

Detection of Enterovirus in Human Skeletal Muscle From Patients With Chronic Inflammatory Muscle Disease or Fibromyalgia and Healthy Subjects

Fatima Douche-Aourik,¹ Willy Berlier,¹ Léonard Féasson,² Thomas Bourlet,¹ Rafik Harrath,¹ Shabir Omar,¹ Florence Grattard,¹ Christian Denis,² and Bruno Pozzetto^{1*}

¹Laboratory of Bacteriology-Virology (GIMAP), Faculté de Médecine Jacques Lisfranc, Saint-Etienne, France

²Laboratory of Physiology (PPEH), Faculté de Médecine Jacques Lisfranc, Saint-Etienne, France

Enterovirus RNA has been found previously in specimens of muscle biopsy from patients with idiopathic dilated cardiomyopathy, chronic inflammatory muscle diseases, and fibromyalgia or chronic fatigue syndrome (fibromyalgia/chronic fatigue syndrome). These results suggest that skeletal muscle may host enteroviral persistent infection. To test this hypothesis, we investigated by reverse transcription-polymerase chain reaction (RT-PCR) assay the presence of enterovirus in skeletal muscle of patients with chronic inflammatory muscle diseases or fibromyalgia/chronic fatigue syndrome, and also of healthy subjects. Three of 15 (20%) patients with chronic inflammatory muscle diseases, 4 of 30 (13%) patients with fibromyalgia/chronic fatigue syndrome, and none of 29 healthy subjects was found positive. The presence of VP-1 enteroviral capsid protein was assessed by an immunostaining technique using the 5-D8/1 monoclonal antibody; no biopsy muscle from any patient or healthy subject was found positive. The presence of viral RNA in some muscle biopsies from patients exhibiting muscle disease, together with the absence of VP-1 protein, is in favor of a persistent infection involving defective viral replication. **J. Med. Virol. 71:540–547, 2003.**

© 2003 Wiley-Liss, Inc.

KEY WORDS: enterovirus; chronic inflammatory muscle diseases; fibromyalgia; persistence; RT-PCR

mellitus [Frisk, 2001]. The mechanisms by which enterovirus induce a chronic infection are still poorly understood. The tropism of enteroviruses for cardiomyocytes is well established both in mice and in humans, and these viruses were shown to persist in the heart from patients with chronic myocarditis or dilated cardiomyopathy [Rabausch-Starz et al., 1994; Andréoletti et al., 1997; Kandolf et al., 1999]. Skeletal muscle can also be infected persistently by enterovirus, as evidenced in murine models [Tam et al., 1991, 1994]. In man, chronic inflammatory muscle disease, i.e., polymyositis and dermatomyositis, are linked to autoimmune disorders [Miller, 1994] and enterovirus have been proposed among the triggering agents [Rosenberg et al., 1989; Yousef et al., 1990; Dalakas, 1995]. In addition, mice infected with Coxsackievirus B1 (CV-B1) may present a chronic myositis that is very similar to human polymyositis [Ytterberg et al., 1988]. In addition, the role of enterovirus in the pathogenesis of chronic fatigue syndrome and fibromyalgia, noninflammatory muscular syndromes with dominant fatigue, and pain symptoms, respectively [McCluskey and Riley, 1992; Buchwald, 1996], has been largely disputed [Yousef et al., 1988; Abbey and Garfinkel, 1991; Bowles et al., 1993; Lindh et al., 1996]. The detection over a long period of time of enterovirus structural protein VP-1 in the serum [Yousef et al., 1988] or enterovirus RNA in muscle biopsy specimens [Archard et al., 1988] of patients with chronic fatigue syndrome provides evidence of a persistent enterovirus infection, at least in some of these patients. The study by Gow et al. [1994], which investigated a large number of muscle biopsies from patients with either chronic fatigue syndrome or

INTRODUCTION

Infection in humans by Enterovirus, although often asymptomatic, is responsible for a wide range of acute diseases. In addition, these agents are possibly involved in the genesis of chronic enterovirus pathologies, including chronic myocardial diseases, postpoliomyelitis syndrome, and even juvenile-onset (type 1) diabetes

*Correspondence to: Pr. Bruno Pozzetto, Laboratoire de Bactériologie-Virologie, CHU de Saint-Etienne, Hôpital Nord, 42055 Saint Etienne Cedex 2, France.

E-mail: bruno.pozzetto@univ-st-etienne.fr

Accepted 7 July 2003

DOI 10.1002/jmv.10531

Published online in Wiley InterScience
(www.interscience.wiley.com)

neuromuscular disorders, demonstrated the presence of enteroviral RNA by RT-PCR in 26.4% and 19.8% of samples, respectively. In light of these data, it can be assessed that enteroviruses can persist in pathologic muscle. However, the involvement of these agents in chronic inflammatory muscle diseases remains unclear. Furthermore, no data are available regarding the presence of enteroviral RNA in muscle of healthy subjects.

In this study, we investigated the presence of enterovirus by RT-PCR and immunohistochemistry in muscle biopsies taken from three populations of subjects: patients suffering from chronic inflammatory muscle diseases, patients suffering from fibromyalgia or chronic fatigue syndrome, and healthy subjects. No evidence of enterovirus infection was found in the latter group. In contrast, the presence of viral RNA in some muscle biopsies from patients exhibiting muscle disease, together with the absence of VP-1 protein, were in favor of a persistent infection involving defective viral replication.

