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Case reports

Coxsackie B antigen in the central nervous system of a patient with fatal acute encephalitis: immunohistochemical studies of formalin-fixed paraffin-embedded tissue*

S. Kamei[1], S. M. Hersch[2], T. Kurata[3], and Y. Takei[1]

Division of Neuropathology, Departments of [1] Pathology and Laboratory Medicine and
[2] Neurology, Emory University School of Medicine, Atlanta, GA 30322, USA
[3] Department of Pathology, The National Institute of Health, 2-10-35, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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Summary. A case of fatal acute encephalitis due to Coxsackie B_1 virus is described. Confirmation of Coxsackie B virus as the etiological agent of encephalitis was based on identification of the virus antigen in formalin-fixed paraffin-embedded tissue sections. In the past, the diagnosis was obtained by serological studies of peripheral blood and viral isolation. This is the first report in which indirect immunofluorescent and immunoperoxidase methods using rabbit antiserum raised against Coxsackie B types 1–6 was utilized in determining the etiology of encephalitis. It must be emphasized that these methods can be used both on biopsy or autopsy specimens, even retrospectively.

Key words: Central nervous system – Coxsackie B virus – Encephalitis, viral – Immunohistochemistry

Many cases of central nervous system (CNS) infection have been ascribed to Coxsackie B virus since the first report by Javett et al. in 1956 [9]. In most of these cases, however, the etiology was determined by serological studies of peripheral blood and/or viral isolation from tissues other than the CNS, particularly from feces. It is often too presumptous to conclude the exact etiology of an encephalitis based on fecal examination alone. This is especially true with enterovirus infections because these viruses, including Coxsackie group, are frequently isolated from the gastrointestinal (GI) tract of healthy individuals.

In our review of the literature, only ten cases of Coxsackie B encephalitis have been reported in which viral isolation from the CNS established the diagnosis [4–6, 11, 15, 17, 22]. We report a unique case of fatal, acute encephalitis in a 4-year-old boy caused by Coxsacki B_1 virus. The diagnosis was established by immunohistochemical methods demonstrating Coxsackie B antigen in paraffin sections from formalin-fixed cerebral tissue. This case is of additonal interest because of the absence of myocarditis, which has been considered to be the cause of death in most of children infected by Coxsackie-B virus even when concomitant encephalitis is present [6, 10].

Case report

The patient was a previously healthy 4-year-old white male who was admitted to Emory University hospital on September 13, 1982, with suspected diagnosis of encephalitis. He first became ill on September 3, 1982, when he was noted to be irritable and febrile (39.4° C). There were no upper respiratory symptoms, vomiting, or diarrhea and no history of insect bites, toxic ingestion, recent vaccination, or trauma. The fever persisted and by September 8, the patient appeared weak and somnolent. On September 11, he became less responsive and incontinent of urine and feces. The next day, he staggered and fell to the right when walked. At this point, he was admitted to a local hospital where a right-sided tonic-clonic seizure occurred. There he was found to be alert but irritable, responsive to verbal commands, had normal muscle tone and was able to ambulate, but fell to the right. There were no signs of meningeal irritation. Cerebrospinal fluid (CSF) contained: WBC, 14/mm^3 with 13 mononuclear cells and 1 polymorphonuclear leukocyte; protein, 27 mg/100 ml; glucose, 85 mg/100 ml. Bacteriological cultures of CSF were negative. Neurological status deteriorated and by the morning of September 13, he was comatose. Head CT showed bihemispheric white matter edema and slit-like ventricles. A cardiopulmonary arrest ensued and the patient was intubated.

He was noted for generalized flaccidity and unresponsiveness. Pulse was 100, blood pressure 78/56, and there was marked hypothermia. Neurological examination revealed fixed and dilated pupils, absent deep tendon reflexes, and the absence of all brain stem reflexes including respiration. Chest x-ray revealed patchy consolidation of the right upper lobe of the lung and increased interstitial markings consistent with pulmonary edema.

The patient was treated with hyperventilation and mannitol without benefit. The day after admission, severe papilledema was noted and death occured shortly thereafter. Autopsy was performed 3 h post-mortem.

