The possible role of enteroviral persistence in the etiology of the chronic fatigue syndrome (CFS) was investigated by serological testing, VP-1 antigen testing, and polymerase chain reaction (PCR) analysis of stool specimens as well as by viral cultures of stool—both direct and after acid treatment. No differences between 76 patients with disabling unexplained fatigue and 76 matched controls were found by serological or antigen testing. Furthermore, no enteroviruses were isolated from any stool culture. Enterovirus was detected by PCR in one stool specimen from a patient with CFS but was not detectable in a second sample obtained from the same patient 3 months later. All stool specimens from controls were PCR-negative. These results argue against the hypothesis that enteroviruses persist in patients with CFS and that their persistence plays a role in the pathogenesis of this syndrome.

Materials and Methods

Patients. Seventy-six patients and 76 healthy controls were examined. Patients were randomly chosen from a database of 298 patients with CFS. (This group, which was self-referred, has been described in detail by Vercoulen et al. [9].) All patients reported severe, unexplained, debilitating fatigue of at least 1 year’s duration. The enrolled patients came to our outpatient clinic twice within a 3-month interval. On the second visit, each patient was accompanied by a neighbor of the same gender and within 2 years of the same age; this control was selected by the patient. Patients and controls visited our clinic between December 1991 and April 1992—a period covering months during which the incidence of infections due to enteroviruses is usually low in the Netherlands. Samples of blood and stool were obtained from patients and controls. All patients underwent a physical examination and an extensive laboratory work-up and completed a questionnaire that solicited information on age, sex, and duration and nature of symptoms.

Antibody-capture ELISA. A broadly reactive antibody-capture ELISA was used for the detection of IgG, IgM, and IgA antibodies to enteroviruses [10]. A strongly positive serum sample, a “cutoff” sample, and a negative control sample were included in each test. The cutoff value was based on the assumption that at most 5% of serum samples from 200
healthy blood donors would be reactive. Thus levels were scored as positive (greater than the cutoff value) only when they exceeded those in a healthy population. Serum samples from patients and controls were tested simultaneously in order to minimize the day-to-day variability of the assay.

Complement fixation test. The CF test was performed according to the microtiter technique described by Casey [11]. The enteroviral antigen used in this test (Behringwerke AG, Marburg, Germany) actually consisted of a mixture of antigens, including those of coxsackieviruses B1 through B5; coxsackievirus A9; and echoviruses 4, 6, 9, 14, 24, and 30. The CF titer was expressed as the reciprocal of the highest dilution showing 50% hemolysis. Serum samples from patients and controls were tested simultaneously.

VP-1 test. The VP-1 antigen test was performed at the laboratory of Dr. J. F. Mowbray (Department of Immunopathology, St. Mary’s Hospital Medical School, London). The enterovirus group-reactive monoclonal antibody 5-D8/1 was used [12]. Serum samples from patients and controls were randomly numbered for blind testing.

Isolation of virus from stool specimens. A 100-mL suspension of feces in PBS (20%, wt/vol) was prepared from every stool specimen. Chloroform (10%, vol/vol) was added, and the mixture was shaken vigorously for 20 minutes. Supernatants were clarified by centrifugation at low speed (2,000 g). A 5-mL volume of supernatant was used to inoculate duplicate tubes containing human fetal lung fibroblasts (no. 002; Flow Laboratories, Rockville, MD) and tertiary kidney cells from cynomolgus monkeys [13] (0.1 mL of supernatant and 0.25 mL of culture medium per tube). After absorption of the inoculum for 1 hour at 37°C, the inoculum was decanted and replaced by 1 mL of the following culture medium: minimal essential medium (MEM) with 3% fetal bovine serum (FBS), 20 mM HEPES (pH 7.5), 2 mM glutamine, 50 μg of gentamicin/mL, 100 μg of vancomycin/mL, and 1 μg of amphotericin B/mL. The tubes were incubated at 37°C and were examined for cytopathic effect (CPE) every 3 days for 2 weeks. A blind passage was undertaken after 1 week.

