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Virological Diagnosis of Enterovirus Type 71 Infections: Experiences Gained During an Epidemic of Acute CNS Diseases in Hungary in 1978

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With 1 Figure

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Summary

In 1978 a severe epidemic of acute CNS diseases occurred in Hungary. Enterovirus type 71 (E71) proved to be the main aetiological agent. This could, however, be established only by special investigations, since the usual laboratory tests proved inefficient for diagnosis of E71 infections.

Attempts to isolate virus in tissue cultures were made on 1952 samples from 686 patients. E71 could only be isolated in cultures of a Vero cell line and even then only after 3 to 6 blind passages. Successful isolations were made from 47 stool samples, 5 throat swabs and 6 CNS samples originating from 44 patients. This represented only 13.6 per cent of the total number of E71 cases diagnosed in the course of studies, thus indicating the poor sensitivity of these tests. Suckling mice were unsuitable for the isolation of E71, although E71 tissue culture isolates proved pathogenic to these animals. Isolates could be identified by neutralization technique only after disaggregation of virions by ether treatment.

Blood samples from 1050 patients were tested for E71 antibodies by a radial plaque neutralization technique and those from 593 patients proved positive. A significant change in antibody level could only be detected in 52 out of 286 patients whose paired sera were available. The limited usefulness of this technique for the detection of current E71 infections was, however, indicated by the fact that 66 per cent of patients positive in virus isolation experiments already had antibodies in the early phase of their illness which remained at a constant level thereafter.

Investigation of 684 blood samples from 511 patients for E71-specific IgM antibodies showed this test to be the most reliable for the detection of current E71

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infections. When appropriate blood samples were available, these antibodies could be detected in patients positive in the above mentioned tests, moreover, this method made an aetiological diagnosis possible in 236 cases which otherwise would have remained undetermined.

Infection with E71 was finally confirmed in 323 cases (poliomyelitis-like paralysis, 13; encephalitis, 145; aseptic meningitis, 161; hand, foot and mouth disease, 4).

Introduction

A number of clinical and epidemiological conditions have been shown over the last decade to be associated with infection by a recently recognized enterovirus, type 71 (E71). It has elicited sporadic cases of meningitis, encephalitis, exanthematous and respiratory diseases in the U.S.A. (5, 6, 24), Australia (12) and Sweden (2, 28); extensive mixed epidemics of hand, foot and mouth disease and aseptic meningitis with a predominance of the former in Japan (10, 11, 14) and of the latter in Sweden (2); and a severe epidemic of acute CNS diseases in Bulgaria (4, 13, 25). In Hungary a severe meningitis-encephalitis epidemic occurred in 1978 lasting from May to September. Certain clinical manifestations and epidemiological characteristics were similar to those observed in Bulgarian epidemic. This tended to suggest that E71 might be responsible also for the epidemic in Hungary. There were 826 aseptic meningitis (7.7 per 100,000) and 724 encephalitis (6.8 per 100,000) cases reported (26). The latter category included cases with unusual clinical features such as acute cerebellar ataxia and poliomyelitis-like paralyses (1), as well as fatal cases showing distinct histopathological alterations in CNS (27). The age specific morbidity rates were especially high in age groups under 6 years; 30 out of 47 fatal cases have fallen into these age groups.

It was, however, a laborious and time consuming laboratory exercise to prove that E71 played the main role in the epidemic. As expected, infections due to other aetiological agents were also detected in the epidemic. Laboratory diagnoses of infections with tick-borne encephalitis, LCM, mumps, herpes simplex virus, coxsackievirus types B4 and B5 and adenoviruses were made with ease but of infections due to E71 proved difficult to establish.

The present paper gives an account of experiences gained in the laboratory diagnosis of E71 infections during the epidemic. It is hoped that these may be profitable for those interested in diagnostic virology. Epidemiological and virological analysis of the epidemic will be published elsewhere (9, 26).

