ELSEVIER

Neuroscience Letters 329 (2002) 91–95

Neuroscience Letters

www.elsevier.com/locate/neulet

Micrometer-sized particles in cerebrospinal fluid (CSF) in patients with schizophrenia

Lennart Wetterberg[a,b,*], Rolf Nybom[c], Trond Bratlid[b], Tormod Fladby[d], Berit Olsson[e], Hans Wigzell[e]

[a]Department of Psychiatry, The Karolinska Institute, St. Goran's Hospital, S-112 81 Stockholm, Sweden
[b]Department of Psychiatry, The University of Tromsø, N-9291 Tromsø, Norway,
[c]The University of Stockholm, Wallenberg laboratory, S-10405 Stockholm, Sweden
[d]Department of Neurology, Akershus University Hospital, N-1474 Norbyhagen, Norway
[e]Department of Immunology, The Karolinska Institute, S-17177 Stockholm, Sweden

Received 21 February 2002; received in revised form 17 May 2002; accepted 17 May 2002

Abstract

The etiology of schizophrenia is unknown, but the pathogenetic process involves organic changes in brain tissue, which may alter the composition of cerebrospinal fluid (CSF). For the present study, CSF was obtained by lumbar puncture from 22 schizophrenic patients and 38 control patients. We have used scanning electron microscopy combined with filtration techniques to search for pathogenic correlates and diagnostic biomarkers in the nano-micrometer range. Micrometer-sized spherical particles were isolated from CSF in 20 of the 22 patients with schizophrenia compared to only two of the 38 controls ($P < 0.001$). Reverse transcription-polymerase chain reaction analysis did not reveal bacterial DNA material in the particles. The particles have not replicated in culture. The micrometer-sized particles may serve as biological disease markers in schizophrenia. Hypothetically, they may be involved in development of the disease or may result from the disease process in brains of schizophrenic patients.

Keywords: Micrometer-sized particles; Cerebrospinal fluid (CSF); Schizophrenia; Reverse transcription-polymerase chain reaction (RT-PCR); Scanning electron microscopy (SEM)

Schizophrenia has a lifetime incidence of about 1% worldwide [8]. It is a severe psychiatric disease involving multiple cognitive domains including perception, executive functions, and affective and social behaviour. The etiology remains unexplained, although alterations in brain cytoachitecture, neuropil and regional loss of cortical tissue have been implicated [6,12].

We aimed to look for infectious agents as possible precipitating factors for schizophrenia in some patients. An initial observation, which led to the present study, was made some 10 years ago when we examined the cerebrospinal fluid (CSF) for virus particles, in particular human immunodeficiency virus (HIV), in a 34-year old schizophrenic, treatment resistant, male patient from northern Sweden. We did not find any sign of HIV or other viruses in the CSF of this patient, but instead spherical particles within the micrometer range, somewhat larger than known viruses, but smaller than known bacteria. To test whether similar findings were present in other patients with schizophrenia and/or in non-schizophrenic controls we have now used updated techniques for the analysis of small particles of unknown nature and origin in CSF. Abnormalities in CSF may reflect aberrations in the brain and its function in different disease states [2,5,9]. We have now re-examined CSF of the original patient, and added 21 new schizophrenic individuals and 38 control patients to test if they carry similar small particles in CSF as seen in the pilot patient.

The Ethics Committees of Health Region V in Norway at the University of Tromsø and of the University of Umeå, Sweden approved the study. After oral and written information about the investigation, its aim and possible discomfort reactions, all included subjects gave their informed consent according to the principles of the Declaration of Helsinki. The study was performed according to the guidelines of the local ethics committees.

* Corresponding author. Tel.: +46-8-256-970; fax: +46-8-256-970.
E-mail address: lennart.wetterberg@cns.ki.se (L. Wetterberg).

0304-3940/02/$ - see front matter
PII: S0304-3940(02)00580-3

The original 34-year old schizophrenic male patient was re-examined together with 21 added schizophrenic patients, six females (mean age 41.3, range 22–62 years) and 13 males (mean age 42.6, range 27–60 years) selected from the two northernmost counties in Norway and two male patients from one county in northern Sweden. The mean age of onset for schizophrenia was 23.7 years, range 16–33 years and the mean duration of illness at the time of examination 19 years, range 6–40 years. The patients were diagnosed according to the polydiagnostic computerized diagnostic system for psychosis, Operational Criteria Checklist for Psychotic Illness, that includes eight main diagnostic systems for schizophrenia and three systems for schizophrenic subgroups [14]. All 22 patients with psychiatric symptomatology fulfilled the criteria for schizophrenia according to DSM-III and DSM-III-R as well as the DSM-IV criteria. The patients were both in institutions and living in communities. The patients used different types of antipsychotic medication and one had been without any medication for 2 years.