MATERIALS AND METHODS

Patients and Controls

Muscle biopsies were selected retrospectively from a bank of frozen specimens. Seventeen patients were female and 28 were male. The median age at time of biopsy was 48.5 years (range, 29–82). They were divided in two groups. The first group consisted of 15 patients presenting a chronic inflammatory muscle diseases diagnosed according to acknowledged clinical and histological criteria [Engel et al., 1994], and including cases of polymyositis (7), dermatomyositis (6), granulomatous myositis (1), and inclusions body myositis (1) (group A). The second group consisted of 30 patients suffering from myalgia and/or chronic fatigue diagnosed as fibromyalgia (22) and chronic fatigue syndrome (8), respectively, according to defined clinical criteria [Buchwald, 1996], and whose muscle biopsy exhibited neither histological nor immunohistochemical lesions (group B).

A third group (group C) included 29 healthy volunteers as controls; all of them but one were male. The median age at time of biopsy was 30.9 years (range, 20–84).

Muscle Biopsy

The muscle biopsy was carried out in the vastus lateralis or deltoid muscle, using a Weil-Blakesley

alligator forceps, as previously described [Féasson et al., 1997]. For the two groups of patients, biopsies were taken as part of routine diagnosis investigations. Healthy volunteers were fully informed of the procedures before giving written consent; the protocol had been approved by the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale 1-1 Rhône Alpes Loire, in accordance with the declaration of Helsinki. The specimens were kept frozen in liquid nitrogen until required. The mean time of sample storage was 3 years (range, 0.5–8).

Histological Analysis

The tests were carried out on 10- μ m-thick frozen transversal sections. For diagnosis, histological preparations included hematoxylin-eosin-safran (HES) and Gomori's trichrome. NADH-tetrazolium reductase, myosin ATPase, cytochrome C oxidase, and succinodehydrogenase were used when histological inflammatory lesions were present. Immunohistochemical preparations were carried out using anti-CD4, anti-CD8, anti-CD68, and anti-C₅b₉ antibodies. The biopsies from patients of groups B and C were selected on the absence of inflammatory, myopathic, metabolic, and neurogenic lesions.

RNA Extraction

RNA was extracted from pooled sections of each biopsy sample following the guanidinium thiocyanate-phenol-chloroform procedure [Chomczynski and Sacchi, 1987] with Tri-Reagent[®] (Sigma-Aldrich, Saint-Quentin, France). The resulting pellets were dried, dissolved in diethylpolycarbonate-treated water combined with 15 U of RNase inhibitor (Stratagene Europe, Netherlands) and stored at –80°C. RNA extracted from 150 μ l of cell-free culture supernatant containing about 10²–10³ TCID₅₀ of Coxsackievirus A9, Coxsackievirus B2, or Echovirus 30 was used as positive controls. For each RNA extraction assay, 150 μ l of water was used as negative control.

RT-PCR

In this study, 100–200 ng of total RNA was used for reverse transcription (RT). RNA was copied into cDNA under a volume of 20 μ l containing 1 \times reaction buffer (Stratagene), 0.2 mM of dNTP, 10 pmol of negative sense primer RNC2 (Eurogentec, Angers, France) (Table I),

TABLE I. Primers and Probes Used in the Study

Designation	Sequence	Application	Reference
RNC2	5'-CACCGGATGGCC-3'	EV reverse transcription	Rotbart, 1990
NC1	5'-CTCCGGCCCCTGAATGCG-3'	EV PCR, sense primer	Rotbart, 1990
E2	5'-ATTGTCACCATTAAGCAGCCA-3'	EV PCR, antisense primer	Rotbart, 1990
S08	5'-AAACACGGACACCCAAAGTA-3'	Detection of EV PCR products	Rotbart, 1990
GAPDH1	5'-TGAAGGTCGGAGTCAACGGATTGGT-3'	GAPDH PCR, sense primer	Ercolani et al., 1988
GAPDH2	5'-CATGTGGGCCATGAGGTCCACCAC-3'	GAPDH PCR, antisense primer	Ercolani et al., 1988

EV, enterovirus; PCR, polymerase chain reaction.

10 U of RNase inhibitor (Stratagene) and 25 U of MMLV reverse transcriptase (Stratagene). After a 45-minute incubation at 37°C, the reaction mixture was heated to 95°C for 5 min, and then cooled at 4°C for immediate use. Two μ l of the cDNA was added to 48 μ l of PCR reaction mixture containing 1X PCR buffer (Gibco-BRL, Life Technologies, Cergy Pontoise, France), 3 mM of $MgCl_2$, 0.2 mM of dNTP, 20 pmol of positive sense primer NC1, 20 pmol of negative sense primer E2 (Eurogentec) (Table I) and 5 U of Taq polymerase (Gibco-BRL). PCR amplification was carried out in a 9600 Perkin-Elmer thermal cycler as follows: 94°C for 5 min; 40 cycles at 94°C for 15 sec, 60°C for 15 sec and 72°C for 15 sec; then 72°C for 5 min. The integrity of each RNA sample was tested by amplification of the GAPDH mRNA using primers GAPDH1 and GAPDH2 (Eurogentec) (Table I) and an one-step RT-PCR method carried out in a single tube using a mixture of recombinant Taq DNA polymerase and SuperScript II H-RT[®] (Gibco BRL) as follows: 52°C for 15 min; 95°C for 5 min, 40 cycles including 15 sec at 94°C, 15 sec at 60°C and 15 sec at 72°C; then 72°C for 5 min.

