Infection 2001 Dec;29(6):315-9

Association between multiple sclerosis and cystic structures in cerebrospinal fluid.

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Abstract
BACKGROUND: The aim of the study was to search for infectious agents in the cerebrospinal fluid (CSF) of patients
with multiple sclerosis (MS).
PATIENTS AND METHODS: CSF from ten patients with the diagnosis relapsing remitting MS and from five controls without MS were examined by transmission electron microscopy (TEM), dark field microscopy (DF), interference contrast microscopy (ICM) and UV-microscopic examination of acridine orange staining (AO). All CSF samples from patients and controls were cultured.

RESULTS: Cystic structures were observed in CSF of all ten patients by AO and TEM. DF revealed eight cyst-positive patients out of nine. One of five control persons had such structures in the CSF; this person had suffered from erythema migrans. Spirochete or rod-like structures emerged after culturing two of the MS patient CSF samples and these structures could be propagated.

CONCLUSION: A significant association of CSF cysts and MS was identified in this small study among residents in a coastal area of southern Norway. The cysts could be of spirochetal origin. Our study may encourage other researchers to study larger patient groups.

PMID: 11787831 [PubMed - in process]

Introduction
Multiple Sclerosis (MS) is a serious disease attacking the nervous system, and many hypotheses have been suggested to reveal the cause of the disease. The etiologic agent responsible for the development of MS has not yet been discovered, but MS has been suggested to be triggered by various viral infections, e.g. human herpesvirus 6, rubella, measles, and by Chlamydia pneumoniae [1-3]. Autoimmune disorders have also been proposed as the cause of the disease [4], but no definite verification is given. From 1909 until the 1950s many researchers considered MS to have a spirochetal origin because of its similarity to other spirochetal diseases [5]. Steiner [6] observed Spirochaeta myelophthora in brain lesions from autopsied MS patients in the 1950s. When Adams et al. [7] inoculated Macacus rhesus monkeys with material from cases of disseminated sclerosis, spirochetes were observed in the ventricular fluid after some months. MS has also been proposed to be caused by an oral spirochete [8, 9].
As the course of the disease was apparently not altered by antibiotics and observations of

spirochetes in brains of MS patients could only be evidenced in a few case records, this view was eventually abandoned. However, when entering into the CNS, microorganisms can undergo extensive structural, antigenic and metabolic changes which prevent them from being recognized by serology and other diagnostic test methods [10]. These changes could also protect them from the immune system and reduce the effect of antibiotics.

We recently observed that transformation of the spirochete Borrelia burgdorferi to cystic forms occurs invariably and rapidly after incubation in CSF and that they can reconvert to spirochetes if the conditions become favorable [11]. Using light microscopy and transmission electron microscopy (TEM), we identified morphologically similar cysts in the CSF of one MS patient who had been treated for 4 months with intravenous ceftriaxone (unpublished observations). Therefore, we performed this small controlled study to examine if cystic structures are more commonly found in the CSF of MS patients than in control individuals.

Patients and Methods

Patients

Ten patients were studied. Clinically, all of them had definite relapsing remitting MS according to Poser's criteria (12). All patients stayed in hospital for prednisolone (parenteral) or beta-interferon (intramuscular) treatment. They had been treated in this way for a few days before CSF puncture and blood sampling. The age ranged from 34-53 years, with a mean of 44.4 years. Five males and five females participated. The duration of the disease was in the range of 2-23 years, mean 10.4 years.

Controls

The control group consisted of five persons with the diagnosis of suspected ischialgia. These persons stayed in the hospital for a definite diagnosis. The age ranged from 38-63 years. with a mean of 52 years. Three males and two females participated. One of these persons had had erythema migrans- a disease caused by the spirochete B. burgdorferi. We performed all laboratory tests on the CSF from this person to see the test results in a patient with this spirochetal disease. However, he was excluded from the statistics.
All spinal punctures and blood collections were performed between February and July 1998 at the Department of Neurology, Vestfold Sentralsykehus, Tonsberg, Norway. Serum and CSF were stored in a refrigerator at 4 C, examined within 1 week and then maintained at -70 C.

The examinations were performed as blind studies as the observers did not know whether the sample was from the patient group or the control group.

Ethics

All participants were told about eventual adverse effects of the spinal puncture, and they signed a consent form in accordance with the advisory board of the regional ethical commission.