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Offprint requests to: S. Kamei, Department of Neurology, Nihon University School of Medicine, 30-1, Oyaguchi-Kamimachi, Itabashi-Ku, Tokyo 173, Japan

Material and methods

Tissue from the brain, oropharynx, and GI tract as well as feces were obtained for viral isolation using African green monkey and human embryonic lung tissue. Routine tissue samples were fixed in 10% buffered formalin at the time of autopsy and embedded in paraffin subsequently. Sections were stained with hematoxylin and eosin and with special stains for bacteria, fungi, and acid-fast bacilli. Selected areas of the CNS were prepared for electron microscopic (EM) study in the usual manner.

Detection of viral antigens. An indirect immunofluorescent and immunoperoxidase methods were used to detect viral antigens on paraffin sections of the cerebral, pontine, myocardial, and lung tissue in the following manner: sections from selected blocks were cemented to clean glass slides with 0.1% neoprene (polychloroprene, Merck) and then treated with 0.25% trypsin in phosphate-buffered saline (PBS; pH 7.6) containing 0.02% $CaCl_2$ and incubated at 37° C for 3 h in a moist chamber. After digestion, the slides were washed in PBS for 15 min at room temperature. Our indirect immunofluorescent and immunoperoxidase techniques have been previously reported in detail [7, 20]. In the present study, primary antibodies used were hyperimmunized rabbit serum against Coxsackie B, types 1 – 6 (provided by Dr. J. B. McCormick, Centers for Disease Control, Atlanta, Georgia), Coxsackie group A, Herpes simplex types 1 and 2, Varicella-zoster and cytomegaloviruses. Normal rabbit serum was applied as a negative control at the same dilution. Fluorescein isothiocyanata (FITC)-labeled anti-rabbit IgG (goat; Miles-Yeda, LTD.) was used as the secondary antibody. Formalin-fixed cells and mouse tissues infected with Coxsackie B_1 virus were substituted for positive control. Antibodies (rabbit) had titers of 1:256 – 1:512 to Coxsackie B_{1-6} by complement-fixing test. Anti-Coxsackie B_1 serum lost staining activity for immunofluorescence after mixing with Coxsackie B_1 antigen for 1 h at 37° C. Also viral antigen was stained by immunoperoxidase method using avidin-biotin-peroxidase complex (ABC) kit (Vectastain, Vector Laboratory) with the same antiserum [20].

Results

General pathological examination

Gross findings. The heart weighed 85 g and was grossly normal. The combined weight of both lungs was 300 g. The lower lobes were mildly congested and the other lobes were normal. All other organs were unremarkable.

Microscopic findings. Multiple sections through the heart revealed no abnormalities. Sections of the lower lobes of the lung revealed acute mild bronchopneumonia. Sections of the liver showed mild lymphocytic infiltrates, predominantly in the portal connective tissue, with small numbers of lymphocytes scattered in the parenchyma. Other organs were essentially unremarkable.

Neuropathological examination

Gross findings. The weight of the unfixed brain was 1760 g. The leptomeninges were clear. The brain was moderately swollen showing flattened gyri and choked sulci associated with tonsillar herniation. Coronal sections through the cerebral hemispheres revealed no grossly discernible lesion. The spinal cord was also examined in its entirety and was unremarkable.

Microscopic findings. Sections through the temporal and cingulate cortex revealed foci of encephalitis characterized by marked perivascular cuffing with lymphocytes and histiocytes, microglial proliferation, and microglial nodules (Fig. 1). The lesions were prevalent in the gray matter, but extended focally into the underlying white matter. The leptomeninges also contained moderate numbers of mononuclear inflammatory cells which, at times, extended into the perivascular spaces (Fig. 2). Occasional fibrinoid necrosis of the vessel walls was seen, and some of the vessel walls showed overt vasculitis.

The basal ganglia, particularly the putamina, were also intensely involved (Fig. 3). Similar lesions were also present in the centrum semiovale as well as in the temporal cortex. The changes in the cerebrum appeared to be more pronounced on the left than the right side. In these areas, occasional eosinophils were present within the marked inflammatory infiltrates. Special stains for bacteria, fungi, and acid-fast bacilli were negative. Lipid-laden macrophages were numerous in necrotic foci.

