JOURNAL OF CLINICAL MICROBIOLOGY, May 1985, p. 698–701
0095-1137/85/050698-04$02.00/0

Vol. 21, No. 5

Isolation of Inoue-Melnick Virus and Detection of Antibodies in Coded Cerebrospinal Fluid Specimens from Patients with Disorders of the Central Nervous System

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Inoue-Melnick virus was isolated from coded cerebrospinal fluid samples from 10 of 25 patients with chronic diseases of the central nervous system. Of 13 multiple sclerosis patients and 12 patients with a variety of other symptoms and signs 4 and 6, respectively, were positive. Replicate samples from the same patient yielded the same results, either positive or negative. Neutralizing antibody was present in cerebrospinal fluid at a dilution of 1:25 in the virus-positive patients. Only a single virus-negative patient was positive for antibody at this dilution.

Two years ago, we reported (4) on the isolation of a virus from the cerebrospinal fluid (CSF) of four patients with chronic central nervous system disease (three with multiple sclerosis [MS] and one with amyotrophic lateral sclerosis). Virus was not detected in 27 control CSF specimens from acutely ill patients whose CSF had been taken for diagnostic purposes. The isolates were related to the subacute myelo-optico-neuropathy virus previously recovered in Japan (3). However, until its clinical spectrum is more fully understood, Inoue et al. (2) have suggested that the agent be called Inoue-Melnick (IM) virus.

This report is concerned with the isolation of IM virus from, and the detection of IM antibodies in, the CSF of patients with a variety of chronic neurological disorders. Through the National Multiple Sclerosis Society, New York, N.Y., 31 coded CSF specimens were obtained in 1982 from patients in Connecticut with MS or with a variety of other neurological illnesses. The samples were tested under code for virus and antibody, and appropriately coded virus-positive and virus-negative fluids were included in each test. After all the results were in, they were made known to the society, and the code was broken.

MATERIALS AND METHODS

The culture methods and the identification of the characteristic cellular changes were performed as described previously (4, 5). The specimens were stored at −70°C. For virus isolation, three trials were conducted, several months apart, with dilutions prepared from each original sample. The specific result of any prior test was not known to the investigators. For virus isolation, the CSF samples were diluted with tissue culture medium; the dilutions were 1:10 in trial I, 1:4 in trial II, and 1:5 in trial III. Quantities were not large enough to allow us to test undiluted samples.

Efforts were made to establish a virus passage line from each of the trial samples. When a passage line was established, the passage virus was coded and tested, along with known positive and negative coded samples, against rabbit antiserum prepared against previous isolates of IM virus. If replication of the virus was inhibited by a 1:50 serum dilution, it was considered to be related antigenically to our earlier isolates (2, 5).

Tests were also done with two coded dilutions of each CSF sample (1:10 and 1:25) to detect antibody against the previously isolated Baylor-9 strain, a virus of intermediate antigenicity, which crosses with IM virus types 1 and 2 (2). When tested for antibody, the CSF samples were first heated at 56°C for 30 min, a procedure that inactivates IM virus. CSF dilutions of 1:10 and 1:25 were tested. Portions of CSF (0.5 ml) were mixed with 0.5 ml of IM virus (Baylor-9 strain, tissue culture passage 8, diluted to $10^{-3.0}$). This stock in previous titrations contained $10^{5.5}$ times the 50% tissue culture infective dose per μl. Tubes were coded and incubated for 1 h at 37°C, and then 0.2 ml of each mixture was inoculated into three MRC-5 cultures. The cultures were slowly rolled at 37°C. Two experienced observers separately read the coded cultures on day 5 and recorded their results on separate pages. Readings were made again on day 6 with neither observer having available the readings from the previous day. Again, the cultures were read, and the results were recorded separately. The four pages of readings were decoded by a third person. In addition, negative controls were included as uninfected MRC-5 cultures, and positive controls were included as cultures inoculated with control virus stock at 10^{-3}, 10^{-4}, and 10^{-5}. In satisfactory tests, all the cultures infected with 10^{-3} and 10^{-4} virus stock showed cytopathic changes. Neutralization was considered positive if two (usually three) of the culture tubes containing CSF-virus mixtures showed no cytopathic changes.

RESULTS

Of the 31 coded samples, 12 yielded virus isolates, and passage was obtained (Table 1). In every case, the passage virus was identified as IM virus by neutralization tests. The results of testing the samples for antibody are shown in Table 2 for those that were virus positive and in Table 3 for those that were virus negative. Again, all antibody tests were done under code.

The data are summarized in Table 4 in terms of specimens. Among 11 virus-positive specimens tested for antibody, 8 had antibody at the 1:10 dilution, 8 were positive even at the higher dilution (1:25), and only 2 were negative. On the other hand, of 19 specimens not yielding virus, 13 were also negative for antibody, and in only 4 was antibody detected at the 1:10 dilution. A single specimen was positive at the higher dilution tested (1:25).