Culturing

4 ml CSF was centrifuged at 6,00)g for 30 min and 0.1 ml was transferred to 4 ml BSK-H medium (Sigma no. B3528; Sigma, St. Louis, MO.USA). BSK-H medium consists of several amino acids, vitamins, glucose and bovine albumin [13] and is a medium for culturing the spirochete B. burgdorferi. The concentration of inactivated (56 C. 30 min) rabbit serum (Sigma

R7136) or fetal calf (cat no.14-701 E: Bio Whittaker Boehringer lngelheim Bioproducts Partnership, Verviers, Belgium) the serum in the BSK-H medium was 6% and was free of antibodies against B. burgdorferi. All culture media had been sterile filtered by the manufacturing company (Sigma) ensuring both sterility and the absence of mammalian cells from serum. In addition, we filtered the media with a 0.2 um filter (Schleicher & Schuell FP 030{2, Dassel, Germany) to remove small particles which may resemble cysts in the microscope. All cultures were incubated in sterile 5 mI closed tubes (Nalgene cryovial; Nalge, Rotherwa, UK). BSK-H medium supplemented with rabbit serum was incubated at 30 cC and BSK-H medium supplemented with calf serum at 22 C. After 2 weeks of cultivation the tubes were centrifuged at 2,770 g for 30 min. to a mixture consisting of the sediment and 0.5 ml of the supcmatant, 4 ml of fresh medium was added. For cultures incubated at 30 C this process was continued in cycles of 2 weeks until an age of 3 months was reached and then every month until 14-18 months. For the cultivation medium which was incubated at 22 C the same procedure was performed every 1-3 months. .

Microscopic Examination of Cultures and CSF
All sediments were examined using dark field microscopy (DF) (2(X)--EOOX) and interference contrast microscopy (ICM) (800--2,COOx) (Zeiss Axiophot; Carl Zeiss, Oberkochen, Germany) to determine whether any bacteria or cysts were present.

Acridine Orange Staining (AO)
An air-dried and heat-fixed sediment of cultUred CSF (produced in the same manner as above) was incubated with acridine orange (SO mg/l in phosphate buffer, pH 6.4) for 4 minutes on a glass slide. Slides were subsequently rinsed in distilled water, air-dried and examined in the UV microscope (400-1,200x).

Transmission Electron Microscopy (TEM)
The CSF cultures were examined by TEM after 4-7 months of incubation in BSK-H medium at 22 C.
The examination by TEM was performed according to the following procedure. The culture was centrifuged at 14,(XX) x 9 for 20 min. The medium was removed and replaced with 2% glutaraldehyde in 0.2M cacodylate buffer (pH 73) and the sediment was fixed for 2 hours. The sediment was post-fixed in 1% osmium tetroxide in 02 M cacodylate buffer for 2 h. The pellet was dehydrated. infiltrated and embedded in conventional epoxy resin (LX-112 ; Ladd. Burlington, Vl~ USA) by a method described previously [14, 15]. Ultrathin sections were cut with a diamond knife (Jumdi;Juniper ultra Micro. Stockholm. Sweden) on an ultramicrotome (LKB 2088 UItrotom V) and mounted on 200 mesh copper rim. The sections were stained with 5% uranyl aretate in 30% ethanol for 20 min and with Reynolds lead citrate for 5 min. The sections were examined in a Jeol 1200 EX electron microscope to identify interesting structures.

The CSF samples were also processed for immunoelectron microscopy. The specimens were fixed in 4 '% paraformaldehyde in phosphate buffer pH 7.3 and the embedding was performed with LR- White using a method described previously [16]. After blocking nonspecific labeling [17], immunogold labeling was performed by applying a primary antibody against spirochetes (polyclonal anti-Borrelia with known reactivity to other Borrelia (cat. no: OWYH, Dade Behring. Marburg. Germany). dilution 1 : 2048 in 10% BSA. overnight incubation at 4 c. The

secondary immunoreagent, antibodies coupled to 10 nm colloidal gold particles (goat anti-rabbit IgG. Auroprobe EM Gar Gl0, Amersham, Little Chalfont, Bucks, England) was diluted I: 50 in 3% BSA and incubated 75 min at 22 c. After immunolabeling. the sections were stained with uranyl acetate and lead citrate and examined in the electron microscope as stated above.

Fisher's exact test was applied for the statistical comparison of the occurrence of cysts in MS patients and controls.

PCR
PCR was performed for the CSF from all patients using standard primers for the OspA gene of B. burgdorferi.

Analysis of IgG
Measurements of IgG in the CSF and serum were performed at the clinical chemistry laboratory. Vestfold Sentralsykehus. Tonsberg, Norway, according to established methods.
The IgG index was calculated automatically using the formula

$$\text{IgG index} = \frac{(\text{IgG in CSF}) \times (\text{albumin in serum})}{(\text{IgG in serum}) \times (\text{albumin in CSF})}$$

TABLE 1

Results
The main results are given in table 1. Cystic structures were observed in all the CSF samples from the MS patients. No cysts were detected in the CSF of any of the four controls by any method used. Cysts were detected by DF before cu1turing in eight of the MS CSF samples ($p < 0.007$) and in all the MS patients by TEM and AO ($p = 0.001$). AO and TEM were performed only after culturing. Cysts were also detected with all the methods in the CSF of the patient who had a history of erythema migrans.