PCR Detection Systems

For each GAPDH RT-PCR product, 10 μ l was loaded on a 1% agarose gel (TEBU, Le Perray-en-Yvelines, France) in 1 \times TEA buffer, stained with ethidium bromide (0.4 mg/ml), and visualized on an ultraviolet (UV) transilluminator. The detection of enterovirus products of RT-PCR amplification was carried out by hybridization assay on microtiter plates according to the manufacturer's protocol (HybridowellTM universal, Argene Biosoft, Varilhes, France). Briefly, after sample denaturation, DNA was directly coated on a microtiter plate and hybridized with a hybridization buffer containing the biotinylated oligonucleotide probe S08 (100 ng/ml) (Eurogentec) (Table I). The hybrids were detected by using a streptavidin-peroxidase conjugate and a tetramethylbenzidine substrate. The result was considered positive if the OD₄₅₀ value was ≥ 0.2 , it was confirmed positive if at least two of three determinations were found positive. For the PCR techniques, two independent persons (FAD and WB) carried out the same experiments in parallel.

Immunohistochemistry

The immunohistochemical staining used for the detection of VP-1 antigen [Yousef et al., 1987; Samuelson et al., 1995] was carried out using a polymer/peroxidase conjugate applied on the frozen tissue sections according to a method adapted from Li et al. [2000]. The enterovirus group-specific monoclonal antibody (MAb) 5-D8/1 (DAKO, Trappes, France) directed toward the VP-1 capsid protein and diluted 1:50 (2 μ g/ml) in Tris-bovine serum albumin (BSA), was applied together to the EnVisionTM/HRP detection system (DAKO). An anti-adenovirus group-specific MAb (Argene-Biosoft) diluted 1:250 (2 μ g/ml) was used as negative control. Paraffin-embedded heart muscle tissue sections from

patients with enterovirus-associated dilated cardiomyopathy (as demonstrated by both RT-PCR and detection of VP-1) were used as positive controls.

Statistical Analysis

The Fisher exact test was used for comparing qualitative variables. Means were compared by using variance analysis and Student's *t*-test. Statistical significance was reached for a *P*-value of <0.05 .

RESULTS

In preliminary experiments, different amounts of muscle biopsy were tested using the GAPDH primers to determine the sensitivity of the extraction step. At least 10 sections of each biopsy (corresponding to a total amount of 0.5–1 mg of skeletal muscle) were necessary to amplify successfully a 983-bp fragment corresponding to the GAPDH mRNA (Fig. 1).

To determine the sensitivity of the single-step RT-PCR technique described above, this assay (using primers NC1 and E2, Table I) combined to hybridization was compared to the RT-nested PCR technique followed by a gel electrophoresis described by Kämmerer et al. [1994] on serial dilutions of CV-B2. As both tests exhibited the same sensitivity of $\sim 10^{-2}$ TCID₅₀ (data not shown), the former was chosen in further experiments to avoid contamination known to occur with techniques using nested amplification.

In a further set of experiments, we evaluated the influence of the muscle tissue on the sensitivity of the PCR assay by comparing the amplification of different amounts of CV-B2 diluted, respectively, in water and in a pool of 10 sections from a control muscle biopsy. As illustrated in Figure 2, a tremendous decrease in sensitivity was observed in spiked biopsies as compared with the water control from one log₁₀ on gel electrophoresis to 2 log₁₀ after hybridization of PCR products.

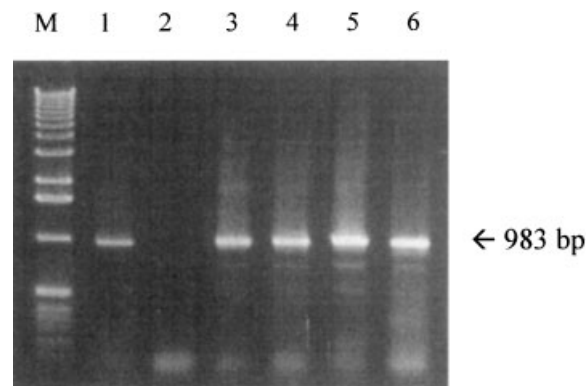


Fig. 1. Amplification of a 983-bp fragment of the GAPDH gene by one-step reverse transcription-polymerase chain reaction (RT-PCR) from skeletal muscle of patients with dermatomyositis (lane 3), fibromyalgia (lane 4), polymyositis (lane 5), and of a healthy subject (lane 6). mRNA of peripheral blood mononuclear cells from another healthy subject was used as positive control (lane 1). Lane 2, water negative control; M, size marker.

