FACTORS DETERMINING PATHOGENICITY OF VARIANTS OF ECHO 9 VIRUS FOR NEWBORN MICE*

By HANS J. EGGERS, M.D., AND ALBERT B. SABIN, M.D.

(From The Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Cincinnati)

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The ECHO (enteric cytopathogenic human orphan) group of viruses was originally distinguished from the Coxsackie viruses not only because they could not be recovered by inoculation of human material into newborn mice but also because they lacked this pathogenicity even after enrichment of the population in tissue culture (1). The prototype strain (Hill) of ECHO 9 virus, originally reported as human enteric or "HE" Type 3 virus (2) was not pathogenic for newborn mice after several passages in tissue culture. Seven other strains of ECHO 9 virus recovered from healthy children in Cincinnati and Mexico (3) as well as the Quigley strain (4) recovered from a patient with aseptic meningitis in West Virginia, also lacked pathogenicity for newborn mice in tests carried out in this laboratory (5) after a varying number of passages in tissue culture.

However, many of the viruses isolated in tissue culture during the extensive epidemics of aseptic meningitis and febrile exanthem in Europe and Canada in 1955 and 1956 that were originally or subsequently shown to be related to ECHO 9 were found to produce paralysis in newborn mice with muscle lesions similar to those associated with the Coxsackie A group of viruses (4, 6–13). These reports indicated that the original human specimens were not paralytogenic for mice; five investigators (6, 7, 9, 13, 14) listed a total of 76 stools or cerebrospinal fluids that were tested with negative results. After one or more tissue cultures passages, Johnsson (6) found only 7 of 20 strains paralytogenic, Godtfredsen and von Magnus (9) only 10 of 21, while Tyrrell and Snell (8) found 9 of 10 and Laforest *et al.* (11) and Brohl *et al.* (13) indicated that all the strains they had tested (13 and 12 respectively) were paralytogenic in mice. Godtfredsen and von Magnus (9) found no correlation between the amount of virus contained in the tissue cultures of various strains and their paralytogenic activity. All of these observations raised the question of the factors that were responsible for the paralytogenic activity of some tissue culture passaged strains of ECHO 9 virus. It was especially desirable to

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determine whether paralytogenic activity was a property of only a few virus particles that might be more readily encountered in large populations in tissue cultures and perhaps be selectively enriched under certain conditions.

Accordingly when many thousands of cases of ECHO 9 virus disease occurred in the United States in 1957 (15), we undertook a quantitative study of the factors which determine the presence or absence of paralytogenic activity for newborn mice in various strains of ECHO 9 virus. The results of this study (16), reported in detail in the present communication, established that while some ECHO 9 viruses lack the capacity to multiply in the muscles of newborn mice, others can multiply slowly so that only very large doses of virus can produce paralysis, and still other virus particles can multiply so fast that even small initial inocula can produce paralysis.

Materials and Methods

Viruses.—The ECHO 9 virus prototype (Hill), purified by the terminal dilution technique' was used in its 14th passage in monkey kidney tissue culture. The history of the other virus strains is described in the text.

10 per cent stool suspensions were prepared in 0.5 per cent lactalbumin hydrolysate in Earle's solution (LHE medium) containing 2000 units of penicillin, 2 mg. of streptomycin, and 25 units of nystatin (mycostatin) per ml. as described before (15). Cerebrospinal fluids were used untreated. Tissue culture fluids from infected tubes were harvested as soon as the cytopathogenic effect was complete and used without further treatment. All virus materials were stored at about −20°C.

Tissue Cultures.—Trypsinized *cynomolgus* monkey kidney tissue cultures were used throughout these studies prepared and maintained as described elsewhere (15). They were inoculated 6 to 12 days after preparation.

Titrations in tissue culture were carried out by inoculating 0.2 ml. of tenfold dilutions in the gassed LHE medium pH 7.5, (the diluent used throughout these experiments), into 2 to 5 tubes per dilution. These tubes were read every 2 days, the final reading being made 8 days after inoculation. Virus titers were calculated according to the method of Reed and Muench.

Mice.—Swiss albino mice—less than 24 hours old, unless otherwise stated—were bought from a local animal supply house. The mice were pooled, redistributed among the mothers, and then inoculated simultaneously with 0.02 ml. intracerebrally and with 0.05 ml. subcutaneously in the interscapular region, each mouse receiving a total of 0.07 ml. In a few instances only 0.02 to 0.05 ml. of cerebrospinal fluid was inoculated subcutaneously in each mouse because no more was available. In the "growth curve" experiments with the Hill and the A. B. strains each mouse received only 0.03 ml. subcutaneously, except in one case mentioned in text.

