997.

Presented in part: International Congress on Lyme Borreliosis, San Francisco, July 1996.

Informed consent was obtained from the patients for the collection of CSF, and the review of patient records was conducted according to procedures approved by the institutional review boards of the New England Medical Center, the Brigham and Women's Hospital, and the Institut Central des Hôpitaux Valaisans.

Financial support: NIH (AI-37241, AI-31610, AI-65308 to M.S.K; AA-09669 to M.E.C.; AR-20358 to A.C.S.), Eshe Fund (to A.C.S.); Earle P. Charlton Award (to G.P.).

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The Journal of Infectious Diseases 1998;177:401–8

0022–1899/98/7702–0018$02.00

chronic neuroborreliosis in the Lyme Disease Clinic at New England Medical Center (Boston) and were characterized earlier [10, 11]. The 8 patients met Centers for Disease Control and Prevention (CDC) criteria for the diagnosis of Lyme disease [24]: 5 had had erythema migrans, and the other 3 had had Lyme arthritis accompanied by a positive serologic test for *B. burgdorferi*. Their ages ranged from 20 to 66 years; 4 were men and 4 were women. From 1 to 11 years after disease onset, they developed neurologic symptoms of headache, memory difficulty, radicular pain, or numbness and tingling in the extremities, which prompted their evaluation. Encephalopathy was characterized primarily by memory impairment and polyneuropathy by spinal radicular pain or distal paresthesias. These classic manifestations of Lyme disease were accompanied by CSF evidence of *B. burgdorferi* infection (either by intrathecal antibody production or a positive PCR test for *B. burgdorferi* DNA). In addition, patients with polyneuropathy had electromyographic evidence of a diffuse axonal polyneuropathy.

For all American patients, serum and CSF samples were frozen in multiple aliquots at −70°C until testing was done. Serum samples were tested for antibodies to *B. burgdorferi* by indirect ELISA and immunoblot [25], and positive results were defined according to the CDC Association of State and Territorial Public Health Laboratory Directors criteria [24]. CSF samples were tested for cells and protein, for *B. burgdorferi* DNA by using 2 different primer-probe sets that target different regions of the plasmid DNA encoding the spirochetal outer surface protein A (OspA) [11], and for evidence of intrathecal antibody production by antibody capture ELISA (>1 is considered positive) [26]. Of the 8 patients, 7 had a positive serologic test for *B. burgdorferi* by ELISA and immunoblot analysis at the time of our evaluation. The final patient, who 1 year previously had had erythema migrans, Lyme meningitis, and a positive serologic test for *B. burgdorferi,* was seronegative when evaluated in our clinic but had a positive PCR test for *B. burgdorferi* DNA in CSF. Four patients had intrathecal antibody production and the other 4 had a positive PCR test for *B. burgdorferi* DNA (table 1).

European patients with chronic and acute Lyme neuroborreliosis. CSF samples were analyzed from 6 patients who were evaluated for neuroborreliosis in the Institut Central des Hôpitaux Valaisans (Sion, Switzerland). Their ages ranged from 25 to 70 years; 2 were men and 4 were women. Two patients had radiculitis, 2 had radiculoneuropathy, and 2 had spastic paraparesis.

The CSF samples were obtained by lumbar puncture and kept at 4°C up to 4 days before freezing. All of the European patients had CSF abnormalities. Three of these patients had CSF pleocytosis and elevated total protein. Five of the 6 patients had positive IgG serology by Western blot analysis. All 6 patients had evidence of intrathecal antibody production, based on an IgG index >2, calculated by the ratio of the EIA value (CSF/serum over the ratio of albumin concentration in CSF/serum) (VIDAS Lyme IgG+IgM; bioMérieux, Lyon, France). The IgG index ranged between 2.6 and 8.8 (table 1).

Nonneurologic controls. CSF from 18 healthy persons was obtained by lumbar puncture as part of spinal anesthesia at the New England Medical Center. Of the 18 patients, 9 were admitted for cesarean section, 2 for tubal ligation, 3 for urologic procedures, and 4 for orthopedic procedures. The CSF of the nonneurologic controls from the New England Medical Center was aliquoted and stored at −70°C within 30 min after lumbar puncture.

