Laboratory Studies of Epidemic Strains of Echovirus 9[1] (35568)

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Epidemics of acute meningoencephalitis due to Echovirus 9 occurred in Central Ohio during the summers, 1967 and 1968. Strains of Echovirus 9 were isolated from the cerebrospinal fluid of 196 children. The magnitude of the epidemics, particularly that of 1968, the high viral recovery rate from CSF, and the occurrence of a severe, rapidly progressive fatal encephalitis in a 5-year-old child led to studies comparing epidemic strains to the prototype Hill strain.

Materials and Methods. One group of patients was seen in the emergency room or clinics of the Columbus Children's Hospital (CCH). From these patients, only a single specimen of cerebrospinal fluid (CSF) was obtained for viral culture. The quantity of CSF was variable. The second group of patients was admitted to CCH with a tentative diagnosis of aseptic meningoencephalitis. One specimen of CSF was available for culture, and in addition, washings of throat and anal swabs, obtained on 2 consecutive days, were processed for recovery of virus. In the latter group, 37 patients had paired acute and convalescent phase sera for antibody studies. Description of the details of the procedures used have been reported elsewhere (1, 2).

The plaque morphology of an epidemic strain recovered from postmortem human brain was compared with the prototype Hill strain, and later, 53 epidemic strains were studied for plaque morphology. Plaque morphology was studied as described by Conant *et al.* (3) using Ionagar and Agarose overlay media.

Mouse pathogenicity was examined by intracerebral inoculation of CSF into suckling mice.

Immunological relationships between epidemic strains and prototype Hill strain were examined. Viruses were plaque purified by three serial passages and seed virus for animal immunization was propagated in continuous African green monkey kidney cells grown and maintained in Eagle's minimal essential medium containing rabbit serum. The method for immunization of guinea pigs was that described by Conant *et al.* (4). In addition to these immune sera, others for the Hill strain, obtained from the National Communicable Disease Center and Microbiological Associates, were also used.

Further antigenic analysis of epidemic and prototype Hill strains was performed using the micromethod of double diffusion gel precipitation as described by Conant *et al.* (5).

Results. The CSF specimens obtained from 351 children with a clinical diagnosis of aseptic meningoencephalitis were inoculated into a variety of cell cultures (Table I). Echovirus 9 was recovered from 196 of these specimens. Primary African green monkey kidney (AGMK) cells were best for recovery of virus. 177 were positive in AGMK cells, 55 in diploid fibroblasts and 3 in continuous epithelial cells, for an overall recovery rate of 57%.

Other investigators have demonstrated a

[1] This investigation was supported by U.S. Public Health Service Research Grant NB 5409 from the National Institutes of Health and by Public Health Service General Research Support Grant FR 5504 from the Children's Hospital Research Foundation.

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TABLE I. Gross Recovery Rate Virus from CSF, 196/351 (57%).

	No. of patients
Clinical diagnosis of aseptic meningo-encephalitis	351
Virus from CSF	196
Cell system located in	
AGMK	177
Human diploid	55
Cont. epithelial	3

relationship between plaque size and virulence. Figure 1 shows the plaque morphology of prototype Hill strain and an epidemic strain (Priess) recovered from postmortem human brain, under both Ionagar and Agarose overlay media.

The Priess strain produced plaques up to 15 mm in diameter under either overlay media. Prototype Hill strain, on the other hand, produced minute plaques under Ionagar and plaques 3 to 5 mm in diameter under sulfated polysaccharide-free Agarose. The inhibition in plaque size of the Hill strain under unpurified agar overlay has also been reported by others (6). A total of 53 epidemic strains were studied for plaque morphology; and all produced large plaques comparable to the Priess strain.

