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Detection of Enterovirus-Specific RNA in Serum: The Relationship to Chronic Fatigue

Geoffrey B. Clements, Francis McGarry, Carron Nairn, and Daniel N. Galbraith
Regional Virus Laboratory, Ruchill Hospital, Glasgow, United Kingdom

The serum of 88 chronic fatigue patients was screened for enteroviral specific sequences by polymerase chain reaction (PCR) assay. The PCR method used was "nested" PCR targetting the 5′ nontranslated region of the enteroviral genome which yielded a final fragment length of 264 base pairs. Samples were obtained from patients during 1990–1991. In addition, buffy coat specimens and stool specimens were examined in some patients. Samples from two cohorts of comparison individuals were also obtained. The comparison groups were firstly, acutely ill individuals with symptoms consistent with a presumed enteroviral infection (matched by age, sex, and date of receipt of specimen) and secondly, healthy individuals (matched by age and date of receipt of specimen). Enteroviral specific sequences were detected in 36 of 88 serum samples from chronic fatigue patients, 22 of 82 acutely ill individuals, and 3 of 126 healthy individuals. The enteroviral PCR positivity did not correlate with any one particular feature of chronic fatigue nor did it reflect any history of illness at onset of fatigue, duration of fatigue, or age of patient. These results provide new evidence for the presence of enteroviral specific sequences in serum, buffy coat, and stool samples in many patients with chronic fatigue. This may reflect a persistent enterovirus infection in a proportion of chronic fatigue patients.

KEY WORDS: polymerase chain reaction, persistent enteroviral infection, serum analysis

INTRODUCTION

Patients commonly present with incapacitating fatigue without there being an identifiable physical cause [Komaroff, 1993]. A number of terms have been used to describe this situation, for example, epidemic neuromyasthenia, benign myalgic encephalomyelitis, and postviral fatigue syndrome among others. The term chronic fatigue (CF) describes the situation most appropriately and guidelines have been established to facilitate diagnosis and research [Holmes et al., 1988; Sharpe et al., 1991]. The main symptom is excessive fatigue for at least 6 months which may be made worse by exercise and cause significant reduction in exercise tolerance. The symptoms are present for more than 50% of the time and a definite time of onset is described. There is also a range of other features which may be present, such as mental fatigue, disturbance of mental functioning, mood/sleep disturbance, or excessive sweating. There is no objective diagnostic test for CF and a diagnosis has to be reached by clinical assessment and exclusion of organic disease. CF has been described worldwide and there are descriptions as far back as adequate medical records are available [Shorter, 1993]. The disorder is endemic with occasional epidemics and an infective causation has been suggested [Manu et al., 1993]. There have been attempts to establish a link with a number of viruses including Epstein-Barr virus, human herpesvirus 6 (HHV-6), human retroviruses, and enteroviruses [Buchwald et al., 1992; DeFreitas et al., 1991; Holmes et al., 1987; Yousef et al., 1988]. Human enteroviruses are established aetiological agents in a range of diseases, many of which involve the musculoskeletal or nervous systems, and are widespread throughout the world [Tracy et al., 1991]. For a number of years there has been accumulating evidence of an association between CF and enterovirus infection [Cunningham et al., 1990]. This association, having been made initially on epidemiological grounds, was subsequently followed up using serology [Bell et al., 1988; Bell and McCartney, 1984; Miller et al., 1991]. The direct detection of specific virus sequences is now possible using the highly sensitive polymerase chain reaction (PCR) which we have previously used to detect enterovirus specific sequences in muscle biopsies of CF patients [Gow et al., 1991]. This paper presents an extension of this work using serum samples from CF patients and two comparison groups, one of hospital inpatients with symptoms of acute virus infection and the other of indi-

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Address reprint requests to Geoffrey B. Clements, Regional Virus Laboratory, Ruchill Hospital, Glasgow G20 9NB, United Kingdom.

Francis McGarry's present address is Department of Rheumatology, Royal Infirmary, Glasgow.

viduals in the community with no recent evidence of virus infection.