Thirty-eight non-schizophrenic control individuals had a lumbar puncture at a neurological department as part of a diagnostic work-up. They were not matched for age. Twenty-three females (mean age 42.5, range 22–62 years) and 15 males (mean age 60.8, range 39–82 years) participated in the study.

CSF was obtained from the 60 subjects through lumbar puncture between vertebras L4 and L5 with the patients in horizontal positions. Most of the patients had travelled for some time from their homes or their treatment facilities before the examination. The skin in the lumbar region was thoroughly washed with sterile cotton swabs and chlorhexidin 5% (Nycomed, Oslo). One of the investigators (TF) performed the lumbar punctures of the patients, and most of the control punctures. Fine disposable needles were used (Becton Dickinson 20 GA 3.5 IN 0.9×90 mm) and identical procedures were used in both schizophrenic patients and controls. About two ml of CSF were sampled for analyzes in each of four plastic tubes (Nunc 10 ml). The samples were processed for analysis of alleged infectious particles, immunological examinations, and protein and cell content. CSF was allowed to drip in 0.5 ml portions in plastic tubes and tightened immediately after sampling with screw tops. The CSF-tubes were sent by air transport to Stockholm for examination. Two types of antipsychotic drugs in oil suspension (clopenthixol decanoate and perphenazine enanthate) used for injections in long-term medication in schizophrenic patients were also tested for possible interference with the CSF results. The preparations of the oil suspensions were examined according to the same method as the CSF samples as described below with completely negative results.

For scanning electron microscopic (SEM) examination a volume of 200 μl of cerebrospinal fluid was used. The CSF samples were added on the surface of a polycarbonate 0.6 μm filter (Nucleopore, Inc.), which was fitted to an airtight gadget (GP Plastindusti AB, Gislaved, Sweden). The filter was dried in room temperature, coated with 40 Å thick layer of ionized gold and analyzed in the SEM (Philips High Resolution SEM 515).

The method has previously been used and reported in studies of cytomegalovirus in urine by Andersson et al. [1] as well as cerebrospinal fluid of patients with HIV infection [13]. One important role of diagnostic electron microscopy, used in this study, is to identify and/or classify novel or emerging pathogens for which sufficient specific and sensitive tests are lacking. Electron microscopy of CSF from 20 of the 22 schizophrenic patients showed micrometer sized particles on the filter (see Fig. 1).

From CSFs where particles were detected, here called 'positive' CSF, 500 μl were centrifuged at 15 000 rpm for 10 min to obtain a pellet. The pellet was diluted in 50 μl distilled H_2O and 3 μl were used for reverse transcription-polymerase chain reaction (RT-PCR) study. The two primers NV1 and NV2, which covers the presence of most bacterial strains, were utilized [3]. Polymerase chain reaction amplification products were visualized on an ethidium bromide-stained agarose gel. The alleged bands were stained with ethidium bromide and the gel exposed to ultraviolet light.

The primers of the 16S ribosomal rRNA were used for direct diagnosis of possible bacterial origin in the spinal fluid after the treatment of the CSF samples as above [7]. The sample was considered bacterial if a DNA band was detected in the ethidium bromide-stained agarose gel.

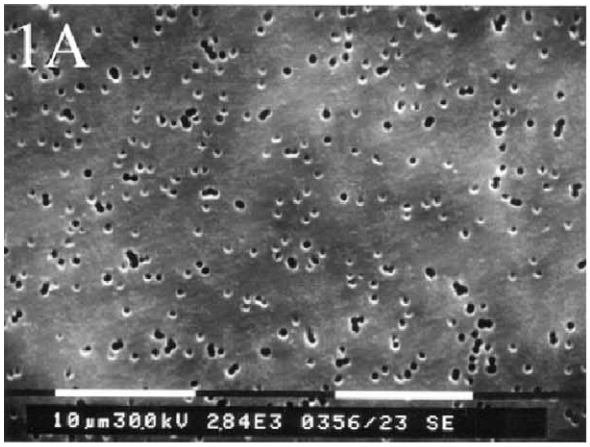
Cultures of CSF were done with 20% of CSF diluted in Dulbecco's modified minimum essential (GIBCO) without antibiotic and filtered through 0.22 μm syringe filters before culturing at +37°C in a CO_2 incubator. To test for proliferation of microbes we used a Leitz Diavert inverted microscope at an enlargement of 300 times. Weekly microscopic inspections during 8 weeks of culture did not reveal replication of the particles. CSF culture fluids of seven patients were also tested with the NV1/NV2 primers with negative results.