Whereas the cerebral peduncle, mammillary bodies and pineal gland were intact, the pontine tegmentum was extensively involved (Fig. 4). The medulla oblongata was much less involved than the pons with the exception of the inferior olivary nuclei, particularly on the right, where marked perivascular inflammatory infiltrates and reactive microgliosis were noted. Sections through the cerebellum were essentially unremarkable except for the tonsils where a necrotic process was obvious because of the herniation. The spinal cord was well preserved throughout.

Electron microscopy. Several paragon-stained semithin sections were used to identify foci of encephalitis. Selected blocks were cut and examined ultrastructurally. An extensive search for viral particles was unsuccessful.

Detection of viral antigen. Coxsackie B antigen was detected in the frontal and temporal cortexes, leptomeninges overlying involved these cortical areas, cinguli gyrus, basal ganglia, and pontine tegmentum, as well as, myocardium and lung. This specific antigen was often identified readily in the cytoplasm of both neurons and glial cells (Fig. 5a, b). The antigen was also detected in some capillary endothelia with or without inflammatory reactions (Fig. 5c, d, e). For comparison, Coxsackie A, Herpes simplex types 1 and 2, Varicella-zoster, or cytomegalovirus antigens was examined in the same specimens. They were all negative. Coxsackie B virus in culture displayed immunofluorescence when our Coxsackie B antibody solution was applied. When sections from normal brains were used in the same procedures as negative controls, no immunofluorescent reaction was found.

Virus isolation. A type of enterovirus was isolated from oropharyngeal, gastrointestinal, and fecal specimens. The virus was later identified as Coxsackie B_1 by neutralization tests with specific antiserum. However, no virus was isolated from any other tissue or fluid including the brain or CSF.

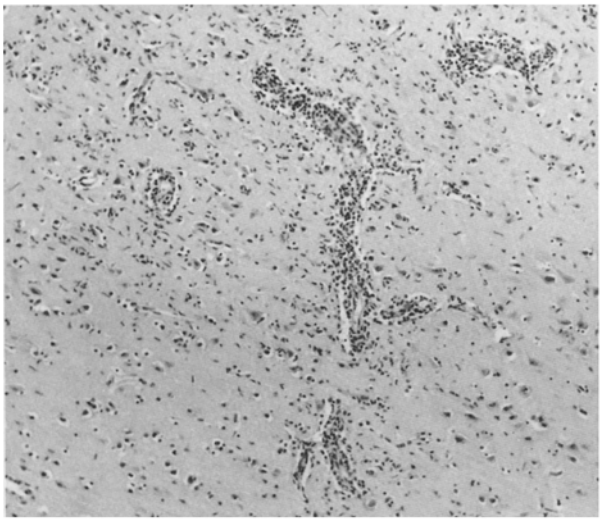

Fig. 1. Gliosis and perivascular cuffing with lymphocytes and histiocytes from a section of temporal cortex stained with hematoxylin and eosin. H&E, ×100

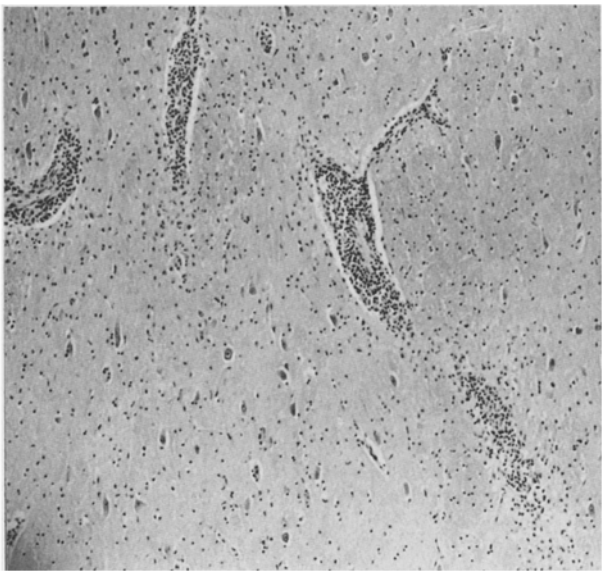

Fig. 3. Section through the basal ganglia showing marked perivascular cuffing and glial nodule formation. H&E, ×100

