Coronaviruses in spinal fluid of patients with acute monosymptomatic optic neuritis

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Acute monosymptomatic optic neuritis (AMON) may occur as an isolated event, but is often considered an initial symptom of multiple sclerosis (MS). About half of the patients will convert to clinically definite MS within 15 years (1). AMON frequently lasts from a few days to several weeks and may affect one or both eyes. The main symptoms are loss of vision, retrobulbar pain and dyschromatopsia. The presence of human coronaviral RNA has been demonstrated in brain tissue and spinal fluid from patients with clinically definite MS and controls (2-4). To investigate the possibility of an infection with human coronaviruses (HCV) in early MS and as a possible cause of AMON we have analyzed cerebrospinal fluid (CSF) from patients with AMON using reverse transcriptase reaction and the polymerase chain reaction (RT-PCR) applying primers specific for HCV.

Materials and methods

CSF was obtained by lumbar puncture from 37 untreated patients with AMON. None of the patients had other symptoms of MS at the time of lumbar puncture. Thirty-four samples were obtained from 6 to 104 days (median 23 days) after the onset of symptoms. Three patients had 2 attacks of AMON and the samples were obtained in relation to the first episode 205, 182 and 71 days before their second episode of AMON. In 2 patients different eyes were involved and in 1 patient the same eye. Thirteen of the patients have later developed clinically definite MS. CSF obtained from 15 surgical patients with protrusion of the intervertebral discs were used as controls. The median age of patients and controls with AMON was 33 (range 13-57) and 51 (range 35-66) years respectively (median test P<0.001). In the AMON group there were 25 female patients and among the controls there were 8 females. In the RT-PCR assay, a pool of 14 HCV negative spinal fluids, from the routine laboratory, were interspersed at every 3 to 4 patient samples and were included throughout the whole procedure starting with the RNA-extraction. Further negative controls, sterile water instead of sample, were added at each enzymatic reaction, starting with one in the RT-reaction ending with 3 in the second PCR with the inner set of nested primers.
As positive controls for the PCR, cell culture supernatant fluids containing HCV strain 229E (ATCC VR-740, TCID\textsubscript{50} 10\textsuperscript{-3}) and HCV strain OC43 (TCID\textsubscript{50} 10\textsuperscript{-3}, ATCC VR-759) were used. All samples were stored at −80°C until analysis.

A multiplex nested RT-PCR as detailed below was used, using primers specific for both HCV-229E and HCV-OC43 (Table 1).

RNA extraction was performed by lysis of 100 μl spinal fluid or serum by vortexing 10 s in 400 μl GuSCN-buffer (4 mM guanidiniumisothiocyanate, 1 mM dithiothreitol, 10 mM TRIS, pH 6). Isopropanol (500 μl) was added, vortexed briefly and spun in a microcentrifuge at 13,000 g for 10 min at room temperature. For the serum samples, complete removal of supernatant after the isopropanol precipitation was important to avoid a sticky protein precipitate following the ethanol step. To prevent a sticky GuSCN-buffer (4 mM guanidiniumisothiocyanate, 1 mM dithiothreitol, 10 mM TRIS, pH 6) was used, using primers specific for both HCV-229E and HCV-OC43 (Table 1).

DNA from each sample was isolated by using the PhotoGene detection kit according to the manufacturer’s instructions (Life Technologies, Rockville, USA). The sequence reactions were separated and analyzed on an Applied Biosystems 373A Automated DNA Sequencer as recommended by the manufacturer. For sequence editing, the Seqed software (Applied Biosystems) was used. Alignment of the sequence results was done using the GCG software package (6).

Table 1. Primers used in this study specific for the nucleocapsid protein gene of the human coronaviruses. The primers COR09, COR10, COR11 and COR12 were designed for this study.

| Primer | Type | Sequence (5’-3’) | Bases | Ref. |
|--------|------|-----------------|-------|------|
| COR01 | f,i  | agggccgaaa attcgaang ccag | 488–521 | (7) |
| COR02 | r,i  | aagggcggc tgaactgcag aagc | 793–806 | (2) |
| COR09 | r,o  | cggcgagcgg ccagccagcgc | 400–418 | (2) |
| COR10 | r,o  | aagggcggc ggctggggcc | 823–842 | (2) |
| COR05 | f,i  | ccagcgagcc gtcatttcgc | 215–238 | (7) |
| COR06 | r,i  | gcgcgctcgg tggcgcgcgtgc | 481–520 | (2) |
| COR11 | f,o  | gcgcgctcgg tggcgcgcgtgc | 103–174 | (7) |
| COR012 | r,o | cgatgctgctc ggctggggcc | 527–540 | (7) |

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The sensitivity of the PCR assay was measured by samples containing 10 fold dilutions of the virus containing cell culture supernatants in coronavirus negative spinal fluid. The sensitivity was 10 times TCID\textsubscript{50} for both HCV-229E and HCV-OC43.