The remaining 95-mL volume of supernatant was centrifuged in two bottles at 150,000 g for 3 hours. The supernatants were removed. One pellet was resuspended in 2.5 mL of MEM with 3% FBS and stored at −70°C for acid elution. The other pellet was stored directly at −70°C for PCR.

Nested PCR. Primers were selected from the 5′ nontranslated region, which is highly conserved among enteroviruses. The primers were 100% homologous to the known enteroviral RNA sequences. RNA was extracted from the concentrated fecal pellet by the acid guanidinium thiocyanate-phenol-chloroform procedure (RNAzol B method; Cinna/Biotexc Laboratories, Houston, Texas), as described by Chomczynski and Sacchi [14].

Reverse transcription was performed essentially as described by Zoll et al. [15], with primer 4 as the reverse primer and primer 1 as the sense primer (see Table 1). The amplified product had a length of 484 base pairs (bp). For nested PCR, a 10-μL volume of the first PCR reaction product was added to a new PCR mixture containing 50 pmol of nested primers 2 and 3 and was subjected to an additional 40 cycles of amplification. A positive amplification resulted in a product of 155 bp. Each test included five controls: a DNA control (cDNA of coxsackievirus B1), an RNA control (RNA of coxsackievirus B1), a negative control (diethyl pyrocarbonate [DEPC]–treated distilled water), and RNA isolated from stool specimens from two healthy controls. The reactions were analyzed by electrophoresis of 25 μL of the PCR reaction product on a 1.5% agarose gel stained with ethidium bromide and subsequent Southern blot hybridization as described previously [15], with use of an end-labeled oligonucleotide probe (table 1).

Isolation of virus after acid elution. Acid elution was performed as described by Yousef et al. [1] with slight modifications. In brief, a 1.5-mL volume of 0.1 M glycine/HCl buffer (pH 2.4) was added to 2.5 mL of concentrated stool extract, and the pH was readjusted to 2.4 with 0.5 M HCl. The mixture was incubated at room temperature for 3 hours and then centrifuged over a 30% sucrose cushion at pH 2.4 for 3 hours at 150,000 g. The pellet was resuspended in 2 mL of MEM with 3% FBS, and the pH was adjusted to 7.0 by the addition of 0.5 M NaOH. The suspension was then inoculated in duplicate onto tertiary monkey kidney cells and human fetal lung fibroblasts (1 mL of concentrated stool extract plus 2.5 mL of culture medium) seeded in 25-cm² flasks (no. 690160; Greiner GmbH, Frickenhausen, Germany). After absorption for 1 hour at room temperature, the inoculum was decanted, and a 5-mL volume of culture medium was added to the flasks, which were subsequently incubated at 37°C. The cultures were examined for CPE every 3 days for 6 weeks. Blind passages were done after 2 and 4 weeks.

Results

Demographic and clinical information. The mean age of the 76 patients was 40 years (median, 41 years; range, 20–66 years); the mean age of the 76 controls was 41 years (median, 42 years; range, 19–67 years). The male-to-female ratio for patients was 1:3. The majority of patients reported myalgia, muscular weakness, impairment of memory, and problems

Table 1. Enteroviral primer sequences used for nested PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence</th>
<th>Location*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-CAAGCACTTCTGTTTCCCCGG-3'</td>
<td>160–180</td>
</tr>
<tr>
<td>2</td>
<td>5'-CTTCTCGGCCCCCTGAATGCG-3'</td>
<td>445–464</td>
</tr>
<tr>
<td>3</td>
<td>5'-ATTGTCACCATAAGCAGCCA-3'</td>
<td>452–465</td>
</tr>
<tr>
<td>4</td>
<td>5'-CACCGGATGGCCAATCCA-3'</td>
<td>482–501</td>
</tr>
<tr>
<td>5</td>
<td>5'-TGTGTCGTAACGGGCAACTCTGCACCGGAAA-3'</td>
<td>509–538</td>
</tr>
</tbody>
</table>

* Positions refer to the coxsackievirus B1 sequence of Iizuka et al. [16].
with concentration and sleeping. In 90% of cases, the criteria for CFS delineated by Sharpe et al. [17] were fulfilled. The average duration of symptoms was 11 years (median, 7 years; range, 2–45 years). Sixty-six percent of patients reported an acute onset of the manifestations of CFS after an infectious illness. Patients were not part of a cluster but came from all parts of the Netherlands (both rural and urban areas).