Neurologic controls. Aliquots of 238 consecutive CSF samples from 101 persons were obtained from the clinical laboratory at the Brigham and Women's Hospital (Boston). Samples were from 58 women and 43 men ranging in age from 18 to 91 years (mean, 52). Patient records were reviewed without knowledge of the results of the zymography. On the basis of chart review, patients were categorized into normal neurologic controls and patients with neurologic diagnoses.

Lumbar puncture was done for 39 patients to evaluate possible meningitis, severe headache, or seizures. All of these patients had normal CSF protein and cell counts and brain imaging studies that were normal or showed old cerebral infarcts (2 patients). These patients were classified as normal neurologic controls.

CSF was obtained by lumbar puncture or ventricular aspiration from 62 patients with established neurologic diagnoses, including Alzheimer's disease (7), Parkinson's disease (1), multiple sclerosis (5), human immunodeficiency virus encephalopathy (6, of which 3 had viral meningitis), bacterial or fungal meningitis (5), metabolic encephalopathy (4), lymphoproliferative disorders with sepsis or encephalopathy (6), primary brain tumors (8), metastatic tumors (2), epidural hematoma (1), ischemic cerebral infarction (4), intracerebral hemorrhage (2), subarachnoid hemorrhage (7), subdural hematoma (2), transverse myelitis (1), and Guillain-Barré syndrome (1).

Samples from the clinical laboratory at the Brigham and Women's Hospital were stored at 4°C after processing for routine cell counts. All specimens were kept for 1–7 days at 4°C and then stored in aliquots at −70°C. There were no appreciable differences in MMP activity in specimens run immediately after lumbar puncture and after storage for 7 days at 4°C (not shown). Duplicate aliquots of CSF run on different days or on different gels produced identical results. When the lumbar puncture was traumatic, the CSF sample from the tube with the least blood contamination was used. Addition of up to 5% human control serum to CSF samples did not modify gelatinase activity (data not shown). All samples used in this study were frozen only once before zymography.

Methods

SDS-PAGE zymography. CSF was subjected to zymography. SDS-PAGE and zymography in 7.5% polyacrylamide gels (0.75 mm thick) containing 0.5% gelatin (unless noted otherwise) were done as described earlier [17, 27]. CSF (16 μL) was supplemented with 4 μL of 5× SDS sample buffer without β-mercaptoethanol, and electrophoresis was done at 4°C. The gels were incubated for 1 h at room temperature with 2.5% Triton X-100 and incubated for 16 or 40 h at 37°C in 20 m*M* Tris-acetate, pH 7.6, and 10 m*M* $CaCl_2$. Incubation for >2 days at 37°C did not increase the intensity of the bands. Gels were then stained with Coomassie blue (0.25% Coomassie brilliant blue, 10% acetic acid, 25% methanol) for 1 h and destained in 10% acetic acid and 5% methanol overnight. Stained gels were photographed with an MP-4 Polaroid system, scanned at 595 nm, and analyzed (Imaging Densitometer GS700; Bio-Rad Laboratories, Hercules, CA). To determine

Table 1. Characterization of CSF from patients with Lyme neuroborreliosis.

No.	Sex	Age	Cells/μL	Protein, mg/dL	Serum ELISA or Western blot result	Index	PCR of CSF	Clinical picture	MMP2	MMP9	130-kDa gelatinase
American patients											
1	F	66	<5	46	6400	1.4	−	PN, ENC	+	−	+
2	M	65	<5	42	3200	1.4	−	PN, ENC	+	−	+
3	F	47	<5	50	51,200	1.0	+	ENC	+	−	+
4	M	54	<5	ND	800	1.0	+	PN, ENC	+	−	+
5	M	52	<5	39	800	3.8	−	PN, ENC	+	−	+
6	F	20	<5	25	3200	0.7	+	ENC	+	−	+
7	M	55	<5	49	−	−	+	ENC	+	−	−
14	F	28	<5	78	1600	1.35	−	PN, ENC	+	−	+
Swiss patients											
8	M	34	1	ND	+	7.7	ND	SP	+	−	+
9	M	25	1	31	+	7.1	ND	SP	+	−	+
10	F	72	93	146	+	6.5	ND	RI	+	−	−
11	M	42	289	89	+	8.8	ND	RNP	+	−	+
12	M	31	5	28	+	2.6	ND	RI	+	−	+
13	F	70	643	267	ND	4.8	ND	RNP	+	+	+