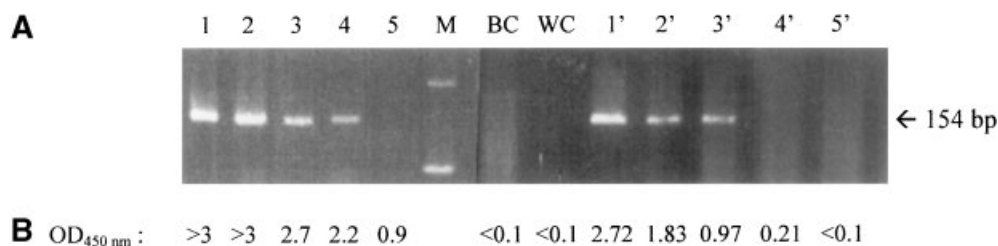


Fig. 2. Sensitivity analysis of the reverse transcription-polymerase chain reaction (RT-PCR) technique used in the study for detecting enteroviral RNA, after electrophoresis on a 3% agarose gel (A) or hybridization assay (B). **Lanes 1–5**, 10-fold serial dilutions of CV-B2 (from 10^6 to 10^{-3} TCID₅₀) in water. **Lanes 1'–5'**, same dilutions of CV-B2 in a pool of 10 sections from a control muscle biopsy. M, size marker; BC, control biopsy without virus; WC, water control.

As a result of the previous experiments, 10 sections of each sample were pooled and analyzed for the presence of enterovirus by RT-PCR using NC1 and E2 as primers and the S08 probe for hybridization. The results of the RT-PCR experiments carried out on muscle biopsies from the three groups of subjects are presented in Table II. The mean age of patients differed significantly among the three groups; group A being the oldest and group C the youngest (Table II). Of 74 tested specimens, enterovirus RNA was detected in 3 (20%) of 15 patients with inflammatory muscle diseases, in 4 (13.3%) of 30 patients with fibromyalgia/chronic fatigue syndrome and in none of 29 healthy volunteers. As a whole, no statistically significant difference was noted among the three groups. However, when each group of patients was compared with the group of healthy subjects, a significant difference was noted for group A ($P = 0.03$) and a trend to significance for group B ($P = 0.06$). In addition, although the PCR test used in this assay was not quantitative, the signals expressed as OD_{450nm} values observed among the three positive patients of group A were higher than those found in the four positive patients of group B (Table III).

The EnVision™ detection system using 5-D8/1 MAb was used to detect the enterovirus group-specific antigen located on the VP-1 capsid protein. Biopsies of cardiac muscle from a patient with dilated cardiomyopathy (Fig. 3E) and from a patient without heart disease (Fig. 3F) were used as positive and negative controls, respectively. Using an anti-adenovirus MAb instead of 5-D8/1 MAb, the specificity of the reaction on the positive control was checked (data not shown). As a

result of these experiments, the VP-1 antigen was detected in none of the 45 muscle biopsies of the study, whatever enterovirus RNA was present or not.

DISCUSSION

The main goal of this study was to investigate the presence of stigmata of enterovirus infection in muscular biopsies in patients suffering from chronic inflammatory muscle diseases or fibromyalgia/chronic fatigue syndrome as compared with healthy subjects. Consequently, RT-PCR and immunohistochemistry tests were carried out on tissue sections of skeletal muscle from these three groups. In contrast with previous studies listed in Table IV, we used a control group consisting of subjects in excellent health condition that had participated in a research project that included muscle biopsy.

From a technical point of view, the main difficulty encountered in this study was the small quantity of specimen available for RT-PCR, since only 10 frozen tissue transversal sections (≈ 0.5 –1 mg) were used in each case to homogenize the sample size. The quality of the extraction process was controlled by the detection of the GAPDH gene found positive for all specimens. Performing all the extraction and amplification experiments by two independent workers, and testing the positive results at least twice, the reproducibility of the molecular detection was assessed. In addition, to avoid false-positive amplification, a sensitive hybridization PCR assay was preferred to nested-PCR. However, muscle tissue impaired the sensitivity of the assay as compared to water control (Fig. 2), possibly in relation

TABLE II. Detection of Enteroviral RNA by RT-PCR in Skeletal Muscle Biopsies From Three Groups of Subjects

Group	Condition	No. of subjects	Age \pm SD in years (range) ^a	% of males	No. (%) of positive samples	<i>P</i>
A	Chronic inflammatory muscle diseases	15	57.0 \pm 17.1 (32–82)	53	3 (20)	0.03 ^b
B	Fibromyalgia/CFS	30	43.7 \pm 11.1 (29–77)	66	4 (13.3)	0.06 ^b
C	Healthy subjects	29	30.9 \pm 16.6 (20–84)	96	0	—

RT-PCR, reverse transcription-polymerase chain reaction; CFS, chronic fatigue syndrome.

^a $P < 0.001$ among the three groups by variance analysis ($P < 0.001$ between groups 1 and 3 and between groups 2 and 3, and $P < 0.01$ between groups 1 and 2 by Student's *t*-test).

^bBy Fisher's exact test, as compared with the group of healthy subjects.

TABLE III. Patients Found Positive for the Presence of Enteroviral RNA in Skeletal Muscle Using RT-PCR Test

Patient age (years)/sex	Group ^a /disease	RT-PCR signal at OD ₄₅₀ ^b
25/F	A/Dermatomyositis	1.07 ± 0.04
58/F	A/Granulomatous myositis	1.12 ± 0.08
53/M	A/Polymyositis	0.78 ± 0.17
33/M	B/Fibromyalgia	0.31 ± 0.10
31/F	B/Fibromyalgia	0.42 ± 0.06
26/F	B/CFS	0.34 ± 0.15
39/M	B/Fibromyalgia	0.27 ± 0.05

RT-PCR, reverse transcription-polymerase chain reaction; CFS, chronic fatigue syndrome; OD, optical density.