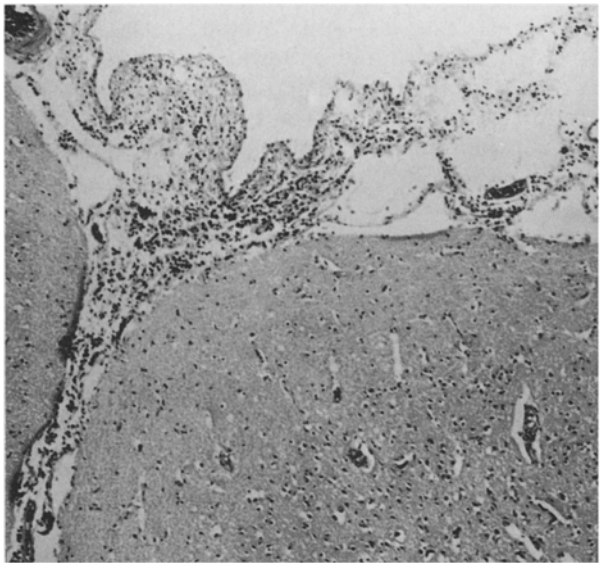

Fig. 2. Section through the surface of temporal cortex showing leptomeningitis with a mononuclear inflammatory infiltration. H&E, ×50

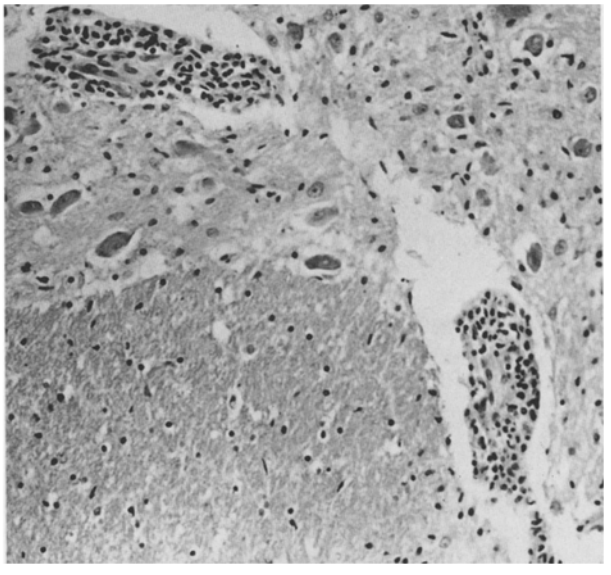

Fig. 4. Section through the pontine tegmentum demonstrating gliosis and perivascular lymphocytic proliferation. H&E, ×200

Discussion

The definitive diagnosis of viral encephalitis due to enteroviruses, including Coxsackie virus, has been problematic. Identifying the virus in extracerebral tissues in patients with CNS involvement would not be satisfactory for determination of the exact etiology of the encephalitis. Enterovirus has frequently been isolated from the GI tract in healthy individuals, particularly during endemic periods [1]. Similarly, it has been reported by Wenner [24] that enteroviruses may be isolated from the CSF of normal individuals. Hence, the diagnosis cannot be established on the basis of viral isolation from the CSF alone either. In addition, enteroviruses have occasionally been isolated in patients with encephalitis associated with other agents [21]. Therefore, reliable diagnosis must depend on the isolation of virus, the detection of viral antigen, or ultrastructural identification of viral particles in the involved brain tissue. Unfortunately, however, technical difficulties may arise during these diagnostic procedures: the sensitivity of tissue culture techniques for virus isolation can be only 65% – 70% for enteroviruses [2]. In cases of encephalitis, this sensitivity for enterovirus isolation may be further decreased. Failure to isolate the