NOTE. MMP2 = matrix metalloproteinase-2; MMP9 = matrix metalloproteinase-9; ND = not determined; PCR = polymerase chain reaction; ENC = encephalopathy; PN = polyneuropathy; RI = radiculitis; RNP = radiculoneuropathy; SP = spastic paraparesis. American patients were tested for *Borrelia burgdorferi* seroreactivity by ELISA; Swiss patients were tested by Western blot.

whether prolonged incubation of the CSF samples at 4°C would cause reduction of the gelatinolytic activity due to autodigestion or due to cell lysis, aliquots from 2 CSF samples containing 88 and 1235 cells/μL were kept for 6 days at 4°C and then subjected to zymography. No significant difference (<20%) in the gelatinolytic activity was observed between these aliquots and aliquots stored at −70°C.

Immunoblot. Immunoblot analysis was done after protein transfer according to Towbin et al. [28] on polyvinyldifluoride membranes as described earlier [29].

Statistics. χ^2 analysis was used to compare results of MMP analysis in different patient groups.

Materials

Mouse monoclonal antibodies against gelatinase A (MMP2) were purchased from Oncogene Science (Uniondale, NY). Gelatin was obtained from Bio-Rad Laboratories. Biotin-conjugated goat anti-mouse and avidin-conjugated alkaline phosphatase were purchased from Vector Laboratories (Burlingame, CA). Polyvinyldifluoride membranes were from Millipore (Bedford, MA). Casein-β was obtained from Sigma (St. Louis). Gelatinase A was isolated from mouse 3T3 fibroblasts as described [17]. Human recombinant gelatinase A was purchased from Biogenesis (Sandown, NH). All other chemicals were of analytical grade and purchased from Sigma.

Results

Quantitative zymography. Human recombinant gelatinase A was used as a standard for quantitative analysis of CSF gelatinase A. Gelatinase A is secreted in a latent form (72 kDa) and is activated in vivo to a 64-kDa form by plasmin or other MMPs [30]. One-third of the recombinant enzyme is present in the activated form and two-thirds in the latent form. Figure 1 shows zymograms of decreasing concentrations of human recombinant gelatinase A. The 72-kDa form has been activated by SDS but retains its electrophoretic mobility. The 64-kDa band represents the constitutively active form. The assay was sensitive to 1–300 ng of gelatinase A (figure 1A). By reducing the concentration of gelatin to 0.03% or 0.01%, we were able to detect 100 and 10 pg of gelatinase A, respectively (not shown). Gelatinolytic activity was linear with gelatinase concentration over a 1-log range, as reported earlier [31]. Over the 1- to 300-ng range, there was a logarithmic-linear relation between the amount of enzyme and the gelatinolytic activity as measured by scanning densitometry (figure 1B).

Gelatinolytic activity in CSF of healthy persons. We first characterized the gelatinolytic activity of CSF in healthy persons. All samples contained gelatinolytic activity with an electrophoretic mobility corresponding to 72 kDa, consistent with gelatinase A (figure 2A). Quantitative densitometry revealed similar amounts of gelatinolytic activity (equivalent to 0.6 ± 0.13 μg/mL human recombinant gelatinase A) in the CSF of healthy persons.