The PCR products were visualized by electrophoresis at 5 V/cm on 1.5% agarose followed by Southern blot on Nytran filter. The blot was performed as described (5), with the exception that the setup was inverted. After overnight transfer, the Nytran filter paper was rinsed briefly in 2×SSC, and nucleic acids were immobilized by baking at 80°C in vacuum for 2 h. Subsequently, the blots were prehybridized at 42°C for 2 h, followed by hybridization overnight with a HCV-229E or a HCV-OC43 DNA fragment. These probes were produced by PCR as described above from the reference strains and labelled with biotin-7-dATP by nick-translation (BioNick, Life Technologies, Rockville, USA). Stringent wash was performed in 0.15 SSC for HCV-229E and in 0.2 SSC for HCV-OC43 at 70°C. Probe detection was performed using the PhotoGene detection kit according to the manufacturer’s instructions (Life Technologies, Rockville, USA).

The PCR products were sequenced in both directions using primers COR01 and COR02 as sequencing primers, cycle sequencing reaction using the primers COR01 and COR02 using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Perkin Elmer Cetus, Connecticut, USA). The sequence reactions were separated and analyzed on an Applied Biosystems 373A Automated DNA Sequencer as recommended by the manufacturer. For sequence editing, the Seqed software (Applied Biosystems) was used. Alignment of the sequence results was done using the GCG software package (6).
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Table 2. Results of nested RT-PCR for HCV-229E RNA

| Patient group                  | RT-PCR on CSF |
|-------------------------------|---------------|
|                               | Positive | Negative | Total |
| AMON                          | 4 (11%)  | 33       | 37    |
| Protrusion of the intervertebral disk | 1 (7%)   | 14       | 15    |
| Total                         | 5 (10%)  | 47       | 52    |

Results

CSF from 4 patients and 1 control (Table 2) were positive on nested RT-PCR using the HCV-229E primers and all samples were negative with the HCV-OC43 primers. The amplified DNA fragments were only visualized after hybridization and not on the ethidium bromide stained gel, indicating that very few RNA copies had been present in the viruses in CSF from patients with AMON has not previously been investigated. In the present study, positive results due to contamination less likely, as the enzymatic step also remained negative. Water controls added for each procedure from RNA extraction to the inner PCR remained negative. Water controls added for each enzymatic step also remained negative.

In the amplified fragments all 261 bases between the primers (Fig. 1) were sequenced. One patient (AMON28) had a frameshift and a base substitution compared to the published sequence (7) used for the design of the primers, but it is however identical to another available sequence of the same gene (8).

Discussion

To our knowledge the presence of human coronaviruses in CSF from patients with AMON has not previously been investigated. In the present study 11% of the patients with AMON and 7% of the controls were positive for HCV-229E and none for HCV-OC43. We consider the possibility of false positive results due to contamination less likely, as uracil-N-glycosylase carry over prevention was used and the negative controls were consistently negative.

On sequencing of the PCR products 2 base substitutions and 1 frameshift mutation were found. Furthermore, 1 control patient had an ambiguous sequencing result. The error rate of PCR using the Taq polymerase has been estimated to be between 1/10^7 and 1/10^8 nucleotides or less depending on the initial number of target copies (9, 10) and the reaction conditions. Also the RT step may incorporate errors into the template for the PCR. In this study 6 PCR products of 261 nucleotides equal to 1566 nucleotides were sequenced (Fig. 1) and 4 differences were found. This gives a frequency of 1 mutation per 392 bases. From these few differences cannot be distinguished between true genomic variation and experimental errors in PCR or DNA-sequencing. The nucleocapsid gene of coronaviruses seems well conserved. There is only one published sequence of the ATCC type strain HCV-OC43. Comparison of HCV-OC43 and bovine coronavirus nucleocapsid gene (6, 11, 12) show a 96.6% homology of the coding sequences of 1347 bases, and only 6 differences over the 258 bases between the inner primers amplified in this study. Thus large genomic variation may not be expected, even in strains originating from different mammal species. Concerning HCV-229E there are two published sequences of the nucleocapsid gene from the same ATCC type strain (7, 8). HCV-229E is not as closely related to other known coronaviruses (7), and the natural sequence variation in clinical strains has not been studied.

Another study (4) has also investigated CSF with PCR-technique for HCV. The CSF from patients with MS and controls with other neurological disease were analyzed for the presence of human coronaliral RNA sequences. The study found the CSF from 10 of 20 patients with MS and 9 of 10 controls positive for HCV-OC43. The CSF from 7 of 20 patients with MS and 2 of 10 controls were positive for HCV-229E. Thus in total more than
80% of the tested patients were virus positive. This very high frequency of positive results also in the control is not consistent with the findings in our study. The difference could be explained by differences in sensitivity of the assays or by differences in the selection of the control group. The study by Cristallo et al. (4) does not describe the level of sensitivity, and the control group in the present study consists of patients with protrusion of the intervertebral disk and not of patients with other degenerative neurological diseases.

Conclusion
HCV-229E RNA was found in the CSF by RT-PCR in 4 of 37 patients with AMON and in 1 of 15 controls. The frequency of positive samples was low and there was no statistical difference between AMON and controls. This study does not provide evidence for an etiological role of human coronaviruses in acute monosymptomatic optic neuritis or in early MS.

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