MATERIALS AND METHODS

Patients and Controls

The 118 CF patients in the study were referred by their general practitioner to the outpatients clinic of the Department of Infectious Disease and Tropical Medicine at Ruchill Hospital where they were examined by a clinician and a diagnosis of CF established. In this group 181 samples were tested. This included a serum and/or buffy coat and/or stool sample for each patient where available. For inclusion in the study the patients had to fulfill Oxford criteria for chronic fatigue syndrome [CFS; Sharpe et al., 1991]. The patient's case history was recorded and samples were taken for analysis at the Regional Virus Laboratory, Ruchill. The demographics of the study population were 41 males (average age 36.5 years, standard deviation 11.0, age range 17–62) and 77 females (average age 36.6 years, standard deviation 12.3, age range 12–65). Each patient included in the study was matched as closely as possible to two comparison individuals. The first comparison group (A) was composed of patients (n = 101) admitted to Ruchill Hospital with symptoms suggestive of an acute enteroviral disease with sudden onset of symptoms such as headache, rash, and/or pyrexia, but no fatigue. In this group 114 samples of serum and or stools from each patient were available for testing. This group was matched by age, sex, and date of specimen receipt. The date of specimen arrival deviated by plus or minus 3 months, and patients age deviated by a maximum of plus or minus 3 years. The second comparison group (B) was composed of individuals (n = 126) from whom serum had been obtained for occupational health or ante-natal screening purposes. This group was assumed to be healthy and was age matched to within plus or minus 3 years and date of specimen received to within plus or minus 1 month. This group was mainly female and therefore it was not possible to completely sex match the group.

Specimens

Specimens taken included venous blood, heparinised blood, and stools. The venous blood was separated into clot and serum. Buffy coat cells were prepared from whole blood by adding 3 ml lymphoprep (Gibco BRL), spinning at 1,500 rpm for 30 minutes, and twice washing the lymphocyte layer with 20 ml phosphate buffered saline (PBS). The cells were resuspended in 2 ml virus transport medium (VTM) and stored at −70°C. Stool extracts were prepared by suspending 2 g of faecal material in 10 ml PBS and spinning at 2,500 rpm for 40 minutes. The supernatant was removed for testing. All samples were requested from each patient, however, some patients did not supply all samples required.

RNA Extraction

RNA was extracted from serum (200 μl), stool extract (1,000 μl), and tissue culture cell material following the methods outlined by Sambrook et al. [1989] and Chomczynski and Sacchi [1987].

PCR

The PCR was carried out using primers from the conserved 5′ nontranslated region that detect a wide range of enteroviral types [Gow et al., 1991; Zoll et al., 1992]. For the first round PCR, primers P1 and P4 were used giving rise to a band of 414 base pairs and for the internal PCR, primers P6 and P9 were used giving a band of 264 base pairs in length. The Ableson tyrosine kinase gene primers, ABL 1 and ABL 2, were used as a confirmation of successful RNA extraction giving rise to a band of 218 base pairs.

P1 5′CGG TAC CTT TGT GCG CCT GT 3′
P4 5′TTA GGA TTA GCC GCA TTC AG 3′
P9 5′TCA ATA GAC TCT TCG CAC 3′
P6 5′GCA CTT CTG TTA CCC C 3′
ABL1 5′CAG CGG CCA GTA GCA TCT GAC TT 3′
ABL2 5′TGT GAT TAT AGC CTA AGA CCC GGA G 3′

All primers were high-performance liquid chromatography (HPLC) purified, supplied by Oswell DNA services (Edinburgh).

The PCR carried out was based on the methods outlined by Gow et al. [1991], except that 1 μl random hexamer oligonucleotides pd (N)6 (Pharmacia) diluted 1:100 in TE buffer (Sigma-Aldrich Ltd.) was used in place of the antisense primers in the reverse transcription step. Experiments were conducted with positive and negative controls (coxsackie A9 infected MRC-5 cells and uninfected MRC-5 cells, respectively) to exclude false-positive and negative results. All samples were renumbered, mixed, and assayed blind.

Data Handling

Patient's previous history, present symptoms, and results of laboratory tests as well as PCR results were stored using the data manager software package DataEase version 4.5 (1992) supplied by DataEase U.K. Ltd.

Statistical analysis was performed on the data using the odds ratio test (P value for alpha error = 0.05) following the methods outlined by Gardner and Altman [1989].

RESULTS

In 93% of CF patients some type of acute illness was described prior to the onset of fatigue. The most common type of illness prior to fatigue was a respiratory/influenza type illness, however other symptoms were reported (Fig. 1). The "other illnesses" category included six cases of glandular fever, four cases of varicella zoster virus (VZV), and four cases of rubella infection. Due to the time interval between the onset of the illness and the time of interview the reliability of this information may be doubtful. All patients fulfilled the minimum criteria of 6 months of fatigue, in many cases the duration of fatigue described was much longer. The