^aThe patient's groups are defined in the Materials and Methods section.

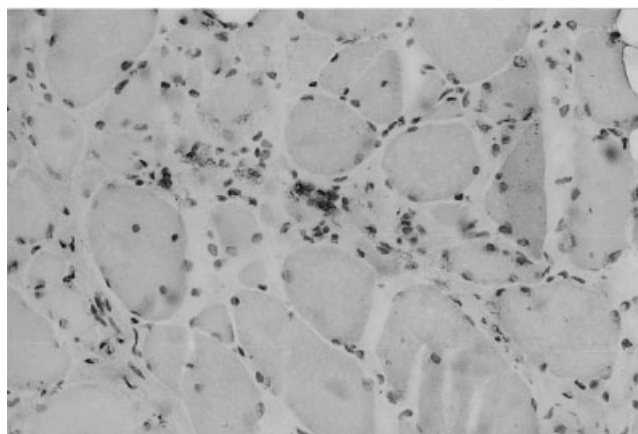
^bThe positivity threshold was 0.2 at OD₄₅₀. Mean ± SD of three independent experiments.

with the presence of inhibitory agents present in skeletal muscle. All these technical difficulties contributed to the heterogeneity of the results reported in the literature (Table IV). They also explain the unsuccessful attempt of nucleotide sequencing of the PCR products generated in this study (data not shown), which would have permitted to identify the enterovirus serotype(s) detected by PCR.

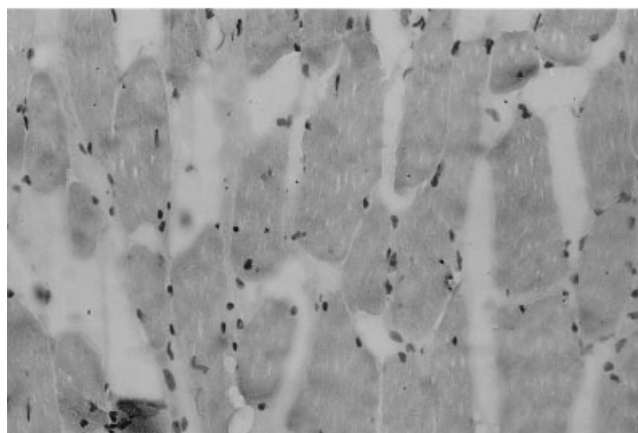
As shown by comparative analysis of different studies dealing with the detection of enteroviral RNA in muscle (Table IV), one of the more original features of this study was the inclusion of specimens from healthy subjects with excellent muscular condition, since most of them were young and involved in training programs. Because of the few control subjects tested and the small size of muscle specimen analyzed, the absence of detection of positive samples cannot exclude definitely the persistence of enteroviral RNA in skeletal muscles of healthy subjects. However, these results are not in accordance, at least for young healthy subjects, with the hypothesis of Arbustini et al. [1997] (Table IV) that skeletal muscle might act as a reservoir of enterovirus persistence. Experimental results from mouse models illustrate that the muscle tropism of enterovirus is highly variable according to the virus serotype. For instance, Coxsackie virus A9 was shown to exhibit a strong tropism for mouse muscle [Harvala et al., 2002], whereas Coxsackie virus B3 had a major heart tropism but a weak muscle tropism, despite the histological similarities between these tissues [Reetoo et al., 2000; Harvala et al., 2002]. Together with the results of Gow and Behan [1991], whose control subjects consisted of patients undergoing surgery for a nonmuscular disease (Table IV), our data suggest that normal muscle is not a good candidate for enterovirus persistence in healthy subjects. An alternative interpretation could be that the accumulation of enterovirus RNA in muscle is linked to the age of patients; actually, the mean age of our control group was significantly lower than that of both groups of patients (Table II).

Another point stressed by this study is the confirmation of a high prevalence of specimens detected positive for enterovirus RNA in the groups of patients with

A



B



C

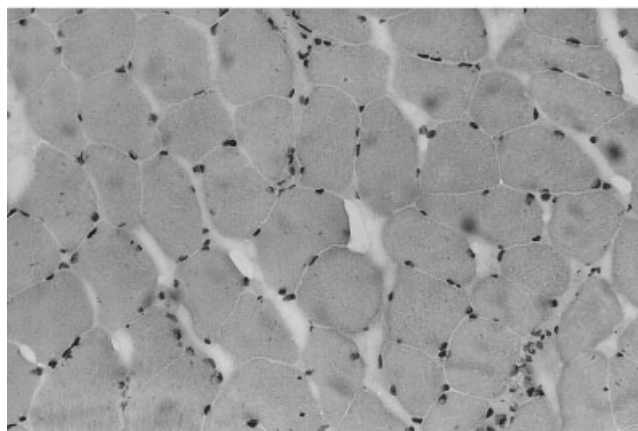


Fig. 3. Immunohistochemical detection of the enterovirus genus-specific VP-1 protein in skeletal (A–D) and heart (E,F) muscle sections using the EnVision™ system (DAKO) from patients with polymyositis (A), dermatomyositis (B), fibromyalgia (C), or dilated cardiomyopathy (E), and from healthy subjects (D,F). Localization of the VP-1 protein corresponds to the black inclusions present in the cytoplasm of the myocytes of the positive control (E). All the other samples were tested negative. ×200.