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TABLE 1. Virus isolation

Code no.	Virus isolation from trial:			Passage from trial:			Neutralization of passage virus
	I	II	III	I	II	III	
1	−	−	−	−			
2	−	−	−	−			
3	−	−	−	−			
4	+	+	+	+	+	+	+
5	−	−	−	−			
6	−	−	−	−			
7	−	−	−	−			
8	±	+	+	±	+	+	
9	−	−	−	−			
10	+	+	+	+	+	+	+
11	−	−	−				
12	−	+	±		+	+	+
13	−	−	−				
14	−	−	−				
15	−	−	−				
16	−	±	+		+	+	+
17	−	−	−				
18	−	+	+		+	+	+
19	+	+	+	+	+		+
20	±	−	−	+			
21	−	−	−				
22	−	−	−				
23	−	±	+		+	+	+
24	+	±	±	+	+		+
25	+	−	−	+			+
26	+	−	−	+			+
27	−	−	−				
28	−	−	−				
29	−	−	−				
30	−	−	−				
31	−	−	−				

When the code was broken, we were told that in five instances CSF from the same patient had been distributed into two or more vials. In one case, a repeat sample was taken almost 2 months after the first, resulting in three vials (and three code numbers) from one patient.

With the information decoded regarding the patients, we correlated (Table 5) the isolation of virus with the detection of antibody in terms of the patients. Among nine patients tested for antibody, whose CSF yielded virus isolates, only two had CSF that was negative for antibody; seven had CSF positive for antibody. In contrast, of 15 virus-negative patients, 11 were also antibody negative. Two virus-negative patients were positive for antibody at the 1:10 dilution; one of these was positive at the 1:25 dilution, but in only one of three specimens. The results on the replicate samples were consistent in both the isolation of virus and the detection of antibody (Table 6).

TABLE 2. Detection of antibody in virus-positive CSF specimens

Virus isolated from specimen no.:	Antibody in CSF at dilution:	
	1:10	1:25
4	+	+
8	+	+
10	+	+
12	+	+
16	±	+
18	+	+
19	+	−
20	−	−
23	+	+
24	+	+
25	Not done	Not done
26	−	−

TABLE 3. Antibody detection in CSF from which no virus was isolated

Virus not isolated from specimen no.:	Antibody in CSF at dilution:	
	1:10	1:25
1	+	−
2	−	−
3	−	−
5	±	−
6	+	−
7	+	+
9	−	−
11	−	−
13	−	
14	−	−
15	−	
17	−	−
21	−	−
22	−	−
27	−	−
28	−	−
29	+	−
30	−	±
31	−	−

DISCUSSION

Virus was detected in 12 of the 31 specimens tested. As in our earlier study, each isolate was passed and then identified by neutralization with rabbit antibody prepared against our previous IM virus isolates. When repeat isolations were made from the same specimen, the virus from virtually every isolation trial was passed and neutralized.

The results of our tests with the coded specimens that later proved to be replicates were consistent when virus isolation and antibody results on only the replicate specimens were compared (Table 6). This demonstrates the reliability of our methods developed to detect this elusive virus and its antibody.

It should be noted that the IM virus is difficult to work with. Its cytopathic effects are so subtle that, to ensure reliability, we have always conducted our testing on a blind, coded basis (5). Thus, not only was information on the clinical test results and diagnoses of these patients held by others outside our laboratory and the specimens sent to us only with code numbers, but also, when either isolation or

TABLE 4. Association of virus and antibody in coded CSF specimens

Virus isolation from specimen	No. of specimens[a]	No. of specimens with antibody in CSF at dilution:		
		1:25	1:10	Negative
+	11	8	8[b]	2
−	19	1	4	13[c]

[a] In addition, virus was isolated from one specimen that was not tested for antibody.

[b] In addition, one specimen was plus/minus at a dilution of 1:10. This specimen was positive at 1:25.

[c] In addition, one specimen was plus/minus at a dilution of 1:10 and negative at 1:25. Another specimen was plus/minus at 1:25 and negative at 1:10. The other 13 specimens listed were negative at both 1:10 and 1:25.

TABLE 5. Association of virus and antibody in terms of patients

Virus isolation	No. of patients[a]	No. of patients with antibody in CSF at dilution:		
		1:25	1:10	Negative
+	9	7	6[b]	2
−	15	1[c]	2	11[d]

[a] In addition, one specimen yielded a virus isolate but was not tested for antibody.

[b] In addition, the CSF of one patient was plus/minus at a dilution of 1:10. This specimen was positive at 1:25.

[c] Two specimens were taken from one patient about 1 month apart, with the first specimen distributed into two coded vials. All three coded vials (specimens 6, 7, and 29) were negative for virus isolation, all three were positive for antibody at a dilution of 1:10, but only one was positive when diluted to 1:25. This was the only virus-negative patient whose CSF was clearly antibody positive at 1:25.