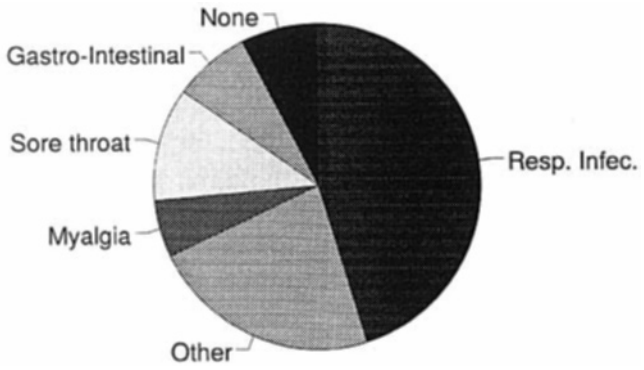

Fig. 1. The proportion of patients describing symptoms prior to fatigue.

average reported for males was 35.4 months and 26.6 months for females, within a range of 6 months to 11 years. In five cases no starting date for the fatigue was available.

Specific features of the clinical presentation were selected for detailed analysis. These were distinctive and were largely as defined at the Oxford consensus meeting [Sharpe et al., 1991]. The features reported by the patients are listed in Figure 2 in which there is a wide range of reporting frequency of each symptom ranging from 4% (difficulty in passing urine) to 64% (poor concentration). The seven features most frequently complained of were poor concentration, sleep disturbance, poor memory, stiffness in arms or legs, headache, and depression. All of these features were noted in over 40% of CF patients. These seven features can be divided into two general headings: firstly, neurological impairment including poor memory, poor concentration, headache, sleep disturbance, and depression; and secondly the musculoskeletal features of stiffness in arms/legs and myalgia. Consideration was also given to the degree of fatigue as patients frequently describe variation in this symptom. This was categorised into severe, moderate, or mild. The majority (67%) of CF patients fell into the moderate category, with 11% describing their fatigue as severe and 12% as mild. All CF patients indicated how they perceived their current status in respect of their overall illness. The majority (66%) reported no change, there was a tendency towards improvement in 22% of patients, while the remaining 12% had a slight or severe worsening of features.

A specimen was deemed positive by PCR if a band of 264 bases in length was visualised after 'nested' PCR, and if a band of 214 base pairs was identified by the ABL PCR (Table I). There was no demographic or clinical difference between the patients who provided specimens of each sample type. In 41% of serum, 27% of buffy coat samples, and 48% of the stool samples of CF patients there were detectable enterovirus specific sequences. Where patients provided both serum and buffy coat specimens (44 samples from 22 patients), 53% of the PCR results were in concordance. In the comparison groups 27% of the serum samples from acutely ill patients (comparison group A) and 2% of the healthy patients (comparison group B) were positive. In comparison group A, 28% of the stool samples were positive for enteroviral specific sequences. Analysis of the data revealed there was a significant difference at the 95% level between the findings on CF serum samples and either comparison groups A (odds ratio = 2.01, 95% confidence interval [1.06–3.82]) or B (odds ratio 30.8, confidence interval [2.4–43.0]). There was, however, no significant difference between the CF stool results and comparison group A stool results. In the CF group the results using serum (88) and buffy coat (62) samples were pooled. Patients were thus categorised as PCR positive if a serum or buffy coat sample was positive by enterovirus specific PCR. Further analysis of the group was performed on the basis of the CF patients being either positive or negative. On this basis, of the 118 patients, 50 (42%) were enteroviral PCR positive.

The enterovirus PCR status and history of some type of acute illness at onset of fatigue are correlated in Table II. In both the enterovirus PCR positive and negative patients the largest numbers of patients were those complaining of a general respiratory infection prior to the onset of CF. There is no significant difference between the patients who were enterovirus PCR positive and negative with respect to the history of an acute illness at onset.

The method of PCR used in this study is not quantitative, but an indication of the initial quantity of enteroviral sequences can be inferred from the presence of a band in the first round of the PCR using only P1 and P4 primers. None of the comparison group samples yielded a first round positive sample (Table I). Thirteen first round bands were observed from samples from CF patients suggesting that in these samples there was a high starting number of enteroviral sequences compared with the other samples which were only positive after the second round of PCR. Twelve CF patients were identified with positive first round analysis, one patient had both stool and serum first round positive results. The feature most commonly complained of was stiffness or pain in the joints, back, or chest of which eight patients complained. Two patients had noted a recent slight worsening of their features whereas the others did not report any recent change or recent illness. The patients who demonstrated enteroviral sequences in their stool samples were found to have similar features of CF to the general CF study group. It would have been expected that the proportion of PCR positive and negative patients complaining of each symptom be the same as the overall ratio of positives and negatives, i.e., 42:58. The numbers for each feature fell broadly into this ratio (Fig. 2). For some symptoms, e.g., rash, the numbers of PCR positive and negative patients are equal, whereas with other symptoms, e.g., poor concentration, the number of PCR negative patients exceeds the number of positive patients. These differences were however not statistically significant. Of the seven most common features, the five which