The gelatinolytic activity was inhibited by 1,10-phenanthroline, an MMP-specific inhibitor, suggesting that the enzyme is an MMP (not shown). To determine whether the 72-kDa gelatinase found in the normal CSF was gelatinase A (MMP2), we performed immunoblot analysis of CSF proteins (figure 2B). A monoclonal antibody raised against gelatinase A identi-

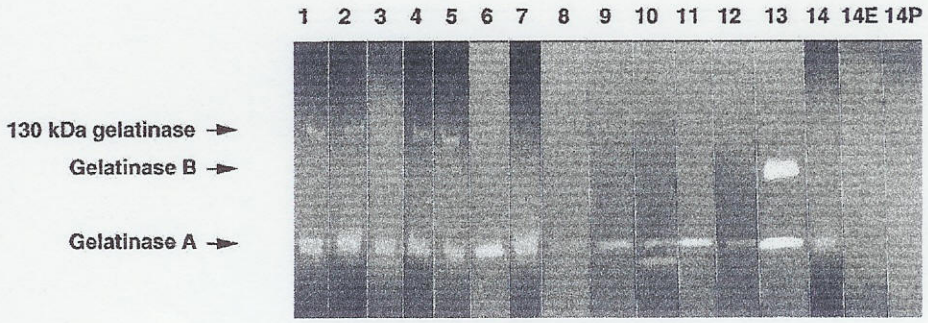

Figure 3. MMPs in CSF of persons with Lyme neuroborreliosis. 16 μL of CSF from 14 persons with Lyme neuroborreliosis was subjected to zymography in 7.5% polyacrylamide gels containing 0.5% gelatin. Samples from 12 patients contained gelatinase with electrophoretic mobility corresponding to 130 kDa in addition to gelatinase A. 1 sample contained gelatinase with electrophoretic mobility corresponding to 92 kDa (gelatinase B) in addition to gelatinase A. 16 μL of CSF from patient 14 with Lyme neuroborreliosis was subjected to zymography in presence of 20 m*M* EDTA 14E or in presence of 1 m*M* 1,10-phenanthroline (14P).

Gelatinase A was present in CSF samples from all 100 neurologic control subjects. Four samples from 38 normal neurologic controls contained the 92-kDa gelatinase (gelatinase B), and 3 samples contained the 130-kDa gelatinase without gelatinase B.

Of the 62 patients with neurologic disorders, there were 7 patients with dementia, including 6 with presumed Alzheimer's disease and 1 with Parkinson's disease. Like the patients with chronic Lyme neuroborreliosis, most of these patients (6/7) had cognitive dysfunction without CSF pleocytosis. The CSF of 3 of 7 patients with dementia (including 1 with CSF pleocytosis) contained gelatinase B (figure 4; lanes 16, 17, and 19), whereas only 1 sample contained the 130-kDa gelatinase without gelatinase B (figure 4; lane 22). The CSF of 5 patients with multiple sclerosis, including 2 with acute exacerbations, contained only gelatinase A (figure 4). None of the multiple sclerosis patients had CSF pleocytosis.

Gelatinase B was detected in 21 of 48 samples from patients with CNS neoplasms, inflammatory neurologic disease, or acute strokes. The gelatinolytic activity at 130 kDa was present in 11 of these 21 patients and the gelatinolytic activity at 250