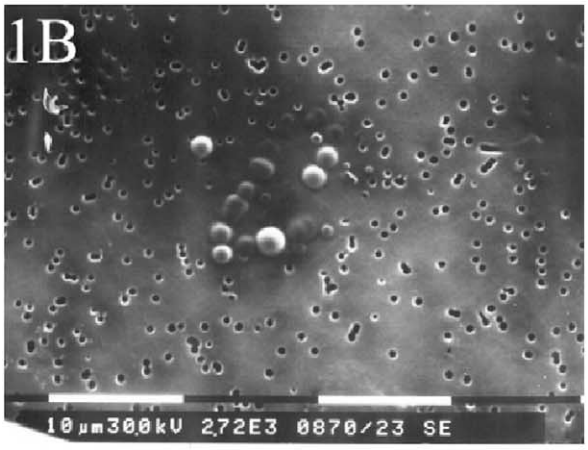
In addition a molecular spectral analysis of the possible oxygen content in the particles was performed using a JEOL-5900LV electron microscope combined with an INCA Energy 400 EDS-system from Oxford Instruments for quantification of molecules. The results show a clear correlation between increased presence of oxygen content and the location of the spherical particles as seen in Figs. 2A,C.

The main result is that 20 of 22 schizophrenic patients (91%) displayed micrometer-sized particles in CSF compared to only two of the 38 individuals in the non-schizophrenic control group of neurological patients (5%) ($P < 0.001$).

The isolation of the particles of CSF showed spherical shaped elements predominately of a size of 1 μm as seen in Fig. 1.

The 22 CSF samples of the schizophrenic and control





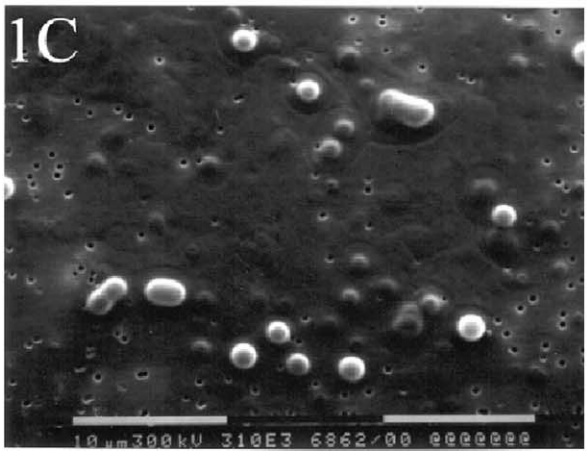
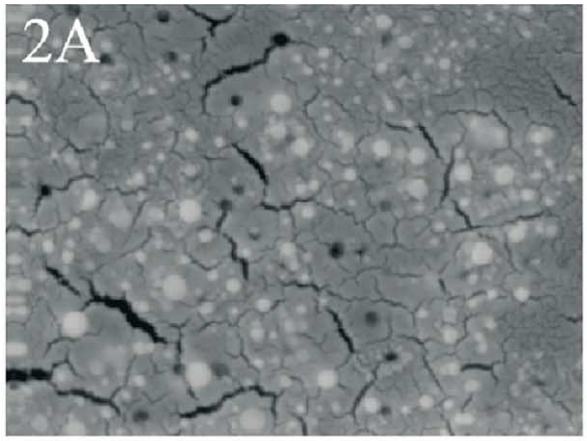
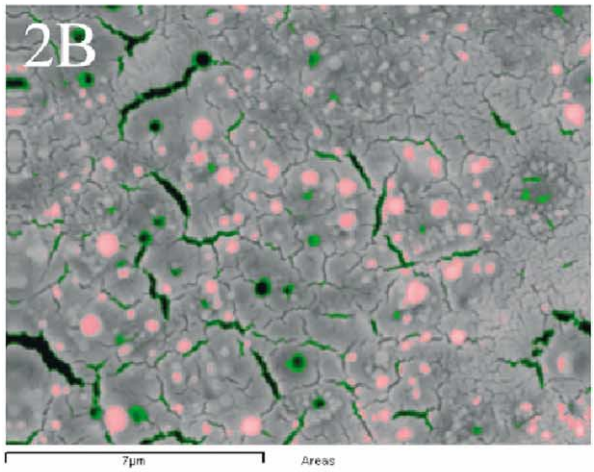


Fig. 1. (A) A polycarbonate filter with 0.2 ml CSF of a non-schizophrenic control person in whom no particles were detected (×2840). The diameter of the holes in the filter is 0.6 μm. A SEM was used (see text). The scale of the white and the black bars shown in the photos 1A–C is 10 μm. (B) Micrometer-sized spherical particles (×2720) directly isolated from CSF sampled in a Norwegian patient with schizophrenia are seen in the middle of the picture. (C) Spherical particles of similar size (×3100) as in Fig. 1B were found in CSF of a Swedish patient with schizophrenia.