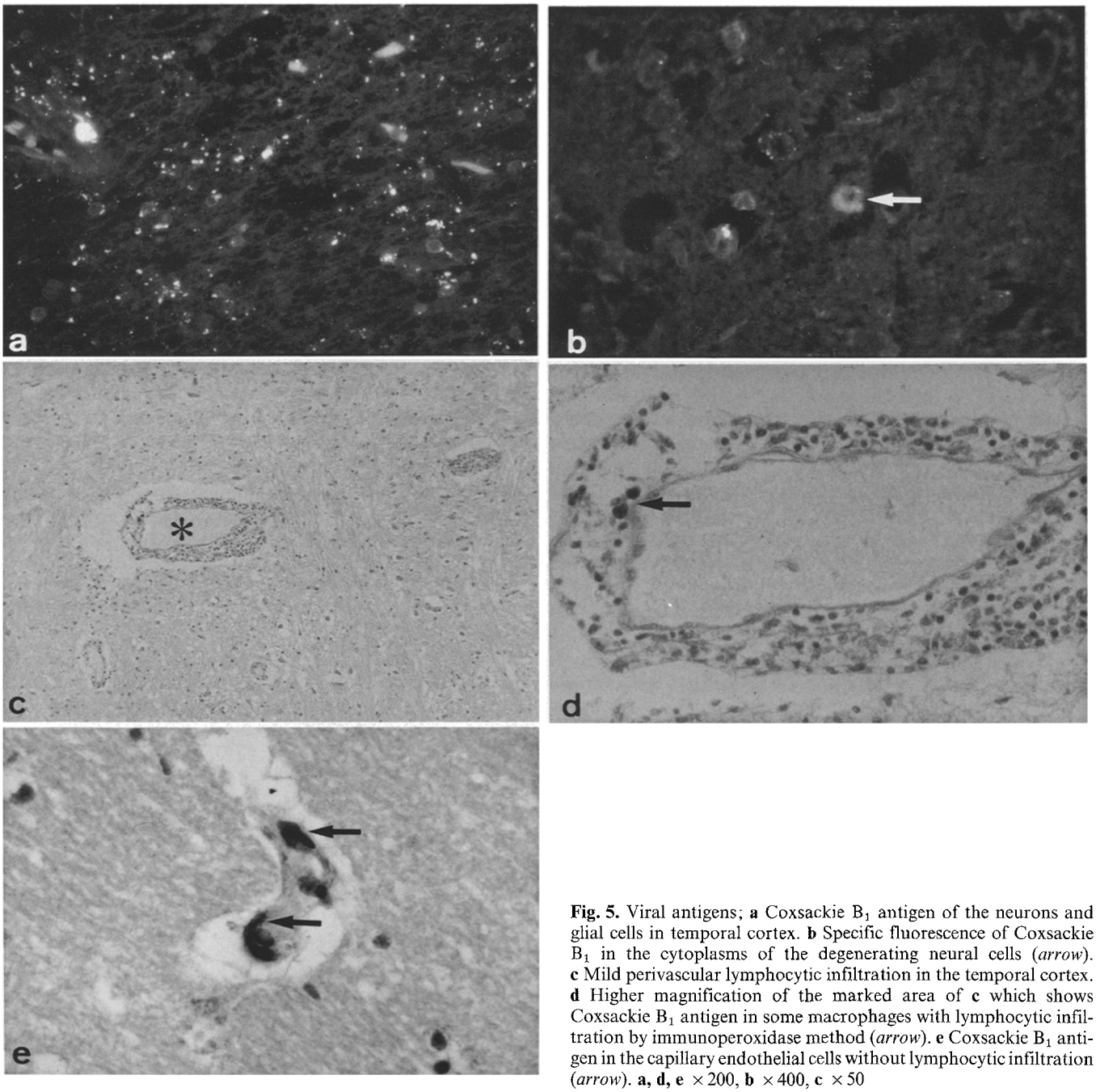

Fig. 5. Viral antigens; **a** Coxsackie B_1 antigen of the neurons and glial cells in temporal cortex. **b** Specific fluorescence of Coxsackie B_1 in the cytoplasms of the degenerating neural cells (*arrow*). **c** Mild perivascular lymphocytic infiltration in the temporal cortex. **d** Higher magnification of the marked area of **c** which shows Coxsackie B_1 antigen in some macrophages with lymphocytic infiltration by immunoperoxidase method (*arrow*). **e** Coxsackie B_1 antigen in the capillary endothelial cells without lymphocytic infiltration (*arrow*). **a, d, e** $\times 200$, **b** $\times 400$, **c** $\times 50$

virus may also be due to topographical error in tissue sampling or to the stage of the illness during which tissue is sampled. We have encountered similar difficulties in attempts to diagnoses Herpes simplex encephalitis.