Mouse pathogenicity was examined by intracerebral inoculation of cerebrospinal fluid into suckling mice. Of the strains inoculated, three, Priess, 8-1201, and 8-1204 produced a fatal flaccid paralysis similar to that observed with the Coxsackie A viruses. We then examined the effect of cell culture passage on mouse pathogenicity. Eleven epidemic strains and the Hill strain were inoculated into mice after 3 to 10 passages in cell culture. The results can be seen in Table II. The $\log_{10}$ TCD_{50} of cell culture material varied between 3 and 7.5. The Priess strain was highly neurotropic for mice, as were strains 8-1201 and 8-1204. Strains 8-1142 and 8-1955 appeared to be less neurotropic. Virus was recovered from the brains of all dead mice and identified as Echovirus 9. The remaining epidemic strains and the Hill prototype

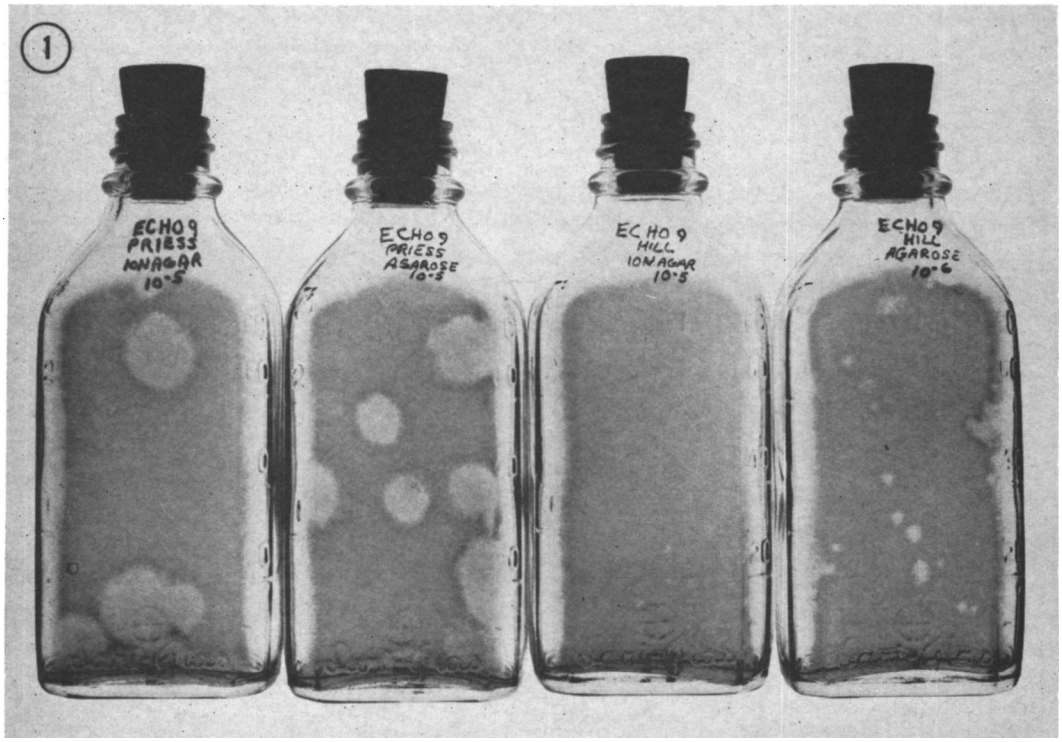

Figure 1

TABLE II. Pathogenicity of Echovirus 9 Strains for Suckling Mice.

Virus strain	Passage level	Log_{10} TCD_{50}/ml; 0.02 ml inoculated ic/mouse							
		7.5	7.0	6.0	5.5	5.0	4.5	3.5	3.0
Priess	6	8/8[a]	—	—	—	—	8/8	—	—
8-1201	4	8/8	—	—	8/8	—	—	—	—
8-1204	4	—	8/8	—	—	8/8	—	—	—
8-1142	10	—	—	3/8	—	—	—	—	0/8
8-1955	10	—	—	—	—	1/8	—	—	0/8
8-1920	9	0/8	—	—	—	—	0/8	—	—
8-2586	6	0/8	—	0/8	—	—	—	—	—
8-1783	9	—	—	—	—	0/8	—	—	—
8-1079	6	—	—	0/8	—	—	—	0/8	—
8-1015	7	—	—	0/8	—	—	—	—	0/8
8-1523	3	0/8	—	—	0/8	—	—	—	—
Hill	20	0/8	—	0/8	—	0/8	—	—	—

[a] 8/8 = number of deaths/number of mice inoculated.

strain did not produce disease at the concentrations used. No blind passages were made.