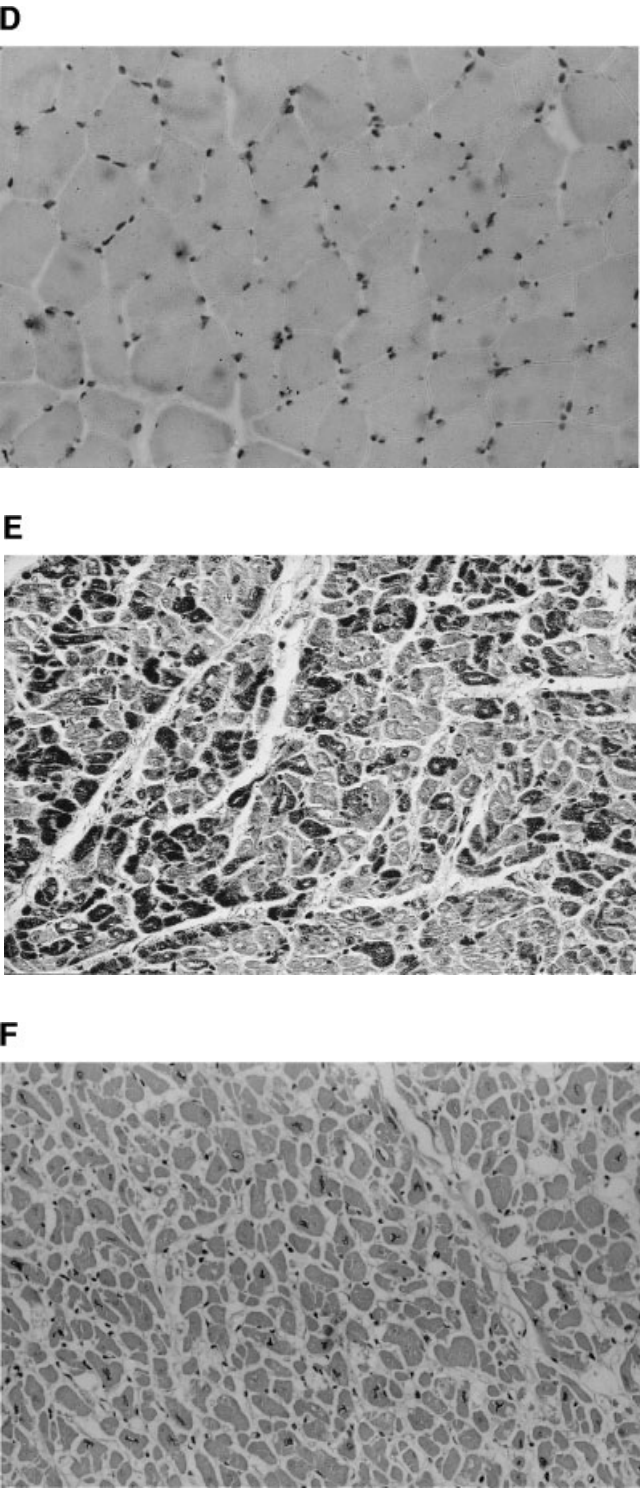


Fig. 3. (Continued)

muscular disorders: patients with chronic inflammatory muscle diseases exhibited the higher prevalence and the higher PCR signals (Table III), as compared with fibromyalgia/chronic fatigue syndrome patients, although the difference was not statistically significant and the PCR test used only qualitative. Conversely,

TABLE IV. Comparative Analysis of the Results of Nine Studies (Including This One) Dealing With Detection of Enterovirus in Muscular Biopsy Samples

Reference	Method for detection of enterovirus in muscular samples	Chronic inflammatory muscle diseases		Fibromyalgia and chronic fatigue syndrome		Suspected neuromuscular diseases ^a		Idiopathic dilated cardiomyopathy		Other cardiac conditions ^b		Other subjects	
		Total	No. (%)	Total	No. (%)	Total	No. (%)	Total	No. (%)	Total	No. (%)	Total	No. (%)
Rosenberg et al., 1989	In situ hybridization	20	3 (15.0)	—	—	13	0	—	—	—	—	—	—
Yousef et al., 1990	In situ hybridization	13	6 (46.2)	4	0	9	0	—	—	—	—	—	—
Gow and Behan, 1991	RT-PCR	—	—	60	32 (53.3)	—	—	—	—	—	—	41 ^c	6 (14.6)
Leon-Monzon and Dalakas, 1992	RT-PCR	39	0	—	—	16	0	—	—	—	—	—	—
Bowles et al., 1993	Slot-blot hybridization	96	25 (26.0)	158	41 (25.9)	152	2 (1.3)	—	—	—	—	—	—
Gow et al., 1994	RT-PCR	—	—	121	32 (26.4)	101	20 (19.8)	—	—	—	—	—	—
Fox et al., 1994	RT-nested PCR	32	0	—	—	36	0	—	—	—	—	—	—
Arbustini et al., 1997	RT-nested PCR	—	—	—	—	—	—	—	—	—	—	—	—
This study	RT-PCR	15	3 (20)	30	4 (13.3)	—	—	31	12 (38.7)	24	2 (8.3)	29 ^d	0