Recently, an immunohistochemical technique has been shown to be useful in demonstrating viral antigens in formalin-fixed paraffin-embedded specimens after treatment with proteolytic enzymes [8, 20]. We have successfully applied this technique and detected Herpes simplex antigen on sections cut from 18-year-old paraffin blocks and from paraffin blocks made of autopsied specimens soaked in formalin for as long as 8 years. Varicella-zoster [7] as well as *Rickettsia rickettsii* antigen [23] were also demonstrated in similarly stored materials.

Since 1958, only ten cases of Coxsackie B encephalitis [4–6, 11, 15, 17, 22] have been reported with a firm diagnosis based on viral isolation from the brain (Tables 1, 2). Whereas, neuropathological findings of these cases have been reviewed by Moossy and Geer [16], all of them had myocarditis concomitantly and was thus labeled an "encephalomyocarditis" or a generalized infection with CNS involvement. Because of this constant myocardial involvement, it has been questioned whether the encephalitis alone can be responsible for the fatal outcome [10]. Indeed, in two of the ten cases, it was suggested that severe myocarditis was the cause of death [6]. Our report, however, clearly establishes that Coxsackie B_1 can cause fatal encephalitis without signifi-

Table 1. Fatal cases of Coxsackie encephalitis and organs of involvement

Case no. [ref.]	Age	Sex	Organ					
			CNS	Myocardium	Liver	Lung	Pancreas	Other
1 [11]	13 h	f	+	+	+	+	+	
2 [17]	14 days		+	+				
3 [4]	48 h	f	+	+		+		
4 [15]	4 days	m	+	+				
5 [6]	9 days	m	+	+	+			Spleen
6	9 days	f	+	+		+	+	
7 [5]	7 days	f	Meningitis	+				
8 [22]	12 days	m	+	+	+	+	+	
9	7 days	f	+	+			+	Skeletal muscle
10	9 days	f	+	+		+	+	
11 (pres. study)	4 years	m	+		+	+		

Table 2. Coxsackie virus type and organs from which identification was made

Case no.	Viral type	Organ					
		CNS	Myocardium	Liver	Lung	Pancreas	Other
1.	B-4	+	+	+			
2.	B-4	+	+	+			Kidney
3.	B-4	+	+				
4.	B-5	+	+	+	+		
5.	B-4	+	+	+			
6.	B-4	+	+		+	+	Kidney, spleen
7.	B-2	+	+		+		Meninges
8.	B-2	+	+				
9.	B-2	+	+				
10.	B-4	+			+		Stool
11.	B-1	+[a]	+[a]		+[a]		Pharynx, GI tract, stool

[a] Viral antigen detected; virus isolated in all other cases
GI, Gastrointestinal tract

cant myocarditis. In previous reports, fatal cases of Coxsackie B encephalitis were only found in infancy, 14 days old being the oldest [17]. To the best of our knowledge, no fatal cases in older children or adults has been reported with confirmed diagnosis by viral isolation from the CNS.

Disseminated disease in the neonate, which is responsible for the majority of confirmed fatal Coxsackie infections, is most likely due to intrauterine or parturient exposure caused by maternal infection. The severe outcome of the infection in newborn infants contrasts with the relatively benign course in older individuals in whom the primary CNS manifestation is usually that of aseptic meningitis [14]. Interestingly, experimental studies have reproduced this clinical pattern on mice [3, 18]. Our case is, thus, unique in that the fatality occurred at the age of 4 without myocardial involvement. The question arises if fatal Coxsackie B encephalitis, when occurred later in life, may be undetected by conventional virus isolation studies. The immunofluorescent and immunoperoxidase methods described here should be able to uncover such cases, even retrospectively.

We were unable to demonstrate viral particles by electron microscopy in this case. Electron microscopic studies, including the pseudo-replica method, have been established as useful techniques in the detection of some types of viral encephalitides, e.g. Herpes simplex encephalitis [13]. The Coxsackie virus measures 25 to 30 nm in diameter as compared to the 145 to 200 nm range of the Herpes simplex virus. This relatively small size is probably the reason for our negative findings.

Two additional methods for detecting Coxsackie virus in CSF have recently been reported. One makes use of a dot hybridization assay in CSF [19], while specific intrathecal IgM antibody to Coxsackie B_2 virus was measured in the other method [12]. The effeciveness of these methods in diagnosing Coxsackie encephalitis is yet to be proven, however.

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