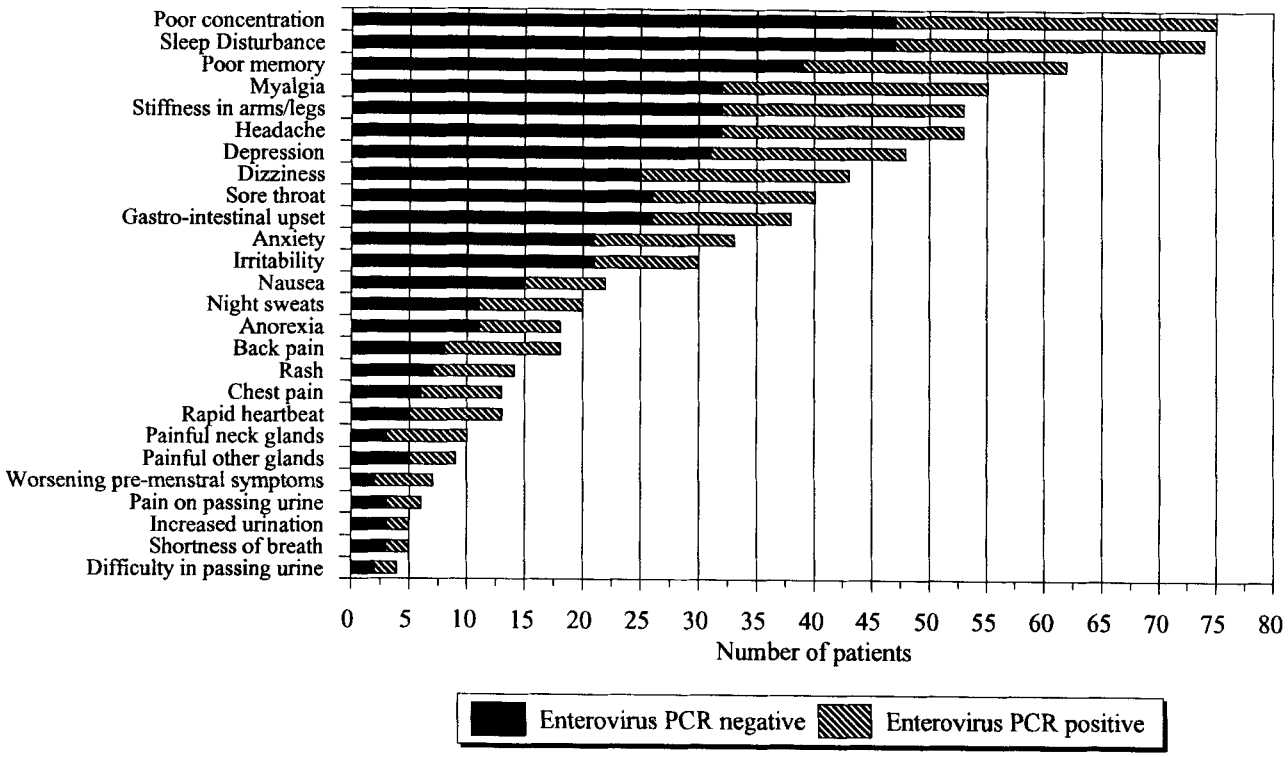

Fig. 2. Proportion of patients demonstrating particular features.

TABLE I. Results of PCR on Specimens From CF Patients and Comparison Groups

	First round PCR			Second round PCR	
Specimen	Number	Positive	Negative	Positive	Negative
CF serum	88	5	83	36	52
Comparison A serum	82	0	82	22	60
Comparison B serum	126	0	126	3	123
CF stool	31	2	29	15	16
Comparison A stool	32	0	32	9	23
CF buffy coat	62	6	56	17	45
Total	421	13	408	102	319

were neurological in origin had a higher number of PCR negative patients than expected; whereas the two musculoskeletal features were close to the expected ratio.