RT-PCR, reverse transcription-polymerase chain reaction.
^aMuscular biopsy performed for diagnostic purpose in patients without fibromyalgia or chronic fatigue syndrome and showing either no lesions or non inflammatory lesions.
^bPatients with ischemic or valvular heart diseases.
^cPatients undergoing surgery for a nonmuscular disease.
^dHealthy subjects.

despite the small size of the effective, a significant difference was noticed in patients with chronic inflammatory muscle diseases versus healthy subjects and a trend to significance in fibromyalgia/chronic fatigue syndrome patients versus controls (Table II). These results confirm the observation made by several other investigators [Rosenberg et al., 1989; Yousef et al., 1990; Bowles et al., 1993] about the link between chronic inflammatory muscle diseases and chronic enterovirus infection. However, the high prevalence of enterovirus RNA in skeletal muscles from patients with fibromyalgia/chronic fatigue syndrome [Gow and Behan, 1991; Bowles et al., 1993; Gow et al., 1994; this study] or with neuromuscular disorders [Gow et al., 1994] suggests that the presence of enterovirus RNA in muscle is not specific to chronic inflammatory muscle diseases.

Using a sensitive immunohistochemical technique developed in our laboratory and having shown positive results in cardiac muscle specimens from patients with dilated cardiomyopathy [Li et al., 2000], the VP-1 protein was not detected in skeletal muscle specimens of any subjects from the three groups. In skeletal muscle, the nondetection of the VP-1 protein suggests a defective viral replication, as evidenced by previous results obtained both in chronic fatigue syndrome patients [Cunningham et al., 1990, 1991] and in a murine model of chronic inflammatory myopathy [Tam and Messner, 1999] about the persistence of viral RNA under double stranded conformation and the presence of plus and minus strands at nearly equivalent levels.

The results of this study confirm previous data on the persistence of enterovirus RNA in skeletal muscle of patients with chronic inflammatory muscle diseases and, at a less extent, with fibromyalgia/chronic fatigue syndrome. The absence of detection of VP-1 protein in positive RNA samples reinforces the concept that enterovirus RNA is in a nonreplicative state in muscle of patients with chronic muscular disability. In addition, the absence of enterovirus RNA in samples from healthy subjects is not in favor of a role of skeletal muscle as a reservoir for enterovirus persistence.

ACKNOWLEDGMENTS

Nadine Vincent is acknowledged for her help in the redaction of the manuscript. This study was supported in part by a grant from the Délégation à la Recherche Clinique from the University Hospital of Saint-Etienne.

