Sequences specific for enterovirus detected in spinal cord from patients with motor neurone disease

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Abstract

***Objective*—To investigate the association of enteroviruses with motor neurone disease, also known as amyotrophic lateral sclerosis.**

***Design*—Analysis by enterovirus polymerase chain reaction of wax embedded material from spinal cords taken at necropsy from subjects with motor neurone disease and from age and sex matched controls.**

***Setting*—Specimens were collected in the west of Scotland and in London between 1982 and 1992.**

***Results*—Sequences specific for a non-poliovirus type enterovirus were detected in spinal cord tissue from subjects with motor neurone disease. Amplification of a 414 base RNA target sequence in the conserved enterovirus 5′ untranslated region from wax embedded tissue sections was successful in tissue from eight of 11 cases of sporadic motor neurone disease, one of two cases of familial motor neurone disease, and the one case of poliomyelitis, but not in the six matched controls or one case of antecedent poliomyelitis. In addition, sequences were detected in spinal cords from one monkey infected with wild type poliovirus and one monkey infected with polio vaccine. Comparison of sequences from cases of motor neurone disease with sequences of corresponding regions of the 5′ untranslated regions of known picornaviruses showed them to be tightly grouped within the enterovirus genus closely related to coxsackievirus type B but not to polioviruses. Sequences derived from different parts of the spinal cord of the same subjects were identical, but sequences differed between individual subjects.**

***Conclusions*—Conserved enteroviral sequences closely related to coxsackie B virus sequences were detectable in spinal cords from subjects with sporadic motor neurone disease and from one subject with possible familial motor neurone disease.**

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Introduction

The enterovirus genus is responsible for various conditions including acute myopathologies and neuropathologies and pancreatitis in humans.[1] The genus includes polioviruses, which have been candidates as causative agents in motor neurone disease[2] because of similarities in histopathology between poliomyelitis and motor neurone disease[3] and clinical and epidemiological observations that relate poliomyelitis with subsequent motor neurone disease.[4-7] Poliovirus has not been isolated from the spinal cord of patients with motor neurone disease,[6 8 9] but picornavirus sequences may have been located by in situ hybridisation.[10] The chronic progressive nature of motor neurone disease suggests that if a persisting viral infection is responsible, the levels of actively replicating virus at any one time would be relatively low and thus not detectable by conventional means.[11] Our recent development of RNA polymerase chain reaction from wax sections[12] with wide range primers[13] enabled the study of enterovirus sequences from blocks of spinal cord taken at necropsy from subjects with motor neurone disease.

TABLE I—*Summaries of clinical diagnosis and histories of cases* and controls**

Sample No	Age/Sex	Clinical diagnosis and case history before death
1	59/F	36 Month history with 12 month difficulty in swallowing and muscular weakness
2	47/F	8 Month history. Upper and lower signs
3	62/M	17 Month history of rapid progressive muscle weakness
4	83/F	12 Month history of difficulty in swallowing, dysarthia
5	64/M	12 Month history of progressive weakness
6	75/F	8 Year history of progressive trunk and limb weakness
7	69/F	Motor neurone disease diagnosed for 25 months
8	56/F	3 Year history of bulbar and limb signs. Family history
9	NA	Motor neurone disease diagnosed
10	NA	Antecedent poliomyelitis
11	NA	Acute poliomyelitis
12	38/F	Motor neurone disease diagnosed for 6 months. Father died of motor neurone disease at age 33 years
13	68/M	Motor neurone disease diagnosed for 5 months
14	61/M	Motor neurone disease diagnosed for 2 years
15	50/M	Motor neurone disease diagnosed for 5 months
16	74/F	Normal
17	48/M	Normal
18	78/F	Physical injury
19	40/F	Physical injury
20	48/F	Normal
21	76/F	Normal
Monkey A		Poliovirus infected
Monkey B		Poliovirus vaccine infected

NA=data not available.
*Samples from cases 1-8 and all controls (16-21) were obtained from Southern General Hospital, Glasgow; samples from cases 12-15 were obtained from National Hospital for Nervous Diseases, London; remaining samples obtained from the London Hospital Medical College.