Serological results. The results of the antibody-capture ELISA for IgG, IgM, and IgA antibodies to enteroviruses were similar for patients and controls ($P > .05$, Wilcoxon rank sum test). Likewise, the CF test detected no significant difference between patients and controls ($P = .69$, $\chi^2$ analysis). VP-1 antigen was detected in serum samples from 16 patients and 17 controls. There was no correlation between the presence of VP-1 antigen and the presence of enteroviral IgG, IgM, or IgA (figures 1–3); of the eight patients whose sera were positive for enteroviral IgG, IgM, and/or IgA (table 2), only one had serum positive for VP-1 antigen. Moreover, no correlation was found between the outcome of virological tests and the duration, nature, or acuteness of onset of symptoms.

Nested PCR. Nested PCR was used for the analysis of concentrated stool extracts from all 76 patients and all 76 controls. An amplification product was visualized by gel electrophoresis in one sample from a patient with CFS and in no samples from controls; the single positive result was confirmed by Southern blot hybridization (figure 4). The patient for whom this result was obtained had a negative result in the VP-1 antigen test and in serological tests. No enteroviral RNA was detected in a second stool specimen obtained from this patient 3 months later.

Isolation of virus from stool specimens. All direct cultures of stool for enteroviruses gave negative results. Acid elution with subsequent culture was undertaken for 23 patients and 22 controls who had either a high antibody titer (i.e., a titer higher than those of 95% of healthy blood donors) or a positive result in the VP-1 antigen test; for the patient with a positive PCR result; and for a randomly selected group of 22 of the remaining 52 patients. The virological findings for these patients are presented in table 2. No virus was isolated.

Discussion

To investigate the possible role of enteroviral persistence in CFS, we examined serum and stool specimens from 76 patients with CFS and 76 controls matched for gender, age, and neighborhood. (We used neighborhood controls to correct for the potential effects of local epidemics of infection due to enteroviruses.) We found no differences between patients and controls in either the prevalence or the titer of enteroviral antibodies, whether assayed by the CF test or by an antibody-capture ELISA broadly reactive with different serotypes [10]. These results are in agreement with those of Miller et al. [8], who found no difference between the IgM and IgG serum antibody titers of patients with CFS and those of controls. Like Lynch and Seth [6] and Halpin and Wessely...
Table 2. Virological findings for patients and controls with cultures performed after acid elution.

<table>
<thead>
<tr>
<th>Test: value given</th>
<th>Random patients (n = 22)</th>
<th>Selected* patients (n = 24)</th>
<th>Selected* controls (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody-capture ELISA: total no. positive</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>IgG: mean ratio (no. &gt; cutoff)</td>
<td>0.45 (0)</td>
<td>0.70 (5)</td>
<td>0.84 (5)</td>
</tr>
<tr>
<td>IgM: mean ratio (no. &gt; cutoff)</td>
<td>0.55 (0)</td>
<td>1.11 (5)</td>
<td>0.70 (4)</td>
</tr>
<tr>
<td>IgA: mean ratio (no. &gt; cutoff)</td>
<td>0.26 (0)</td>
<td>0.37 (2)</td>
<td>0.43 (3)</td>
</tr>
<tr>
<td>CF test: geometric mean (median) titer</td>
<td>39.5 (32)</td>
<td>51.3 (32)</td>
<td>53.6 (32)</td>
</tr>
<tr>
<td>VP-1 antigen: no. positive</td>
<td>0</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Culture: no. positive</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCR: no. positive</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Selected on the basis of a high antibody level and/or a positive VP-1 test.
† Extinction of test sample divided by extinction of cutoff serum sample.

[7], we failed to find differences in the prevalence of VP-1 antigen in serum samples from patients and controls. These results are in contrast to those obtained by Yousef et al. [1], who found VP-1 antigen in the serum of 44 of 87 patients with postviral fatigue syndrome (PVFS) but in no serum samples from controls.