The mice were observed at least once a day and clinical manifestations as well as deaths were recorded. In order to permit a proper follow-up of individual mice, each "suspect" or sick mouse was appropriately marked with a dye. The observation period was 21 days.

Suspensions for further work were prepared as follows: whole mice or mouse carcasses (*i.e.*, whole mouse without skin, legs, and intestines) that had been frozen at −20°C., were ground in a mortar with alundum to a 20 per cent suspension in LHE medium as used for preparation of stool suspensions. The suspensions were centrifuged in an International centrifuge (model U) at 2000 R.P.M. for 20 minutes and the supernatant fluids were stored at −20°C. until used for titration or passage.

EXPERIMENTAL

Mouse Pathogenicity of Original Cerebrospinal Fluids or Stool Specimens and of First Tissue Culture Passages in Relation to Concentration of Virus in Inoculum

From the large number of specimens studied during the ECHO 9 epidemic in Milwaukee and Cincinnati (15) we selected 14 cerebrospinal fluids and 25

TABLE I

Mouse Pathogenicity of Original Cerebrospinal Fluid and of First Tissue Culture Passage in Relation to Concentration of Virus in Inoculum

Patient	CSF—original		CSF—KP_1*	
	TCD_{50} inoculated Log 10	Result	TCD_{50} inoculated Log 10	Result
1. D. T.	2.4	0/6‡	7.0	8/8
2. W. R.	2.0	0/8	7.5	8/8
3. S. K.	1.9	0/6	6.5	8/8
4. A. B.	1.5	0/8	7.5	8/8
5. M. M.	1.0	0/6	6.5	8/8
6. P. M.	0.4	0/6	6.5	8/8
7. D. S.	0.4	0/6	7.0	8/8
8. A. H.	0.1	0/6	7.0	8/8
9. C. M.	?§	0/7	5.5	6/7
10. J. S.	1.5	0/8	6.0	8/8
11. P. H.	1.0	0/8	7.0	8/8
12. M. H.	0.4	0/6	6.0	8/8
13. C. McL.	0.4	0/8	6.9	0/9
14. R. B.	0.0	0/8	6.0	8/8

Note.—Patients 1 to 9 were from Milwaukee during a large epidemic; Nos. 10 to 14 were from Cincinnati where only sporadic cases were encountered.

* KP_1, first passage in monkey kidney tissue culture.

‡ $0/6, \frac{\text{No. of mice paralyzed}}{\text{No. inoculated}}$.

§ Virus present but not enough CSF available for titration.

stools which produced cytopathogenic effects in tissue culture so rapidly that they appeared to have the highest original concentrations of virus. These specimens (cerebrospinal fluid undiluted, stools as 10 per cent suspensions) as well as the undiluted first passage tissue culture fluids were inoculated into groups of newborn mice and simultaneously titrated in monkey kidney tissue culture. The results of these tests are presented in Tables I and II.

Only 1 of the 39 original specimens was paralytogenic for suckling mice, but this specimen (Table II, No. 25) had to be excluded from our study, because

the paralytogenic effect was caused by a non-cytopathogenic Coxsackie A virus and not by the ECHO 9 virus that it contained. Actually then none of 38 stool or cerebrospinal fluid specimens produced paralysis in newborn mice. On the other hand, after one passage in monkey kidney tissue culture, only 6

TABLE II

Mouse Pathogenicity of Original Stool Suspensions and of First Tissue Culture Passage in Relation to Concentration of Virus in Inoculum

All specimens from patients in Milwaukee

Patient	Stool—original		Stool—KP_1	
	TCD_{50} inoculated Log 10	Result	TCD_{50} inoculated Log 10	Result
1. F. M. B.	2.1	0/7	6.0	7/7
2. L. M. A.	2.0	0/8	4.0	0/7
3. D. S.	1.8	0/10	4.7	4/10
4. Th. G.	1.5	0/8	5.0	2/9
5. A. K.	1.5	0/5	5.0	3/8
6. D. C.	1.5	0/6	5.5	6/9
7. M. T.	1.0	0/9	4.2	0/10
8. A. R.	1.0	0/8	5.0	6/8
9. Sc. N.	1.0	0/10	5.5	10/10
10. J. P.	1.0	0/5	6.0	8/8
11. Fr. J. Jr.	1.0	0/6	8.0	6/7
12. J. Hu.	0.5	0/6	4.5	9/9
13. T. K.	0.5	0/4	4.9	0/9
14. J. H.	0.5	0/9	5.1	0/10
15. M. S.	0.5	0/9	5.5	9/10
16. M. P.	0.5	0/9	5.5	9/9
17. R. W.	0.5	0/8	6.5	8/9
18. J. G. A.	0.0	0/9	4.5	7/9
19. E. K.	0.0	0/9	4.6	6/9
20. W. G. L.	0.0	0/8	4.9	0/8
21. L. S. Jr.	0.0	0/9	5.0	8/9
22. R. Z.	0.0	0/6	6.0	9/9
23. H. G.	0.0	0/8	6.5	8/8
24. A. N.	0.0	0/6	6.5	7/8
25. E. M.	0.0	9/9*	4.5	9/9
			(6.3)‡	4/8

Legends same as in Table I.