Subjects and methods

MOTOR NEURONE DISEASE CASES AND CONTROLS

Cases were referred to neurologists and the diagnosis confirmed histopathologically at necropsy. Table I describes the case histories. Necropsy revealed atrophy of the ventral spinal roots, which were grey and reduced in size; this was most distinct in the cervical region of the spinal cord and in the cauda equina (with the exception of case 9, for which no necropsy data were available). Microscopy of the ventral horns showed variable loss of motor neurones and associated astrocytosis most easily seen in the ventral horns of the cervical and lumbar segments. There was variable loss of myelin in the corticospinal tract, less demyelination in the anterior columns, and preservation of the posterior columns. There was evidence of neurogenic atrophy of voluntary muscle.

Spinal cords from controls were not obtainable retrospectively from necropsies performed in the same time period as cases. These spinal cords were either histopathologically normal or showed cord compression and were obtained from subjects with no clinical evidence of neurodegenerative disease of an age and sex distribution at death similar to cases (table I). These samples control for persistent enterovirus infections in spinal cords of patients with similar age and sex distribution to cases of motor neurone disease that originate from the same geographical area and from the same necropsy sites as most of the cases. They act as controls for both contamination of spinal cord samples by enteroviruses at necropsy and for persistent infection in the population at risk.

TABLE II—*Analysis of wax sections from cases with motor neurone disease and poliovirus controls by enterovirus and Abelson polymerase chain reaction. Numbers of replicate sections analysed shown in parentheses*

Sample No	Enterovirus polymerase chain reaction	Abelson polymerase chain reaction
1	+ ND +	ND†
2	+ + +	
3	+ + +	
4	+ + +	
5	ND ND	ND† +
6	ND ND	+ +
7	ND	+
8	ND	+
9	+	
10	ND	+
11	+ +	
16	(5) ND	(5) +
17	(5) ND	(5) +
18	(5) ND	(5) +
19	(5) ND	(5) +
20	(6) ND	(6) +
21	(6) ND	(6) +
Monkey A	ND +	+
Monkey B	ND +	+

ND=not detected.
*RNA isolated from single wax sections was analysed by polymerase chain reaction for enterovirus and constitutively expressed proto-oncogene Abelson sequences (to check quality of RNA in samples negative for enterovirus). Each result represents analysis of a single section from a block of spinal cord tissue for each motor neurone disease case. Spinal cord necropsy material of age and sex matched subjects (samples 16-21) was obtained from the same necropsy sites as motor neurone disease samples. RNA isolated from 5-6 replicate wax sections from each control spinal cord block was analysed. Additional amplification of each control sample replicate by nested enterovirus polymerase chain reaction detected no enteroviral sequences.
†Sections where quality of extracted RNA was inadequate.

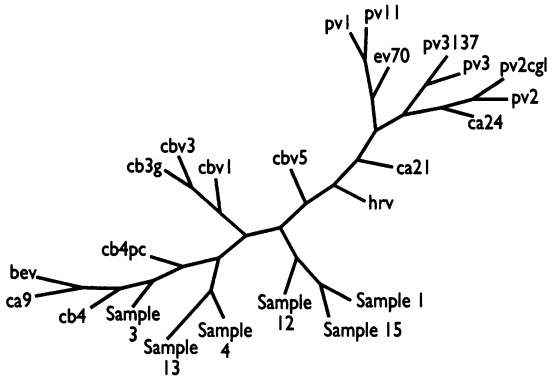
analysis

Isolation of RNA from wax sections and RNA polymerase chain reaction for proto-oncogene Abelson and enteroviruses and detection of polymerase chain reaction products are described elsewhere.[12-14] Care was taken to prevent cross contamination of sections during preparation.[15] Results of enterovirus polymerase chain reactions were analysed blind. Replicate samples of spinal cord from controls were analysed separately in the same way and by nested enterovirus polymerase chain reaction. Repeat analysis of mixtures of samples of spinal cord from controls and cases with motor neurone disease positive for enterovirus reproduced the results obtained when they were analysed separately (data not shown).