Culturing
From the CSF of two MS patients, spirochete-like structures emerged after 5-7 months incubation and were visualized by DF and TEM (Figure 1). None of these structures could be cultivated on blood or chocolate agar plates incubated at 35 C in 5% CO2,microaerobic or anaerobic atmosphere for 5 days. DF,ICM. and AO By direct DF (Figure 2), ICM microscopy (after centrifugation) and AO, single cysts (1-5 fJm) and cysts in clusters were observed, some of which had core structures. When DF was performed before the culturing process relatively few cysts were observed, while the concentration of cysts was much higher after cultivation.
Figure 1. Curved spirochete-Iike bacteria (large arrow) have emerged after cultivating CSF in BSK-H medium. Cystic forms (medium size arrows) and blebs (small arrows) are also present. rEM. Bar = 500 nm.[Unable to display image]

Figure 2. Cysts from an MS patient examined by OF. Bar = 5 um. TEM

TEM revealed cystic structures in the spinal fluids of all MS patients and the patient with a history of erythema migrans (Figure 3). As seen in Figure 4, we observed cystic structures that were intensely imrnunolabeled with a polyclonal antispirochetal antiserum along the cell envelope. This antiserum was produced against B. burgdorfen, but is also known to react with other spirochetes (Treponema pallidum, Borrelia hermsii and Borrelia parkerii).

PCR

All CSFs gave a negative PCR-result with primers for the OspA gene of B. burgdorferi. The corresponding PCR for known spirochetal forms of B. burgdorferi was positive.

Figure 3 [Unable to display image] cysts from an MS patient observed nn the electron microscope. The cyst is filled with biological substances and some pycrIotic structures. TEM. Bar = 500 nm.

Discussion

Cysts were significantly associated with MS patients using each of the three different methods. This multi-method approach strengthens the association between CSF cysts and MS in a well-defined coastal area of southern Norway. There are two possible explanations for the association between MS and CSF cysts: the cystic structures are either the agents causing MS or they have appeared in the CSF as a consequence of MS.

The positive reaction with antispirochetal antiserum, the similarity of the cystic structures with cystic forms of spirochetes and the similarities between the cysts in the erythema migrans patient and the MS patients suggest that the patients were infected with a spirochete. The appearance of rod-like,slightly curved bacteria and spirochetes after culturing two of the CSF samples in BSK-H medium suggests the same. Spirochetes may vary in appearance and may sometimes emerge as rod-like structures [18]. The fact that only two spinal fluids gave rise to spirochete-like structures after culturing may be caused by the fact that cystic forms of spirochetes may often be difficult to convert to normal bacteria {11) and the BSK-H medium is not necessarily optimal for this possible unknown spirochete.

Figure 4a [Unable to display image] A cyst from an MS patient which is immunogold labeled with anti-Bonelia. There is a distinct and specific immunolabelng along the envelope of the cyst. TEM. Bar = 500 nm. Figure 4b. Larger magnification of the envelope of the cyst to illustrate the immunogold labeling. TEM. Bar = 200 nm.

It could be argued that the damage which MS caused in the brains of the patients had made them more vulnerable to spirochetal infection. But this does not seem a probable explanation. since all the MS patients had these cystic structures in their CFS. Other researchers have proposed that spirochetes could be the agents responsible for MS [5, 6, 8, 9]. For instance. Steiner [6] found spirochetes and granular bodies in brain autopsies of MS patients. These were proposed to belong to the genus BorreIia and were named Spirochaeta myelophthora [6].

We previously studied spirochetes (B. burgdorferi) that have converted from spirochetes to cystic forms in CSF in vitro using the same methods as mentioned above [11]. With all these methods used in this study (TEM, AO, DF), the cystic structures observed in the CSF of the MS patients are morphologically similar to cystic forms of spirochetes. We found that cysts which are produced by inoculating B. burgdorferi in CSF at 37 C can be PCR negative using conventional

DNA extraction and OspA primers (unpublished observation). This is either because the cyst wall inhibits the entrance to the genome or because the genomes of spirochetes have been changed. We have also to keep in mind that PCR detection of B. burgdorferi spirochetes often may give false-negative results [19).

The positive IgG index associated with MS in our patient cohort proves that the patients had an active inflammatory process in the CNS (Table 1). Inflammatory processes in the b~ and spinal cord of virtually any cause are usually less intense than inflammation in peripheral tissues and some microbiological agents, including spirochetes, provoke a very gentle inflammatory response [20, 21]. Considering the nature of MS, this disease could very well be a chronic infection and the clinical picture of MS has repeatedly been confused with neuroborreliosis [22-26). Therefore, we have both microbiological and some clinical support for the hypothesis that the cystic structures found in the CSF of the MS patients may originate from spirochetes which could be the causative agents of MS.

Considering the negative PCR for B. burgdorferi, the search for the etiology of MS should continue within the family of spirochetes. This study will encourage the efforts to identify the etiology of MS in different parts of the world and in larger patient cohorts.

Acknowledgments
We would like to thank Rolf Komeliussen and Frank Rl}rstad, Vestfold Sentralsykehus,Norway, for excellent technical assistance. The PCR analysis was performed by Andrew Jenkins PhD, A/S Telelab, Skien, Norway.

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