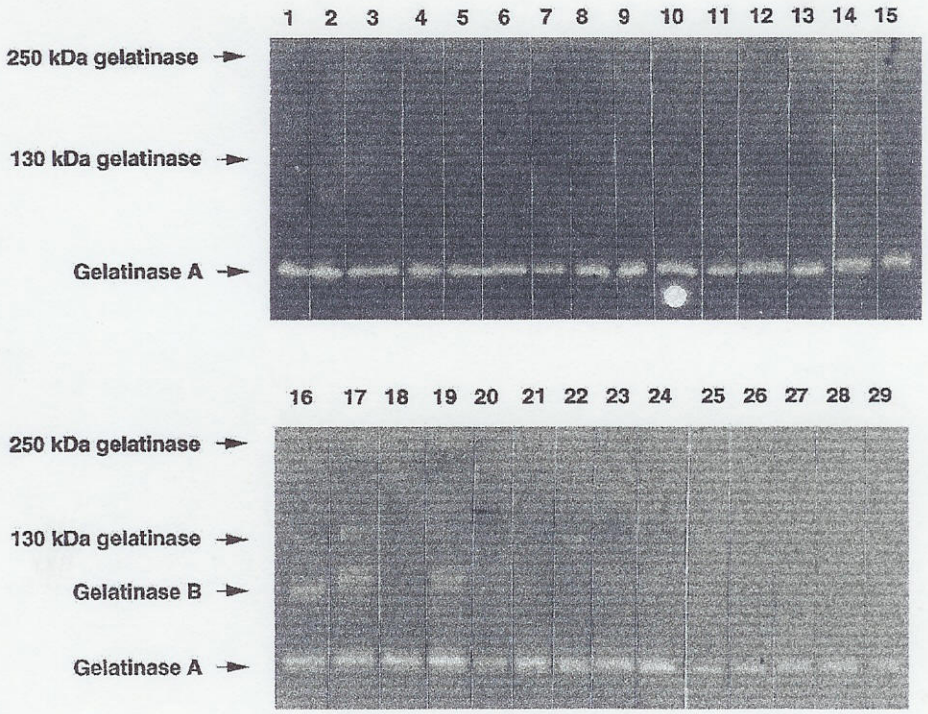

Figure 4. MMPs in CSF of persons with neurologic symptoms. 16 μL of CSF from neurologic controls (lanes 1–15) or from patients with Alzheimer's type dementia (lanes 16–22), Guillain-Barré syndrome (lane 23), Parkinson's disease (lane 24), and multiple sclerosis (lanes 25–29) were subjected to zymography. All samples tested contained gelatinase A at concentrations similar to those of healthy subjects (0.7 μg/mL). Several samples contained 130-kDa (lanes 17 and 22) and 250-kDa (lanes 17 and 19) gelatinases along with gelatinase B (lanes 2, 16, 17, and 19). Lane 22 contains 130-kDa gelatinase without gelatinase B.