Cross neutralization tests were performed using prototype Hill strain, Priess strain, and other epidemic isolates. Guinea pig antisera prepared in this laboratory against Priess and prototype Hill strains, as well as rabbit antiprototype serum from Microbiological Associates, were used (Table III). Anti-Priess serum neutralized epidemic strains at a level comparable to the homologous titer. The prototype strain was poorly neutralized by anti-Priess serum and conversely, Priess virus was poorly neutralized by prototype antiserum. Similarly, 23 other epidemic isolates were poorly neutralized by antiprototype serum. Somewhat better neutralization of epidemic strains was obtained by a commercially prepared rabbit antiprototype serum but, nevertheless, the homologous titer was significantly higher. A summary of these results is shown in Table IV.

When paired human sera from 37 patients were examined using the prototype strain and homologous isolate, no differences in neutralizing antibody responses were noted (Table V). Serologic conversion, as evidenced by a fourfold or greater rise between acute and convalescent sera, was demonstrated with 10 patients using either prototype or homologous isolate. Serum from 4 additional patients demonstrated rises preferentially, 1 with prototype only and 3 only with homologous iso-

TABLE III. Reciprocal Relationships Between E9 Epidemic Isolates and E9 Prototype Virus.

Virus epidemic isolates[a]	Antiserum		
	Priess GP	Prototype GP	Prototype MBA-rabbit
8-2456	768	16	512
8-1639	512	16	256
8-2762	768	6	512
8-1523	768	16	256
8-3619	384	12	512
8-2385	1024	24	384
8-1079	1024	12	128
8-1015	768	8	384
8-1460	192	6	128
8-1741	1024	16	512
8-2586	1024	32	512
8-2924	1024	24	384
8-1395	1024	24	512
8-2387	1024	96	512
7-1342	1024	24	512
7-1555	512	12	512
7-2184	768	24	512
8-1142	512	24	512
8-1664	256	16	384
8-1787	192	8	512
8-2334	192	8	192
8-1103	512	24	512
8-1421	384	24	384
Priess	1024	12	256
Prototype	8	1536	3072

[a] Antiserum titers against 30 to 300 TCD_{50} of each virus.

TABLE IV. Reciprocal Relationships Between E9 Epidemic Isolates and E9 Prototype Virus.

Virus	Antiserum		
	Priess GP	Prototype GP	Prototype MBA-rabbit
Priess	1024	12	256
Prototype	8	1536	3072
Epidemic isolates[a]	742 (192 to 1024)	20 (6 to 32)	420 (128 to 512)

[a] Antiserum titers represent the average titer obtained in tests employing 23 distinct epidemic isolates. The numbers in parentheses are the range of antiserum titers against 30 to 300 TCD_{50} of each virus.

late. The remaining 23 patients possessed comparable levels of antibody in both samples using either strain.

Antigenic analysis of epidemic and prototype Hill strains using the micromethod of double diffusion gel precipitation further characterized the epidemic strains.

The specificity of the reactions was demonstrated when Echovirus types 9 (E-9) and 4 (E-4) were diffused simultaneouly against an animal immune serum containing antibody for both (ANTI-E9 and E4) Fig. 2. A reaction of nonidentity occurred. Further evidence for specificity is seen also in Fig. 3.

TABLE V. Antibody Detected in Paired Sera by Prototype Virus and Isolated Strain.