Materials and Methods

Specimens for Virus Isolation

Specimens for isolation experiments were collected from 352 patients with encephalitis, from 330 patients with aseptic meningitis and from 4 patients with hand, foot and mouth disease. Details of the specimens tested are given in Table 1. They were prepared for isolation experiments as previously described (7).

Virus Isolation Experiments

Specimens from 620 patients were tested in primary rhesus monkey kidney (RMK) cell cultures and in cultures of a Vero cell line (termed Vero A line) originating from

The National Institute for Biological Standards and Control, London. Specimens from some patients were only tested in one of these cell systems, 18 in RMK cells and 48 in the Vero A line. Selected samples from the first group of patients were also inoculated into cultures of the following cells; a Vero cell line originating from The Institute of Microbiology and Virology, University of Bologna and termed Vero B line, an HEp-2(C)-line and primary human embrionic fibroblasts. Cultures were inoculated in triplicate with 0.1 ml of inocula. They were maintained in Parker's 199 medium. RMK cultures were incubated for 14 days at 37° C with a medium change on the 7th day after inoculation, whilst the other cultures mentioned were incubated for 7 days at the same temperature. Every sample tested was subjected to a minimum of 3 blind passages in each cell system used. The inoculated cultures were examined microscopically at least every other day.

Virus isolations were also attempted in 1 day old suckling mice from 90 specimens obtained from 52 patients. These were performed as described previously (7).

Identification of the Isolates

Viruses isolated in cell cultures were identified either by macro- or micro-neutralization techniques using the Vero A cell line. The procedure advocated by SCHMIDT (21) was followed for both techniques. Identification of virus in suckling mice was performed according to the method described previously (7). Type sera used in these experiments were as follows. Monkey antiserum (Lot No. S-2415) prepared against the E 71 prototype BrCr strain (24) was kindly supplied by Dr. E. H. Lennette (Berkeley, U.S.A.) and a rabbit antiserum prepared against the Bulgarian E 71 isolate No. 258 (3, 13) by Dr. M. K. Voroshilova and Dr. S. G. Drozdov (Moscow). Type-specific rabbit antisera to poliovirus types 1 to 3, to coxsackievirus types A 7, A 9 and B 1 to B 6, to echovirus types 1 to 9, 11 to 27 and 29 to 33, to enterovirus type 70, as well as mouse antisera to coxsackievirus types A 1 to A 24 were prepared in this laboratory by methods described previously (8). Enterovirus 70 and 71 type sera were used individually in neutralization tests, whereas all other sera were applied according to the "Intersecting Serum Scheme" described by SCHMIDT *et al.* (22).

Attempts to overcome difficulties encountered in the neutralization tests were made by subjecting the isolates to various traetments (1 per cent sodium deoxycholate, ether, ultrasonic irradiation). The aim of this was to eliminate virus aggregates present in the suspensions. Ether treatment, as described by GOBARA *et al.* (10), finally proved successful.

Serum Specimens from Patients for Antibody Determinations

Altogether 1669 blood samples taken from 1050 patients were tested for the presence of neutralizing antibodies to E 71. Only a single serum sample was available from 632 patients. Two or more samples, taken at different stages of the illness were available from 418 patients. All sera were inactivated at 56° C for 30 minutes before examination. The collected sera were stored at −10° C until use.

Tests for Neutralizing Antibodies Against E 71

Neutralizing antibody assays were carried out by the radial plaque neutralization method as previously described (17). In brief, sera were administered by 5 µl wire-loops to wells cut into the agar overlay of E 71 (strain No. 258) infected monolayers of secondary vervet monkey kidney cells grown in Linbro FB-4 TC multidishes. Radial neutralization zones were formed with a size directly related to their specific antibody contents. Parallel with each series of tests, twofold dilution series of rabbit antiserum to strain No. 258, ranging from undiluted to 1:512, were tested. The neutralizing antibody titre of test sera could be estimated by comparing the diameter of the protected zone to those of the rabbit antiserum dilution series; the specific antibody titre of the latter was previously determined in a microtitre system. Sera producing protecting zones with a diameter corresponding to an antibody titre of 1:10 or higher were considered positive for E 71 neutralizing antibodies.