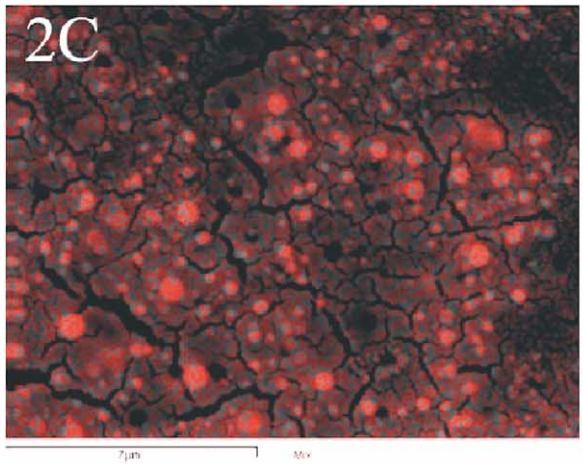


Fig. 2. (A) Scanning electron microscopy with X-ray spectrum processing of atomic elements of filtered CSF in a patient with schizophrenia. The spherical particles shown in white are enriched on the polycarbonate filter. (B) The area of the spherical particles, indicated in pink color, is calculated to 6% of the total microscopic field. (C) The weight-% of the oxygen (O) spectrum is shown in light red color. There is a clear correlation between increased presence of oxygen content and the location of the spherical particles (compare with Fig. 2A). The scale of the bars seen below the photos is 7 μm.

patients that contained micrometer-sized particles were analyzed for bacterial DNA content with primers of the 16S ribosomal rRNA in a random manner [3]. None of these samples were positive for bacterial DNA in the PCR amplification analysis. As a methodological control in the same experiment a culture of *Escherichia coli* bacteria was used as reference of the sensitivity of the assay. *E. coli* bacteria were diluted in 100 μl of H_2O and nine different dilutions were used from 1:10, 1:100, etc., up to 1:100 000. Even 100 000 times dilution of the *E. coli* culture gave clear bands when the NV1- and NV2-primers were used proving the efficacy of the method to detect small amounts of bacterial DNA. Cultures of CSF of the 20 samples with micrometer-sized particles at +37°C for more than 8 weeks failed to demonstrate replication.

The two CSF samples of the 38 non-schizophrenic patients displayed similar micrometer-sized spherical particles as the CSFs of 20 of the 22 schizophrenic patients. Neither of these two patients had any apparent complaints or signs or symptoms of psychotic disease. One of these control patients, a 39-year-old female had a lumbar puncture as part of an investigation of paraesthesias, urinary retention and neurogenic bladder dysfunction and the other, a 55-year-old male was investigated for vertigo, paraesthesias and reduced motor control (for details see below).

Control patient (1) was a female, 39 years old and had two sisters with Bechterews' disease. She had an appendectomy in 1981. She had a history of urinary retention and abdominal pain since 1993. Repeated investigations with clinical testing, lumbar puncture, brain computerized tomography (CT) scan and magnetic resonance imaging of cerebrum and spinal cord did not reveal signs of disease. During the year preceding the last lumbar puncture, she developed paraesthesias in hands and feet. A neurological examination revealed a left patellar hyperreflexia. Clinically, she also was judged to be slightly depressed presumed to be due to long-lasting pain.

Control patient (2) was a male, 55 years old, with no history of previous disease except a gastrointestinal infection (*Shigella flexneri*) aged 52 and repeated episodes of low back pain on one occasion radiating in a right S1 distribution. Apart from this, complaints suggesting central nervous system affection started at 51 years of age with vertigo (positional), numbness in the right foot and (subjectively) reduced control of his right foot and left hand. He had a normal neurological examination, normal brain CT scan, and neurophysiological testing (Visual Evoked Potentials, Brainstem Auditory Evoked Responses). The electroencephalogram-registration showed slightly increased theta-wave activity with a bilateral temporal distribution.

In the present study we demonstrate that the majority of schizophrenic patients (91%) have micrometer-sized particles in CSF compared to 5% in the non-schizophrenic controls ($P < 0.001$). Among the present 38 controls were patients with degenerative and inflammatory diseases of different kinds. The two control patients without schizophrenic symptoms who had similar micrometer-sized particles in their CSFs as the schizophrenic patients had pronounced neurological symptoms, but essentially normal supplementary investigations.