Enteroviral RNA was detected by PCR in a concentrated stool specimen from only one patient with CFS and in no such specimens from controls. A stool sample obtained 3 months later from the patient with a positive result was negative for enteroviral RNA. Several research groups have shown that the limit of detection of enteroviruses in fecal samples by PCR is as low as 1 pfu [18, 19]. The sensitivity of the PCR technique described by our group [15] was found to be as low as 10 genome equivalents in a model system but was ~20 pfu in biological samples. Use of nested PCR increased the sensitivity to <1 pfu [20, 21]. However, we and other investigators [21, 22] have found that fecal samples may inhibit the reverse-transcription or the amplification step. Negative PCR results for fecal samples should therefore be interpreted with caution.

After an acid dissociation step, Yousef et al. [1] isolated enteroviruses from stool specimens from 17 of 76 patients with PVFS; these patients' direct cultures had been negative. However, we isolated no enteroviruses from stool specimens from 46 selected patients and 22 selected controls by the same technique. Even the patient with a positive PCR result had a negative culture after acid elution. Thus, either the enterovirus involved in this infection is difficult to isolate in cell culture, or PCR is generally more sensitive than culture after acid elution. Consistent with a low incidence of enteroviral infections in the winter, all direct cultures were negative.

Several investigators have found enteroviral RNA sequences in muscle-biopsy specimens from patients with PVFS [2-4]. In these studies PVFS was defined as severe fatigue starting after an acute episode of an apparently viral illness and lasting for >1 year [1-4] without laboratory evidence of an initial infection as required by the definition of Sharpe et al. [17]. In cooperation with Drs. J. W. Gow and P. O. Behan (Department of Neurology, Southern General Hospital, Glasgow, United Kingdom), we recently tested muscle-biopsy specimens from their patients with PVFS and from controls. We detected enteroviral RNA in several samples. However, the significance of such findings is difficult to interpret since not only patients but also a number of controls had positive results ([4] and authors' unpublished results). The discrepancy between the latter findings and those

Figure 4. Gel electrophoresis and Southern blot hybridization of stool specimens subjected to nested PCR in a study of patients with CFS (lanes 1–4) and matched controls (lanes 5 and 6). Lane 7 depicts diethyl pyrocarbonate–treated water; lane 8, coxsackievirus B1 RNA positive control; lane 9, coxsackievirus B1 cDNA positive control; and lane 10, size marker pBR322 digested with HindIII. The larger fragments in lanes 1, 8, and 9 are the results of amplification with primers 1 and 4. Arrows indicate the 155-bp fragment specific for amplification of enterovirus.
obtained in the study reported herein may be explained by epidemiological differences in viral incidence between the United Kingdom and the Netherlands. Alternatively, it may reflect differences in methods of patient selection and in case definition.

We used the case definition of CFS on which consensus has been reached in the United Kingdom [17]; this definition is different from that of PVFS. Still, 66% of our patients reported an acute onset of their symptoms after an infectious illness and would qualify as having PVFS as defined by Yousef et al. [1] and Gow et al. [4]. The only difference between our patients and those in the studies from the United Kingdom is the longer average duration of illness in the former. It is unlikely that this difference accounts for the negative results obtained in our study, however, because there was a considerable overlap in the duration of illness documented in the three studies. There was no correlation between the duration of symptoms or the acuteness of onset of symptoms and the outcome of virological tests in our study. Gow et al. found no correlation between the duration of PVFS and PCR positivity [4]. A similar discrepancy is apparent in the incidence of enteroviruses in association with the inflammatory myopathies polymyositis and dermatomyositis: investigators in the United Kingdom have reported the persistence of enteroviruses in patients with these conditions [3, 23, 24], whereas researchers from other geographic areas (including our group in the Netherlands) have been unable to detect enteroviral RNA in muscle-biopsy specimens from these patients [25–27].

In summary, we conclude that persistence of enteroviruses is unlikely to play a role in the development of CFS.

Acknowledgments

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References