Demonstration of E71-Specific IgM Antibodies

The ion exchange batch technique employed for the separation of IgM antibodies from human sera has been described in a previous paper (16). This technique was slightly modified for the present experiments. A thick slurry of QAE-Sephadex A-25 gel, previously equilibrated in 0.03 M Tris-HCl buffer, pH 8.8, and sterilized by incubating in boiling water for one hour, was added to a glass tube until the settled volume reached 1.0 ml. Serum (0.1 ml) was then added and the stoppered tube was rotated end-to-end for 30 minutes. In order to remove unadsorbed IgG the gel was resuspended in 4—5 ml of 0.12 M Tris-HCl buffer, pH 8.1, and mixed for 1 minute. The tube was then centrifuged at 1000 rpm for 1 minute and the supernatant discarded. After repeating this procedure with the same buffer, practically all IgG antibodies were eliminated from the gel. In order to elute IgM from the gel 0.2 ml of 1.0 M Tris-HCl buffer, pH 6.6, was added to the tube and thoroughly mixed. The eluate was obtained by draining the supernatant. This eluate was tested for E71 antibodies by the plaque neutralization method described above. IgM fractions producing protecting zones with a diameter corresponding to an antibody titre of 1:10 or higher were considered positive for E71 specific IgM antibodies. Typical examples of the test are shown in Fig. 1.

The method was regularly controlled by testing selected eluates for IgM, IgG and IgA content in immunodiffusion tests as described previously (15).

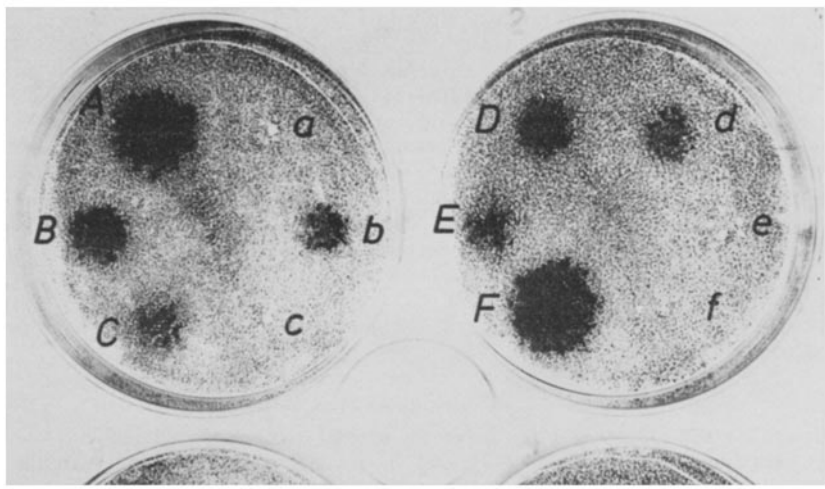

Fig. 1. Results of radial plaque neutralization tests to E71 carried out with 6 human sera and with their IgM fractions. Capital letters on the left (*A* to *F*) indicate the individual serum samples and small letters on the right (*a* to *f*) their corresponding IgM fractions. As may be seen, all the sera contained neutralizing antibodies to E71, but specific IgM antibodies were present only in IgM fractions of B and D sera (*b* and *d* fractions)

Results

Isolation and Identification of E71 Strains

Althogeter 58 E71 strains were isolated. Successful isolation was only achieved in the Vero A line and even in this system the virus could only be recovered after blind passages. An indistinctive cytopathic effect was usually observed after the 2nd or 3rd passage and a further 2 or 3 passages were required to produce a clear

characteristic effect. Thus isolation was a laborious procedure usually taking more than a month. Two isolations were made by alternating RMK and Vero A cells in passages, but otherwise the RMK cells proved insensitive as were the Vero B, HEp-2(C) and human embryonic fibroblast cells.