The majority of negative control-CSFs suggest that the micrometer-sized particles are not debris of unspecific type, formed as part of any general infective, degenerative or inflammatory process. At the present level of specificity, our findings are consistent with the broad range of organic brain disturbances suggested to play a role in the development of schizophrenia [10,11]. The size of the particles in the present study is at least an order of magnitude larger than the previously described 'nanobacterias' [4]. Neither culture nor RT-PCR analysis for 16S rRNA revealed microbiosis. Though the control of *E. coli* bacteria diluted 100 000 times gave clear results, the particles may still be of a microbiotic origin not detected by the primers we used.

The present study indicated that spherical formed micrometer-sized particles are found significantly more often in CSF of schizophrenic patients compared to controls. This finding suggests that the particles may serve as biomarkers for schizophrenia specifically or indirectly reflecting the pathogenic processes in the brain of schizophrenic patients.

The present study was supported by a grant from the Swedish Medical Research Council (MFR) to Hans Wigzell. The advice with RT-PCR by Gudmunder Gudmundsson and with SEM spectroscopy by Michael Andersson, Link Nordiska AB, Lidingö, Sweden is gratefully acknowledged.

[1] Andersson, J., Nybom, R., Larsson, P., Andersson, U., Britton, S. and Ehrnst, A., Rapid detection of cytomegalovirus using immune scanning electron microscopy, J. Virol. Methods, 16 (1987) 253–262.
[2] Davson, H., Hollingsworth, G. and Segal, M.B., The mechanism of drainage of the cerebrospinal fluid, Brain, 93 (1970) 665–678.
[3] Head, I.M., Hiorns, W.D., Embley, T.M., McCarthy, A.J. and Saunders, J.R., The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences, J. Gen. Microbiol., 139 (1993) 1147–1153.
[4] Kajander, E.O. and Ciftcioglu, N., Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation, Proc. Natl. Acad. Sci. USA, 95 (1998) 8274–8279.
[5] Karlsson, H., Bachmann, S., Schroder, J., McArthur, J., Torrey, E.F. and Yolken, R.H., Retroviral RNA identified in the cerebrospinal fluids and brains of individuals with schizophrenia, Proc. Natl. Acad. Sci. USA, 98 (2001) 4634–4639.
[6] Lawrie, S.M., Whalley, H.C., Abukmeil, S.S., Kestelman, J.N., Donnelly, L., Miller, P., Best, J.J., Owens, D.G. and Johnstone, E.C., Brain structure, genetic liability, and psychotic symptoms in subjects at high risk of developing schizophrenia, Biol. Psychiatry, 49 (2001) 811–823.
[7] Ludwig, W., Bauer, S.H., Bauer, M., Held, I., Kirchhof, G., Schulze, R., Huber, I., Spring, S., Hartmann, A. and Schleifer, K.H., Detection and in situ identification of representa-

tives of a widely distributed new bacterial phylum, FEMS Microbiol. Lett., 153 (1997) 181–190.
[8] Mortensen, P.B., Pedersen, C.B., Westergaard, T., Wohlfahrt, J., Ewald, H., Mors, O., Andersen, P.K. and Melbye, M., Effects of family history and place and season of birth on the risk of schizophrenia, N. Engl. J. Med., 340 (1999) 603–608.
[9] Nilsson, C., Lindvall-Axelsson, M. and Owman, C., Neuroendocrine regulatory mechanisms in the choroid plexus-cerebrospinal fluid system, Brain Res. Rev., 17 (1992) 109–138.
[10] Sawa, A. and Snyder, S.H., Schizophrenia: diverse approaches to a complex disease, Science, 296 (2002) 692–695.
[11] Schultz, S.K. and Andreasen, N.C., Schizophrenia, Lancet, 353 (1999) 1425–1430.
[12] Selemon, L.D. and Goldman-Rakic, P.S., The reduced neuropil hypothesis: a circuit based model of schizophrenia, Biol. Psychiatry, 45 (1999) 17–25.
[13] Sonnerborg, A., Nybom, R., Britton, S., Ehrnst, A., Forsgren, M., Larsson, P.H., Strannegard, O. and Andersson, J., Detection of cell-free human immunodeficiency virus in cerebrospinal fluid by using immune scanning electron microscopy, J. Infect. Dis., 159 (1989) 1037–1041.
[14] Wetterberg, L. and Farmer, A.E., Clinical polydiagnostic studies in a large Swedish pedigree with schizophrenia, Eur. Arch. Psychiatry Clin. Neurosci., 240 (1991) 188–190.