DISCUSSION

This study took place in 1990–1991 and during this time a survey on the diagnosis of CF in the West of Scotland by general practitioners (GP) was carried out [Clements, 1991]. The overall frequency of the diagnosis was 0.1% (1,154 CF cases out of just over 1 million patients on the GP's lists) with 70% being female and 30% male, a figure which is in line with other surveys [Sumaya, 1991; Archard et al., 1988]. The sex ratio of the patients in the current study was almost identical with a predominance of females and there was a broad age distribution with a predominance in the 30–40-

TABLE II. Enteroviral PCR Results From CF Patients and a Previous History of Illness at Onset of Fatigue*

	Enterovirus PCR positive	Enterovirus PCR negative
No previous illness	1	7
Respiratory infection	21	27
Gastrointestinal upset	3	5
Myalgia	2	4
Sore throat	6	6
Other infections	11	13
No history given	6	6
Total	50	68

*In the PCR amplification technique a specimen was deemed positive if a band of 264 bases in length was visualised after 'nested' PCR, and if a band of 214 base pairs was identified by the ABL PCR. In the CF group the results using serum(88) and buffy coat(62) samples were pooled to categorise patients as either PCR positive or negative. Patients with either serum or buffy coat positive by enterovirus specific PCR were called positive.

year-old group. When the patients in this study were examined by sex and the duration of symptoms, it became apparent that the excess of females was only seen in patients with a history of less than 2 years of fatigue. There was no overall difference in the symptoms described by the males and females. The reason for this sex difference is unclear. It may be trivial, for example females may have more contact with GPs for general family reasons and may be more ready to complain. However, there are well-documented sex differences in response to some infectious agents, e.g., tuberculosis. This difference is being further examined as part of a prospective study. The pattern of symptoms described by the patients in this study was similar to those described elsewhere, e.g., Buchwald and Komaroff [1991]. The majority of patients described symptoms commencing after an acute infection which categorises their condition as a postinfectious fatigue syndrome. The most common symptoms were myalgia, sleep disorders, depression, and headache.

The epidemiology of CF has suggested that an infective agent (or agents) may be the cause and it is well documented that infectious mononucleosis and influenza may lead to a protracted convalescence. The possible involvement of enteroviruses particularly coxsackie B has been pursued for a number of years initially using serological markers of infection [Bell et al., 1988] and more recently by nucleic acid hybridisation [Cunningham et al., 1990; Buchwald and Komaroff, 1991]. Recently PCR became available and in a previous study using muscle biopsies 53% of CF patients were positive for enteroviral sequences compared to 15% of controls [Gow et al., 1991]. In the study described here using serum specimens as well as some stool and buffy coat samples, enteroviral sequences were found in significantly more CF patients than in the two comparison groups. The presence of the enteroviral sequences in a significant number of the patients points to some role in CF. Relating any particular symptom to the presence or absence of enteroviral sequences did not yield conclusive results. The absence of enteroviral sequences in some of the CF patients at the time of sampling may be due to episodes of virus infection or activity or alternatively the fatigue in these patients may have another aetiology. The sensitivity of the PCR system used is such that approximately 10 copies of the genome would be expected to be detected [Gow et al., 1991]. It is considered that enteroviruses are normally eliminated from the body quickly in normal healthy individuals. A variety of immunological disturbances have been reported for CF patients which may relate in some way to the enteroviral persistence [Buchwald et al., 1992; Holmes et al., 1987; Caligiuri et al., 1987; Klimas et al., 1990]. The findings in the comparison groups were as expected. The higher proportion of enterovirus positive patients with acute viral illness (group A) compared with the healthy patients (group B) probably relates to their presenting illness. The background level in group B is evidence of a low level of enteroviral activity which may or may not be associated with overt illness. Subsequent studies have indicated that this background level of approximately 1–2% is uniform without much seasonal variation over a period of years.

The primers used for the PCR analysis are specific to the 5′ nontranslated region of the enteroviral genome [Gow et al., 1991]. This region has a high degree of conservation within the enterovirus family. The PCR therefore detects coxsackievirus, poliovirus, and echovirus—most members of the enteroviral family—but does not detect rhinoviruses. A sequence analysis of the PCR products will be presented separately.

This study provides evidence for the involvement of enteroviruses in just under half of the patients presenting with CF and it confirms and extends previous studies using muscle biopsies. Further information which may lead to the ability to predict outcome may become available from a longitudinal study on these patients which is currently in progress. At present it is unclear what the mechanism leading to the fatigue and other symptoms in these patients is. There may be involvement of muscle directly or of the immune system or a direct effect on the central nervous system. The previous study using muscle biopsies could not differentiate between the presence of virus in muscle and serum or circulating lymphocytes. We provide evidence for the presence of viral sequences in serum in over 40% of CF patients and also in some buffy coat cells and stool samples.

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