[d] In addition, the CSF of one patient tested as plus/minus at a dilution of 1:10 but negative at 1:25. One specimen from another patient was negative at 1:10 and plus/minus at 1:25. A duplicate sample from the same patient was negative at both 1:10 and 1:25.

neutralization tests were carried out, the individual vials or individual dilutions were coded in our laboratory, and the code was kept in a sealed envelope until after the test was completed. Two of us conducted the readings independently, and the successive readings on days 5 and 6 were each done on a blind basis, without reference to the readings of the cultures from each vial or each dilution from the previous day. Although this makes the process painfully time consuming and slows down our investigations, we believe it is essential for reliable results. The results obtained under these stringent conditions support our view that—whatever the clinical significance of the IM virus—the agent and its antibody are indeed present in CSF from a number of patients with neurological disorders.

There was clearly an association of virus isolation with the presence of antibody in the CSF. Of 9 virus-positive patients, 7 were also antibody positive at a CSF dilution of 1:25, whereas among 15 patients who were virus negative, only 1 was positive for antibody at this dilution.

Sera from these patients were not available to us, and therefore we could not test them for antibody. However, in other studies (J. L. Melnick, submitted for publication) we have found that antibody against IM virus is present in the serum of patients whose CSF yielded virus. In such patients, the level of antibody in the CSF was similar to that in the serum, an indication of local production of antibody in the central nervous system.

TABLE 6. Virus isolation and antibody from replicate specimens

Code no.[a]	Virus isolation from trial:			Passage from trial:			Neutralization of passage virus	Antibody in CSF at dilution:	
	I	II	III	I	II	III		1:10	1:25
6	−	−	−	−				+	−
7	−	−	−	−				+	+
29	−	−	−					+	−
18	−	+	+		+	+	+	+	+
19	+	+	+	+	+		+	+	−
21	−	−	−					−	−
22	−	−	−					−	−
23	−	±	+		+	+	+	+	+
24	+	±	±	+	+		+	+	+
30	−	−	−					−	±
31	−	−	−					−	−

[a] Replicate vials from the same CSF specimen are bracketed together. Specimens coded as 6, 7, and 29 were all from the same patient; two of the vials from one CSF specimen and a third vial from a CSF specimen were obtained almost 2 months later.

TABLE 7. Diagnoses and virus isolation

Code no.[a]	Sex	Clinical diagnosis	Virus isolation
1	F	MS, chronic progressive	−
2	F	MS, exacerbation	−
3	M	MS, stable	−
4	M	Headache (viral meningitis 6 mo previously)	+
5	M	Demyelinating mononeuritis	−
6, 7, 29	F	Pseudotumor, optic neuritis, possible systemic lupus erythematosus	−
8		Granulomatous angiitis of the CNS	+
9	F	MS, exacerbation	−
10	M	Alcohol withdrawal, cerebral atrophy	+
11	F	MS, stable	−
12	F	Postpartum cerebral vein thrombosis	+
13	F	Tumor-induced encephalitis? Demyelinating disease?	−
14	F	MS, stable	−
15	F	Amyotrophic lateral sclerosis	−
16	M	Cervical spondylosis, myelopathy	+
17	F	MS, exacerbation	−
18, 19	F	MS, exacerbation	+
20	F	MS, chronic progressive	+
21, 22	F	MS, chronic progressive	−
23, 24	M	MS, active	+
25	F	MS	+
26	F	Hysteric rheumatoid arthritis	+
27		Guillain-Barré syndrome	−
28	F	Postherpetic neuralgia	−
30, 31	M	MS, exacerbation	−

[a] Two or three code numbers together indicate that replicate samples from the same person were sent.

IM virus was found in patients with a variety of disorders of the central nervous system (Table 7). No pattern could be seen in regard to the association of virus isolation with any specific syndrome or disease of the central nervous system in these patients.

In relation to the four MS patients from whom virus was isolated, there was no particular association of virus with patients in remission or exacerbation. However, since there is not a specific test for MS, the diagnosis based on clinical symptoms alone may be loose. It is becoming increasingly clear that not only may MS be confused with other neurological syndromes or coexist with them, but there is a wide range of severity, which includes those so minimally affected as not to come to medical attention for MS-related problems for decades. Atypical or clinically silent MS is illustrated by 12 such cases discovered unexpectedly at necropsy by Phadke and Best (6). These investigators divided their cases into three groups: (i) cases with no known previous history of neurological illness; (ii) cases with a history of neurological illness, in whom a diagnosis of MS was never considered; (iii) cases with a history of neurological disorder in whom a diagnosis of MS was rejected or considered unlikely. In a large series of autopsies studied by Gilbert and

Sadler (1), five patients with various medical problems were found unexpectedly at autopsy to have typical MS plaques, although no neurological disease had been suspected in life. Obviously, more work is necessary to determine whether IM virus plays a significant role in the initiation or the development of chronic disorders of the central nervous system.

ACKNOWLEDGMENT

The specimens from patients in Connecticut were kindly made available to us through the Research Division of the National Multiple Sclerosis Society.

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