Selected enterovirus polymerase chain reaction products from cases with motor neurone disease were further amplified with internal primers and sequenced. The relatedness of sequence was measured independently by DNADIST and NEIGHBOR (maximum likelihood method) on PHYLIP[16] from multiple sequence alignments generated by "PILEUP" run on the University of Wisconsin Genetics Computing Group Package for Vax.[17]

Results

Amplification of enteroviral 5′ untranslated regions from single wax sections of spinal cord blocks was successful in nine of 13 cases with motor neurone disease and consistently showed the presence of these sequences when several sections were analysed from each block or several blocks analysed from each case (tables II and III). Conversely, enteroviral sequences were not detected in six matched control spinal cords which were either normal or showed cord compression (table II). Enterovirus sequences were detected along the length of the spinal cord, but not all blocks gave a positive result (table III). In particular, case 13 was positive in the sacral, lumbar, and cervical regions but not the intervening thoracic region. Positive results were also obtained from a case with poliomyelitis and from spinal cords from a monkey infected with wild type poliovirus and from one recently infected with a vaccine strain of poliovirus but not from one case with antecedent polio with an intervening period of more than 20 years.



Dendrogram of relation between 5′ untranslated region picornaviral polymerase chain reaction sequences from cases with motor neurone disease and corresponding regions of known enteroviruses; samples 1, 3, 4, 12, 13, and 15 are polymerase chain reaction products from motor neurone disease; cb4pc is polymerase chain reaction product from control positive for coxsackievirus B4; cbv1, 3, 4, and 5 are coxsackievirus B1, B3 , B4, and B5; cb3g is coxsackievirus B3 (Nancy); ca9, 21, and 24 are coxsackievirus A9, A21, and A24; pv1, 2, and 3 are poliovirus 1 (Sabin), 2 (Lansing), and 3 (Leon 12a-1-b); pv11 is poliovirus 1 (Mahoney); pv2cgl is poliovirus 2 (W-2); pv3l37 is poliovirus 3 (P3/Leon/37); ev70 is enterovirus 70; hrv is human rhinovirus 14; bev is bovine enterovirus. Sequences of 5′ untranslated region (position range 200-400 bp) taken from first recorded complete genome sequences from known isolates were obtained from GenBank-European Molecular Biology Organisation sequence libraries

TABLE III—*Analysis of single sections from serial wax blocks taken from spinal cords of motor neurone disease cases. Blocks from cervical (C4 or C5), thoracic (T2, T4, T6, T8, T10, and T12), lumbar (L4) and sacral (S3 or S4) regions of spinal cords of four cases of motor neurone disease, samples 12, 13, 14, and 15 were analysed by sampling single sections from each block*

Sample No	Block position	Enterovirus polymerase chain reaction	Abelson polymerase chain reaction
12	C4	+	
	T2	+	
	T4	ND	ND†
	T4	ND	ND†
	T6	+	
	T8	+	
	T10	+	
	T12	ND	+
	S4	+	
	S4prg*	+	
13	C5	+	
	C5prg*	+	
	T2	ND	+
	T4	ND	+
	T6	ND	+
	T8	ND	+
	T10	ND	+
	T12	ND	+
	L4	+	
	S3prg*	+	
12	C5	+	
	T2	+	
	T4	ND	ND†
	T6	ND	ND†
	T8	ND	+
	T10	+	
	T12	+	
	L4	ND	+
	S3	+	
	S4	ND	+
15	T2	+	
	T4	+	
	T6	+	
	T8	+	
	T10	+	
	T12	+	
	L4	+	
	S4	ND	+

ND=not detected.
*Posterior root ganglion.
†Sections where quality of extracted RNA was inadequate.

Enterovirus polymerase chain reaction products from cases 1, 3, 4, 12, 13, and 15 were sequenced. Sequences when aligned and compared showed overall homologies ranging from 83% to 98%. Unique sequences were obtained from each case. Furthermore, sequences from different parts of the spinal cords of individual cases—for example sequences from C5 and L4 in case 13 and from T2, T4, T10, and T12 in case 15—were identical in each respective subject.