Athough E71 was detected in cell cultures in 6 out of 90 clinical specimens, 2nd or 3rd passage and a further 2 or 3 passages were required to obtain a clear attempts to isolate the virus from these specimens in suckling mice failed. However 5 isolates grown in Vero A cells proved pathogenic for suckling mice causing symptoms characteristic of coxsackie A viruses.

Enterovirus type sera incorporated into intersecting serum pools (see Materials and Methods) failed to neutralize the isolates, and even E71 type sera only delayed virus multiplication in tissue cultures. However, complete neutralization was achieved by E71 sera after suspensions of isolates were treated with ether. It is of note that 2 tissue culture isolates inoculated into suckling mice did not require disaggregation in order to be neutralized by E71 sera.

Results of virus isolation experiments are summarized in Table 1.

Table 1. *Isolation of E71 from different materials*

Kind of specimens	Patients			Samples		
	Tested No.	Positive		Tested No.	Positive	
		No.	%		No.	%
CNS	13	4	31	23	6	26
Stool	454	38	8.4	892	47	5.3
Throat swab	369	5[a]	1.4	615	5	0.8
CSF	401	0	0	422	0	0
Total	686	44	6.4	1952	58	3.0

[a] In the case of 3 patients E71 was isolated simultaneously from the stool samples

E71 could be isolated from 47 stool samples, 5 throat swabs and 6 CNS samples originating from 44 patients (34 with encephalitis, 8 with aseptic meningitis and 2 with hand, foot and mouth disease). Positive CNS samples originated from 4 fatal cases. In two cases brain specimens were tested and found positive; in one case samples of brain and medulla were available but only the latter yielded virus; and in a further case medulla, as well as two spinal cord specimens from different segments were investigated and all proved to be positive. It is of note that all the tests carried out on 422 CSF samples from 401 patients proved negative. Most isolations were from stool samples, however, the isolation rate was only 5.3 per cent. Throat swabs were taken from 26 patients at a time when virus could be detected in their stools but only 3 were found positive for E71.

E71 Antibody Determinations

Blood samples taken from 1050 patients were investigated for E71 neutralizing antibodies. Their presence could be demonstrated in samples taken from 593 patients (Table 2). Two or more samples, taken at different stages of illness, were

available from 286 patients with positive sera. A significant titre increase was found in 46 cases whilst 6 cases showed a significant titre decrease. Paired sera from the appropriate stages of illness were available from 27 patients positive in virus isolation experiments. A significant increase in antibody titre was demonstrated in 9 of the cases. The titre of E71 antibodies measured in the early stages of illness were maintained at a constant level in the rest of the cases. These findings made it clear that verification of E71 infections could not be based on the detection of a significant rise in neutralizing antibody level during the course of the illness.

Thus attempts were made to use E71-specific IgM antibody determinations for the verification of current E71 infections. It was hoped that this might make laboratory diagnosis possible when only a single serum sample was available, or when the timings of serum pair collections were inappropriate. Altogether 684 blood samples positive for E71 neutralizing antibodies were used in these investigations.

The results of E71-specific IgM antibody and E71 neutralizing antibody tests are shown in Table 2.

E71-specific IgM antibodies could be detected in blood samples of 47 out of 52 patients, whose diagnosis was verified by a significant neutralizing titre change.

Table 2. *Results of E71 antibody tests in 1050 patients*

	E71 neutralizing antibodies			
	Positive			
E71 specific IgM	with titre	without[a] change	Negative	Total
Positive	47	260	—	307
Negative	2	202	—	204
Not tested	3	79	457	539
Total	52	541	457	1050

[a] This group includes patients whose antibody titre was repeatedly determined in the course of the illness and in whom no significant change could be detected; patients from whom only a single serum sample was available are also included

Table 3. *Persistence of E71 specific IgM antibodies based on tests on multiple serum samples received from 44 patients*

	No. of patients		
	Tested	Positive	Negative
Weeks after onset		for IgM antibodies	
1— 4	44	44	0
5— 8	11	7	4
9—12	14	9	5
13—16	14	3	11
17—20	13	0	13
≥21	12	0	12

Three patients' sera were not available for IgM determinations since they were exhausted in former investigations. In two further cases no IgM antibodies were found in the blood samples tested. These samples, however, originated from the late convalescent phase of the illness and a significant titre decrease was actually observed.