	No. of patients
4-Fold or greater rise with:	
Both prototype and isolated strain	10
Prototype only	1
Isolated virus only	3
Both show antibody—no significant increase with either virus	23
Total	37

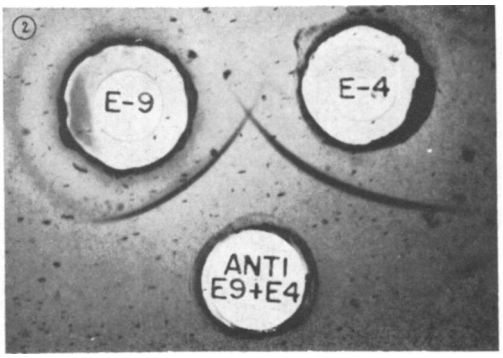

Figure 2

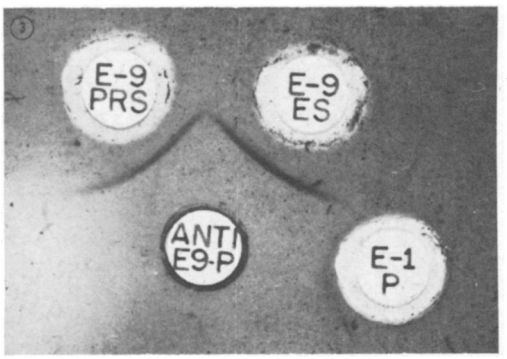

Figure 3

Echovirus 9 epidemic strains (E-9 PRS and E-9 ES) and Echovirus 1 (E-1P) were diffused simultaneously against an anti-Echovirus 9 prototype serum (ANTI-E9-P). A reaction of complete identity occurs between the two Echovirus 9 strains while no visible reaction can be seen with the Echovirus 1 antigen. The Echovirus 1 antigen was reactive in the same test with type specific antiserum.

Although antiprototype serum detected a common antigen when diffused against epidemic strain antigens, distinct antigenic differences were observed when these strains were compared with the prototype using homologous immune sera. Figure 4 shows a reaction of partial identity when prototype (E-9P) and Priess antigens (E-9 PRS) were diffused simultaneously against antiprototype serum (ANTI-E9-P). The spur extends from the prototype line over the Priess line. Similarly, Fig. 5 shows spur formation obtained when

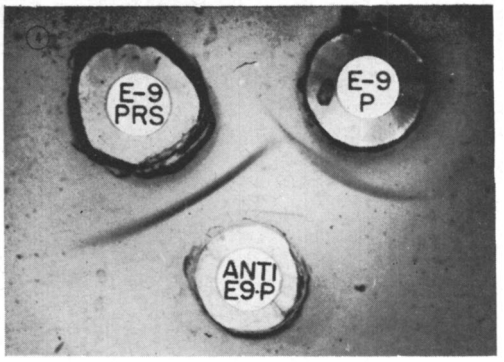

Figure 4

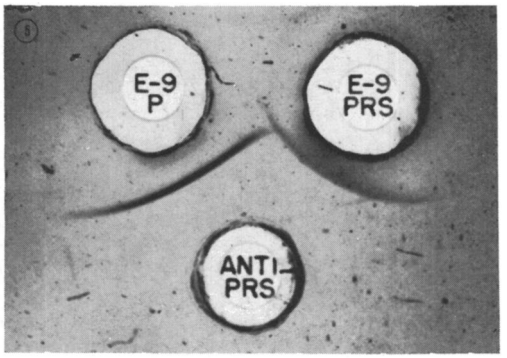

Figure 5

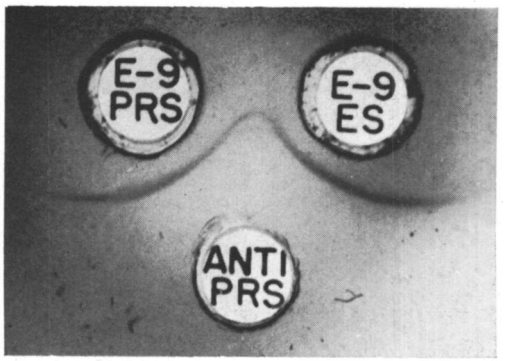

Figure 6

anti-Priess serum (ANTI-PRS) was employed. Each virus was antigenically richer when homologous immune serum was used. Figure 6 reveals that when either of the epidemic strains (E-9 PRS and E-9 ES) was diffused against anti-Priess serum (ANTI-PRS) they reacted in identity. These results confirm the antigenic differences observed in the cross neutralization tests.