REFERENCES

- Abbey SE, Garfinkel PE. 1991. Chronic fatigue syndrome and depression: cause, effect, or covariate. *Rev Infect Dis* 13(suppl 1): S73–S83.
- Andréoletti L, Hober D, Becquart P, Belaich S, Copin MC, Lambert V, Wattré P. 1997. Experimental CVB3-induced chronic myocarditis in two murine strains: evidence of interrelationships between virus replication and myocardial damage in persistent cardiac infection. *J Med Virol* 52:206–214.
- Arbustini E, Grasso M, Porcu E, Bellini O, Diegoli M, Fasani R, Banchieri N, Pilotto A, Morbini P, Dal Bello B, Campana C, Gavazzi A, Vigano M. 1997. Enteroviral RNA and virus-like particles in the skeletal muscle of patients with idiopathic dilated cardiomyopathy. *Am J Cardiol* 80:1188–1193.
- Archard LC, Bowles NE, Behan PO, Bell EJ, Doyle D. 1988. Postviral fatigue syndrome: persistence of enterovirus RNA in muscle and elevated creatine kinase. *J R Soc Med* 81:326–329.
- Bowles NE, Bayston TA, Zhang HY, Doyle D, Lane RJ, Cunningham L, Archard LC. 1993. Persistence of enterovirus RNA in muscle biopsy samples suggests that some cases of chronic fatigue syndrome result from a previous, inflammatory viral myopathy. *J Med* 24:145–160.
- Buchwald D. 1996. Fibromyalgia and chronic fatigue syndrome: similarities and differences. *Rheum Dis Clin North Am* 22:219–243.
- Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
- Cunningham L, Bowles NE, Lane RJ, Dubowitz V, Archard LC. 1990. Persistence of enteroviral RNA in chronic fatigue syndrome is associated with the abnormal production of equal amounts of positive and negative strands of enteroviral RNA. *J Gen Virol* 71(Pt 6):1399–1402.
- Cunningham L, Bowles NE, Archard LC. 1991. Persistent virus infection of muscle in postviral fatigue syndrome. *Br Med Bull* 47:852–871.
- Dalakas MC. 1995. Enterovirus and human neuromuscular diseases. In: *Human enterovirus infections*. American society for microbiology. p 387–398.
- Engel AG, Hohlfeld R, Banker BQ. 1994. Inflammatory myopathies. In: *Myology*. New York: McGraw-Hill. p 1335–1486.
- Ercolani L, Florence B, Denaro M, Alexander M. 1988. Isolation and complete sequence of a functional human glyceraldehyde-3-phosphate dehydrogenase gene. *J Biol Chem* 263:15335–15341.
- Féasson L, Gautheron V, Mosnier J, Antoine J, Damon G, Michel D, Denis C. 1997. Advantage of alligator forceps muscle biopsy in neuromuscular disease diagnosis. *Ann Readapt Med Phys* 40: 527–533.
- Fox SA, Finklestone E, Robbins PD, Mastaglia FL, Swanson NR. 1994. Search for persistent enterovirus infection of muscle in inflammatory myopathies. *J Neurol Sci* 125:70–76.
- Frisk G. 2001. Mechanisms of chronic enteroviral persistence in tissue. *Curr Opin Infect Dis* 14:251–256.
- Gow JW, Behan WM. 1991. Amplification and identification of enteroviral sequences in the postviral fatigue syndrome. *Br Med Bull* 47:872–885.
- Gow JW, Behan WM, Simpson K, McGarry F, Keir S, Behan PO. 1994. Studies on enterovirus in patients with chronic fatigue syndrome. *Clin Infect Dis* 18(suppl 1):S126–S129.
- Harvala H, Kalimo H, Dahllund L, Santti J, Hughes P, Hyypä T, Stanway G. 2002. Mapping of tissue tropism determinants in coxsackievirus genomes. *J Gen Virol* 83:1697–1706.
- Kämmerer U, Kunkel B, Korn K. 1994. Nested PCR for specific detection and rapid identification of human picornaviruses. *J Clin Microbiol* 32:285–291.
- Kandolf R, Sauter M, Aepinus C, Schnorr JJ, Selinka HC, Klingel K. 1999. Mechanisms and consequences of enterovirus persistence in cardiac myocytes and cells of the immune system. *Virus Res* 62:149–158.
- Leon-Monzon M, Dalakas MC. 1992. Absence of persistent infection with enteroviruses in muscles of patients with inflammatory myopathies. *Ann Neurol* 32:219–222.
- Li Y, Bourlet T, Andréoletti L, Mosnier JF, Peng T, Yang Y, Archard LC, Pozzetto B, Zhang H. 2000. Enteroviral capsid protein VP1 is present in myocardial tissues from some patients with myocarditis or dilated cardiomyopathy. *Circulation* 101:231–234.
- Lindh G, Samuelson A, Hedlund KO, Evengard B, Lindquist L, Ehrnst A. 1996. No findings of enteroviruses in Swedish patients with chronic fatigue syndrome. *Scand J Infect Dis* 28:305–307.
- McCluskey DR, Riley MS. 1992. Chronic fatigue syndrome. *Compr Ther* 18:13–16.
- Miller FW. 1994. Classification and prognosis of inflammatory muscle disease. *Rheum Dis Clin North Am* 20:811–826.
- Rabausch-Starz I, Schwaiger A, Grünwald K, Müller-Hermelink HK, Neu N. 1994. Persistence of virus and viral genome in myocardium after coxsackievirus B3-induced murine myocarditis. *Clin Exp Immunol* 96:69–74.
- Reetoo KN, Osman SA, Illavia SJ, Cameron-Wilson CL, Banatvala JE, Muir P. 2000. Quantitative analysis of viral RNA kinetics in coxsackievirus B3-induced murine myocarditis: biphasic pattern of clearance following acute infection, with persistence of residual

- viral RNA throughout and beyond the inflammatory phase of disease. *J Gen Virol* 81:2755–2762.
- Rosenberg NL, Rotbart HA, Abzug MJ, Ringel SP, Levin MJ. 1989. Evidence for a novel picornavirus in human dermatomyositis. *Ann Neurol* 26:204–209.
- Rotbart HA. 1990. Enzymatic RNA amplification of the enteroviruses. *J Clin Microbiol* 28:438–442.
- Samuelson A, Forsgren M, Sällberg M. 1995. Characterization of the recognition site and diagnostic potential of an enterovirus group-reactive monoclonal antibody. *Clin Diagn Lab Immunol* 2:385–386.
- Tam PE, Messner RP. 1999. Molecular mechanisms of coxsackievirus persistence in chronic inflammatory myopathy: viral RNA persists through formation of a double-stranded complex without associated genomic mutations or evolution. *J Virol* 73:10113–10121.
- Tam PE, Schmidt AM, Ytterberg SR, Messner RP. 1991. Viral persistence during the developmental phase of Coxsackievirus B1-induced murine polymyositis. *J Virol* 65:6654–6660.
- Tam PE, Schmidt AM, Ytterberg SR, Messner RP. 1994. Duration of virus persistence and its relationship to inflammation in the chronic phase of coxsackievirus B1-induced murine polymyositis. *J Lab Clin Med* 123:346–356.
- Yousef GE, Brown IN, Mowbray JF. 1987. Derivation and biochemical characterization of an enterovirus group-specific monoclonal antibody. *Intervirology* 28:163–170.
- Yousef GE, Bell EJ, Mann GF, Murugesan V, Smith DG, McCartney RA, Mowbray JF. 1988. Chronic enterovirus infection in patients with postviral fatigue syndrome. *Lancet* 1:146–150.
- Yousef GE, Isenberg DA, Mowbray JF. 1990. Detection of enterovirus specific RNA sequences in muscle biopsy specimens from patients with adult onset myositis. *Ann Rheum Dis* 49:310–315.
- Ytterberg SR, Mahowald ML, Messner RP. 1988. T cells are required for coxsackievirus B1 induced murine polymyositis. *J Rheumatol* 15:475–478.