Tests for IgM antibody were performed on samples from 462 out of 541 patients whose blood contained neutralizing antibodies but did not show a significant change in neutralizing titre or alternatively, for whom paired sera were unavailable. E71-specific IgM antibodies were found in 260 samples. There was a relationship between the actual neutralizing titre and the presence of IgM antibodies; i.e. 68 per cent of sera with high ($\geqslant 160$), 56 per cent with medium (40—80) and 3 per cent with low ($\leqslant 20$) levels of neutralizing antibodies proved to contain specific IgM.

Persistence of E71-specific IgM antibodies was investigated by testing multiple serum samples from 44 patients (Table 3). There were 26 patients among them whose E71 infection was confirmed by virus isolation and/or the demonstration of a significant neutralizing titre rise.

IgM antibodies were invariably present within 4 weeks following the onset of illness. The number of patients with detectable IgM antibodies, however, gradually decreased between the 2nd and 4th months and no E71-specific IgM antibodies could be found in blood samples taken after the 15th week. This indicates that persistence of E71-specific IgM antibodies is of short duration, thus their presence can be accepted as proof of a current or most recent E71 infection.

Comparative Usefulness of the Techniques Applied

As may be seen in Table 4, positive results were obtained from 323 patients (13 with poliomyelitis-like paralysis, 145 with encephalitis, 161 with aseptic meningitis and 4 with hand, foot and mouth disease). In 11 cases E71 infection was verified exclusively by virus isolation, since no sera were available for investigation. Four fatal encephalitis cases belonged to this group. In addition to virus isolation, a significant change in antibody level and/or the presence of specific IgM antibodies indicated E71 infection in 33 cases. In 279 cases in which there was a failure to recover virus, current infection with E71 was confirmed by; a rise in neutralizing antibody titre in 4 cases, a neutralizing antibody titre rise

Table 4. *Detection of E71 infections by different techniques*

Neutralizing antibodies	Change in titre[a]	Specific IgM	Virus isolation		Total
			Positive	Negative	
+	+	NT	1	4	5
+	+	+	8	39	47
+	—	+	24	236	260
NT	NT	NT	11	.	11
Total			44	279	323

[a] A significant increase; in some cases a significant decrease

together with the presence of IgM antibodies in 39 cases, and the presence of IgM antibodies without a change in neutralizing titre in 236 cases. Thus the tests for specific IgM proved to be the most reliable for verification of E71 infections.

Virus isolation as a diagnostic tool for E71 infections proved very inefficient in comparison to the serological techniques. Table 5 shows that E71 could be isolated from the stool samples of 17 per cent of patients positive in antibody tests. Even in stool samples collected during the first week of illness a virus isolation rate of only 22.7 per cent was achieved.

Table 5. *E71 isolations from stool samples collected at different times after the onset of illness from patients with serologically confirmed infection*

Collection time (days after onset)	Patients		
	Tested No.[a]	Positive	
		No.	%
1— 7	97	22	22.7
8—14	52	8	15.4
≥15	15	1	6.7
Unknown	18	0	0
Total	182	31	17.0

[a] Only the first sample was taken into consideration when multiple samples were collected

Discussion

In addition to the variable pathogenic potential of E71, mentioned in the introduction, considerable variations in experimental host range have also been observed in different countries. RMK cells were unsuitable for isolation in our studies, but in the U.S.A. most of the isolations have been made in this system (5, 6, 24). Cell lines of human origin were not sensitive to the E71 strains circulating in Bulgaria in 1975 (4) and in the epidemic described here, but in Australia a number of isolations were made in HeLa, heteroploidic human epithelial cells and diploidic human fibroblasts (12). Cells of vervet or cynomolgus monkey origin (primary kidney; Vero, GMK, CMK, MEK-3 lines) were used with greatest success for recovery of virus from clinical specimens (2, 4, 10, 11, 12, 14); nevertheless even in these systems the results varied in different laboratories and epidemics. Vero cells were found satisfactorily sensitive to E71 in Sweden and in Japan; but in the same system several blind passages were needed to recover virus both in the Bulgarian (4) and Hungarian epidemics. Our finding that one of the two Vero cell lines available in this laboratory failed to support the growth of E71 together with a report from von ZEIPEL (28) on a GMK cell line, indicates that susceptibility of the same cell line to E71 may vary presumably due to differences in maintenance procedures.