The 5′ untranslated region sequences from cases with motor neurone disease were identified as enteroviral in origin. Comparison of sequence information in this region of published enteroviruses correlated with enterovirus subtype relations as determined by other criteria (figure).[18-20] Two distinct clusters of sequences like poliovirus and like coxsackievirus B formed. Sequences derived from cases with motor neurone disease clustered within the coxsackievirus B group, forming two distinc subgroups (cases 1, 12, 15, and cases 4, 13). The case 3 sequence was closely related to coxsackievirus B4. Sequences from spinal cords of monkeys infected with poliovirus were like those of poliovirus (data not shown).

Discussion

We found enteroviral sequences related to coxsackie B viruses in the spinal cord of eight of 11 cases with sporadic motor neurone disease. Enterovirus sequences were detected in one of two potential cases with familial motor neurone disease (case 12) in this series. It has recently been reported that lesions in cytosolic superoxide dismutase genes that reduce activity of this enzyme are associated with familial motor neurone disease.[21 22]

Clinical implications

- Indirect evidence has previously been available implicating enteroviruses in the aetiology of motor neurone disease
- Nucleic acid sequences specific for enterovirus were detected in the spinal cords of eight of 11 cases of sporadic motor neurone disease and one of two cases of putative familial motor neurone disease
- Sequences show a close relation to coxsackievirus B sequences
- No sequences were detected in samples from six matched controls
- Sequences derived from different parts of the spinal cord of the same subject were identical but they differ between individual subjects

Enterovirus sequences were not detected in control patients of a similar age and sex distribution to some motor neurone disease cases and from the same geographical area (or in one patient with antecedent poliomyelitis), although the prevalence of enterovirus infection would predict that these patients would have been infected with enteroviruses in the past. We conclude that detection of these enterovirus 5′ untranslated region sequences in patients with motor neurone disease is evidence of persisting enterovirus infection in spinal cord tissue. The absence of infectious virus and the presumed long term infection suggests viral genomes in a state of restricted replication analogous to measles virus in subacute sclerosing panencephalitis.[23] Further characterisation of these sequences and possible pathogenic mechanisms are currently under investigation.

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Distal forearm fracture as risk factor for vertebral osteoporosis

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Fractures of the vertebral body. and of the distal forearm are typical manifestations of type I or postmenopausal osteoporosis,[1] but the association between the two fractures in individual women is not clear. In type I osteoporosis vertebral fractures are associated with low bone mineral density of the lumbar spine, and the risk of vertebral fracture increases two to three times with each standard deviation decrease in bone mineral density.[2] We aimed to determine whether a fracture of the distal forearm in postmenopausal women indicated an increased risk of vertebral osteoporosis.

Patients, methods, and results

We identified 127 women aged 50 to 82 (mean 64·7 years) from records in the radiology department and fracture clinic who had a fracture of the distal forearm within the previous three years (62% uptake); 375 women aged 50 to 85 years (mean 64·6 years) were randomly selected from three general practice populations in Sheffield (55% uptake). Twelve women with previous fractures of their distal forearm were excluded from the population based group. Bone mineral density of the lumbar spine was measured using dual energy x ray absorptiometry (Lunar DPX, Lunar Corporation, Madison, WI). Each subject gave informed consent and the study was approved by the ethics committee of this hospital. Differences in the bone mineral density of the lumbar spine were examined by comparing Z scores (normalised for age and weight and expressed as units of standard deviation from the expected value) using analysis of variance with Bonferroni correction. The slopes and intercepts of regression lines were compared using dummy variables according to the method of Kleinbaum and Kupper.[3]

We found no difference between the women with distal forearm fracture and the population based group with respect to age (mean 64·7 *v* 64·6 years), time since the menopause (17·0 *v* 17·9 years), height (mean 1·580 *v* 1·585 m), or weight (mean 65·3 *v* 66·0 kg). Bone