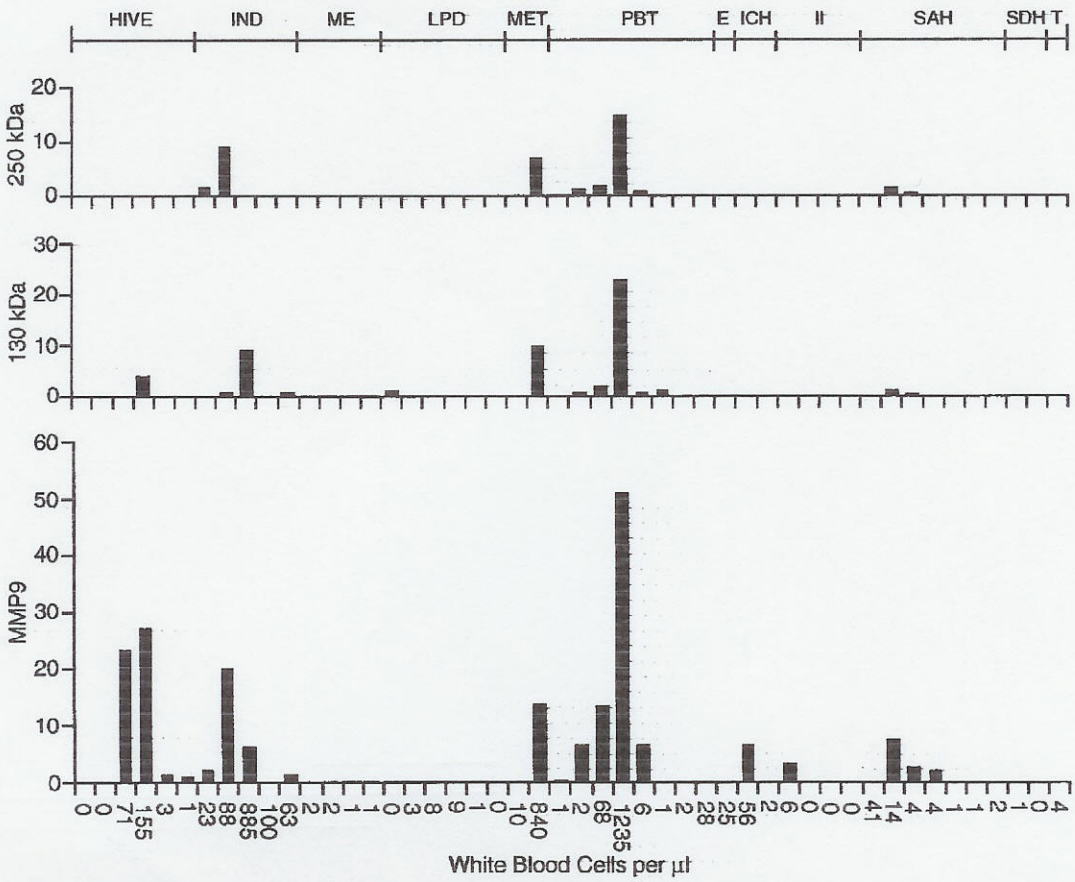

Figure 5. Gelatinolytic activity in patients with various neurologic diseases. 16 μL of CSF of patients with human immunodeficiency virus encephalopathy (HIVE), inflammatory neurologic diseases (IND), metabolic encephalopathy (ME), lymphoproliferative disorders without central nervous system metastasis (LPD), tumors with metastasis to the brain (MET), primary brain tumors (PBT), cerebrovascular diseases (epidural hematoma [E], intracerebral hemorrhage [ICH], ischemic infarction [II], subarachnoid hemorrhage [SAH], subdural hematoma SDH]), and transverse myelitis (T) were subjected to zymography. Amounts of gelatinolytic activity at 92 kDa (MMP9), 130 kDa, and 250 kDa were determined by scanning densitometry and are presented as arbitrary units.

kDa in 9. In CSF samples from 8 patients, the 130- and 250-kDa gelatinases were present with gelatinase B. In most samples, the combined presence of gelatinase B and the 130- or 250-kDa gelatinases was associated with a CSF pleocytosis (figure 5). Only 2 (4%) of the 48 samples in this patient subgroup had the gelatinolytic activity at 130 kDa without the 92-kDa form of gelatinase B.

There were 11 patients with CNS infections. Of 8 patients with meningitis and CSF pleocytosis, 7 had gelatinase B activity and 4 had gelatinolytic activity at 130 kDa. An additional 3 patients had human immunodeficiency virus encephalopathy without CSF pleocytosis. Two of these patients had gelatinase B activity in CSF, and none showed the 130-kDa gelatinase. No patient with nonborrelial CNS infections had CSF showing the 130-kDa gelatinase without gelatinase B.

Altogether, CSF from 6 of 100 patients with neurologic symptoms other than neuroborreliosis contained the 130-kDa MMP without the 92-kDa form of gelatinase B (table 2) (1 patient with a lymphoproliferative disorder, 1 with a meningioma, 1 with Alzheimer's disease, and 3 patients with normal CSF parameters in whom lumbar puncture was done as part of a fever workup). From a total of 118 CSF samples from patients without Lyme neuroborreliosis (100 with neurologic symptoms and 18 healthy controls), only 7 (6%) contained the 130-kDa gelatinase without the 92-kDa gelatinase B. This proportion was significantly lower than in patients with Lyme neuroborreliosis (78%). χ^2 with 1 *df* provides an association with value of 56.1 ($P < .001$).

Discussion

Most manifestations of Lyme disease, such as erythema migrans and arthritis, are easily recognized. In contrast, the symptoms and signs of Lyme neuroborreliosis are often nonspecific.

Lyme arthritis = specific
Lyme neuroborreliosis, non-specific

* Unpublished data - the best kind!

Table 2. Expression of gelatinases in CSF of patients with Lyme neuroborreliosis and controls.

Patient group	No.	No. with gelatinase B	No. with 130-kDa gelatinase	No. (%) with 130-kDa gelatinase without gelatinase B
Nonneurologic controls	18	1	1	1 (6)
Normal neurologic controls	38	4	4	3 (8)
Central nervous system infections	11	8	4	0
Neurologic disorders	51	17	9	3 (6)
All Controls	118	30	18	7 (6)
Neuroborreliosis	14	1	12	11 (78)*

$\chi^2 = 56.1$; $P < .001$.