Discussion. In the summer of 1967 the number of patients at CCH with aseptic meningitis was larger than usual. Some illnesses were caused by Coxsackie B-5, but most were due to Echovirus 9. During the summer of 1968 the number of aseptic meningitis cases due to Echovirus 9 assumed epidemic proportions. Only a small number of these patients required hospitalization. Specimens of CSF from many of these patients were so small that it was necessary to dilute the specimen 10- to 20- fold to have a sufficient quantity to inoculate cell cultures and suckling mice. In addition, CSF specimens frequently stood at room or refrigerator temperatures from 1 to 48 hr prior to inoculation. Even under these adverse circumstances, the gross recovery rate for Echovirus 9 was 57%. This value is somewhat misleading because the total number of patients with a clinical diagnosis of aseptic meningitis includes patients whose meningitis was shown later to be due to other agents (mumps, California encephalitis, partially treated bacterial meningitis) or who definitely did not have CNS disease.

There were 115 patients with a clinical diagnosis of aseptic meningoencephalitis who had one CSF specimen, and two specimens of throat and anal swab washings which yielded Echovirus 9 from at least one of the three culture sites. Ninety-six of these 115 patients (84%) had the virus recovered from CSF. On this basis it was concluded that the epidemic strain of Echovirus 9 was highly neurotropic.

The rapid development of cytopathology in cell cultures suggests that the infectivity titer was high, although titers were never determined precisely.

The large number of patients, the apparent neurotropism, and the ease of isolation of the virus from CSF was, in itself, of interest, but in addition a 5-year-old child had a very severe, rapidly progressive encephalitis which terminated in death. The virus was isolated from an antemortem specimen of CSF and from a postmortem specimen of brain. For these reasons, we compared epidemic strains to prototype Hill strain.

These studies demonstrated differences between epidemic strains and prototype Hill strain by reciprocal neutralization tests, path-

ogenicity for suckling mice, double diffusion gel precipitation, and plaque morphology. However, these strains do not demonstrate the classical prime relationship to the prototype virus as described for Echovirus type 6 (7). The antigenic relationships observed are similar to those described for Echovirus type 4 variants (8). Accordingly, the diagnostic laboratory should be aware of the possibility of variants of Echovirus 9 in order not to miss identifying isolates.

It is very unusual for Echovirus type 9 to produce a fatal encephalitis. This patient, to be reported later in detail, is of particular interest because the fatal encephalitis was accompanied by fatty metamorphosis of the liver consistent with Reye's syndrome.

Summary. The characteristics of epidemic strains of Echovirus type 9 were compared with the prototype Hill strain. Definite antigenic differences were demonstrated; however, they are not the classical *prime* relationships, but correspond more closely to the variant relationships demonstrated with Echovirus type 4. The virus was highly neurotropic, and was easily recovered from specimens of CSF. African green monkey kidney cells were most sensitive for recovery of virus.

1. Haynes, R. E., Azimi, P. H., and Cramblett, H. G., J. Amer. Med. Ass. **206**, 312 (1968).

2. Haynes, R. E., Cramblett, H. G., and Kronfol, H. J., J. Amer. Med. Ass. **208**, 1657 (1969).

3. Conant, R. M., Somerson, N. L. and Hamparian, V. V., Proc. Soc. Exp. Biol. Med. **168**, 51 (1968).

4. Conant, R. M., Thomas D. C., and Hamparian, V. V., Proc. Soc. Exp. Biol. Med. **134**, 677 (1970).

5. Conant, R. M., Barron, A. L., and Milgrom, F., Proc. Soc. Exp. Biol. Med. **121**, 307 (1966).

6. Wallis, C., and Melnick, J. L., J. Virol. **2**, 267 (1968).

7. Melnick, J. L., N.Y. Acad. Sci. **5**, 365 (1957).

8. Barron, A. L., and Karzon, D. T., J. Immunol. **87**, 608 (1961).

Received Dec. 18, 1970. P.S.E.B.M., 1971, Vol. 137.