Our attempts to isolate E71 from clinical specimens in suckling mice failed, although virus isolated in tissue cultures proved pathogenic to these animals.

Similar observations were reported from Australia (12), Sweden (2), and U.S.A. (5, 6). In contrast, some isolates from hand, foot and mouth disease in Japan (11) and 15 isolates from CNS diseases in Bulgaria (4) could be directly recovered in suckling mice or in suckling cotton rats.

Difficulties have regularly been encountered in identification of E71 isolates due to aggregation of virions or to other undefined factors related to the host cell system (2, 12, 24, 28). It is interesting to note that CHUMAKOV *et al.* (4) have not experienced difficulties with the identification of strains isolated and tested in Vero cells, whilst our isolates proved neutralizable by E71 immune sera in the same system only after disaggregation of the virions.

When isolates of different origin were compared in cross neutralization tests some antigenic differences were observed between the Bulgarian strain No. 258 and the prototype BrCr strain (13, 18), as well as between the Japanese Nagoya and the BrCr strains (11). It is of interest to note that two of our isolates, one from the brain of a patient with fatal encephalitis and another from the stool of a patient with poliomyelitis-like paralysis have been tested with anti-BrCr sera in Houston at the WHO Collaborating Centre for Virus Reference and Research. Both isolates, particularly the one from the poliomyelitis-like case, showed notable antigenic difference from the prototype strain.

E71 was isolated with a high frequency from CNS samples of fatal cases observed in Bulgaria (4). The same observation was made in our studies. It seems, however, that E71 occurs in the CSF very infrequently. There are only 6 cases published to date (2, 4) of the isolation of E71 from the CSF. In our studies none of the 422 samples tested yielded virus. Similar findings have been reported from other laboratories (6, 12, 14).

In our studies E71 was rarely isolated from the throat even if it was present in the stool. This is in contrast to other reports (2, 10, 12, 14). The discrepant results may reflect differences in the incubation periods of the diseases investigated. Japanese investigators (14) observed that, in cases where both skin and CNS manifestations were seen, the CNS symptoms occurred 2 to 7 days after the appearence of a rash. This tends to indicate that the incubation period may be generally longer in CNS diseases than in hand, foot and mouth disease caused by E71. If so, it is to be expected that in the case of CNS disease the probability of virus recovery from the throat is much less than from the stool, since following infection virus multiplication is of short duration in the pharynx but prolonged in the intestinal tract (14).

The relatively long incubation period in CNS diseases caused by the E71 may explain why most of the patients tested in our, as well as in other studies (4, 6, 14, 24), have had high antibody titres already at the time of the appearance of clinical manifestations. This made the usefulness of a classical serological approach to aetiological diagnosis questionable.

According to our observations persistence of E71-specific IgM antibodies is transient, as is the case with other enterovirus infections (19, 20, 23). Thus their presence in the blood is a reliable indicator of a current infection. The efficiency of this method as a diagnostic tool is well illustrated by the fact that it alone accounted for the verification of E71 infection in 236 out of 323 cases. An advantage of the specific IgM test is that investigation of a single serum sample may

be sufficient to establish the aetiological diagnosis. The technique applied by us is relatively simple and may also be adapted for diagnosis of other enterovirus infections.

It is of note that in certain studies a high neutralizing antibody level to E71, detected in the course of the illness, has been accepted as evidence of a current E71 infection. In the light of our studies this seems unjustified, since 32 per cent of our patients with high neutralizing titres to E71 had no specific antibodies of the IgM class.

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