In this study, we sought to determine whether MMPs could be useful markers of Lyme neuroborreliosis.

MMPs are expressed in virtually all tissues. Expression of MMPs is increased during physiologic remodeling of tissues, such as mammary gland involution, postpartum uterine muscle contraction, and wound healing [32]. Increased expression of MMPs also occurs in several pathologic conditions, including arthritis [33] and malignant tumors [34]. In the CNS, increased MMP expression has been associated with multiple sclerosis, inflammatory neurologic disorders, amyotrophic lateral sclerosis, brain tumors, and Alzheimer's disease [13, 16, 18, 19, 22]. Gelatinases A and B are expressed by microglia [35] and astrocytes [35–37]. Collagenase (MMP1), matrilysin (MMP7), and stromelysin (MMP3) have been identified in gliomas [16, 38].

The major finding of this study was that CSF of 78% of patients with Lyme neuroborreliosis contained a 130-kDa MMP without the 92-kDa gelatinase B. This pattern was relatively specific, occurring in only 6% of the CSF samples of 118 nonneurologic controls and patients with diverse neurologic disorders. This pattern was not observed in any of 11 patients with nonborrelial CNS infection. The expression of the 130-kDa MMP without the 92-kDa gelatinase B may therefore be a useful laboratory marker for Lyme neuroborreliosis. In most but not all patients, there was a correlation between CSF pleocytosis and expression of gelatinase B, as noted previously [22], whereas gelatinase A was constitutively expressed in all of our patient groups. The CSF of persons without neurologic complaints contained only gelatinase A, as judged by electrophoretic mobility and immunoblot analysis.

We characterized the gelatinolytic activity in the CSF of 100 patients with neurologic symptoms (38 neurologic controls and 62 with documented neurologic diseases). Our observation that the CSF of patients with multiple sclerosis and no CSF pleocytosis contained only gelatinase A and no other gelatinases agrees with the finding of Gijbels et al. [22]. In that study, gelatinase B was found primarily in the CSF of those multiple sclerosis patients with CSF pleocytosis [22], suggesting that gelatinase B is produced by white blood cells. The presence of gelatinase B in the CSF of patients with brain tumors and no pleocytosis suggests that this MMP may also derive from neoplastic and glial cells. The CSF of some patients with presumed Alzheimer's dementia contained only gelatinase A. This was surprising, since it was reported, and we have confirmed, that the brain parenchyma of patients with Alzheimer's disease contains increased amounts of both gelatinase B and the 130-kDa MMP [13].

The mechanism underlying the expression of the 130-kDa MMP in Lyme neuroborreliosis is unknown. Glial expression of some MMPs is regulated by cytokines, and concomitant expression of gelatinase B and interleukin-6 by activated microglia has been reported [39, 40]. Interleukin-6 levels are elevated in the CSF of patients with Lyme neuroborreliosis [41]. Moreover, co-incubation of C6 glioma cells with *B. burgdorferi* induces the expression of interleukin-6 [42]. It is possible that MMP and interleukin-6 expression are regulated by similar, as yet unknown, mechanisms. We have found that primary cultures of rat neural cells infected with *B. burgdorferi* secrete increased amounts of MMPs compared with uninfected cultures (unpublished data). It remains to be seen which cell types mediate this response.

B. burgdorferi is not directly toxic to neurons and is not known to express MMP activity; however, *B. burgdorferi* does bind to glial cells in vitro [43]. Since MMPs can digest myelin basic protein [44], *B. burgdorferi* could promote CNS injury indirectly by inducing the expression of MMPs in neural cells. MMPs also digest at least two proteins of the adult CNS extracellular matrix: the aggregating proteoglycan versican [17] and tenascin [45]. The extracellular matrix helps maintain the structural integrity of the CNS and facilitates cell migration, ion transport, and growth factor delivery. Thus, digestion of the brain extracellular matrix could promote the migration and dissemination of *B. burgdorferi* within the CNS and could contribute to the neuropathology of Lyme neuroborreliosis.

They cultured Bb & neurons + extracted components. Found MMPs.

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