* The paralysis in these mice was caused by a non-cytopathogenic Coxsackie A virus that was present in addition to the ECHO 9.

‡ This culture fluid represents the fourth passage in monkey kidney tissue culture which eliminated the non-cytopathogenic Coxsackie A virus, and the effect in mice was produced by the ECHO 9 virus.

of the 38 strains were not paralytogenic. Histological examination of the affected mice demonstrated muscle lesions similar to those seen after Coxsackie A virus infection.

However, while the original material never had more than 250 TCD_{50} of virus per inoculum, and in many instances it was only 1 to 10 TCD_{50}, the undiluted tissue culture fluids provided inocula with 10^4 to 10^8 TCD_{50} per mouse. Furthermore in tabulating the relation between the amount of virus contained in inocula of undiluted culture fluids of the 38 strains and the incidence of paralysis in newborn mice it was found (Table III) that mouse pathogenicity was generally less with the smaller amounts of virus. Thus, four of the six strains that failed to paralyze mice provided inocula of only 10^4

TABLE III

Relation between Amount of Virus in Inoculum of Undiluted First Passage Culture Fluid of 38 Different Strains and Incidence of Paralysis in Newborn Mice

TCD_{50} of virus inoculated Log 10	No. of strains in indicated range of virus concentration	No. of strains producing indicated effect in mice		
		All mice well	Only some mice paralyzed	All mice paralyzed
4.0–4.9	8	4	3	1
5.0–5.9	10	1	7	2
6.0–6.9	13	1	2	10
7.0–7.9	6	—	—	6
8.0	1	—	1	—

to $10^{4.9}$ TCD_{50}. On the other hand, with one exception all strains providing inocula of 10^6 TCD_{50} or more produced paralysis in newborn mice.

Is Mouse Pathogenicity of First Passage Culture Fluids Due to Larger Amounts of Virus or to Qualitative Differences in Virus?

To answer this question it was first of all necessary to determine the effect in newborn mice of decreasing amounts of virus in first passage tissue culture fluids of various strains. The results of titrations in mice with 5 of our strains are presented in Table IV. It may be seen that the paralytogenic activity disappeared when the concentration of virus in the inoculum fell below the 10^4 to 10^6 TCD_{50} level—different amounts being required for different strains. It is clear, therefore, why the original human specimens containing a maximum of only $10^{2.4}$ TCD_{50} per inoculum were not paralytogenic. Apparently no significant, qualitative change occurred in the virus as a result of a single passage in tissue culture, and the mouse pathogenicity of first passage tissue culture fluids may have been due only to the larger amounts of virus obtained.

TABLE IV

Is Mouse Pathogenicity of First Passage Culture Fluid Due to Larger Amount of Virus or to Qualitative Differences in Virus?

Strain	CSF—original		CSF—KP_1—effect in mice of indicated amount (TCD_{50}) of virus						
	TCD_{50} inoculated	Effect in mice	10^7	10^6	10^5	10^4	10^3	10^2	10^1
1. D. T.	$10^{2.4}$	0/6	8/8	1/8	0/10		0/8	0/8	
2. W. R.	$10^{2.0}$	0/8	8/8*	0/8	0/8		0/8	0/8	0/8
3. S. K.	$10^{1.9}$	0/6	8/8	7/7	4/8		0/8	0/8	0/8
4. A. B.	$10^{1.5}$	0/8	8/8	5/8	1/8		0/8	0/8	0/9
5. J. S.	$10^{1.5}$	0/8		8/8	1/8	0/3	0/8	0/8	

* These mice received $10^{7.5}$ TCD_{50}.

TABLE V

Effect of Multiple Passages in Tissue Culture on Mouse Pathogenicity in Relation to Amount of Virus Inoculated

Strain	Effect in newborn mice of indicated amount (Log 10 TCD_{50}) of virus of indicated passage in tissue culture				
	Passage 1	Passage 5			
1. L. M. A.	4.0* / 0/7	6.0 / 8/8	4.0 / 0/7	2.0 / 0/8	
2. M. T.	4.2 / 0/10	6.3 / 4/8	4.3 / 0/8		
3. D. S.	4.7 / 4/10	6.8 / 5/8	4.8 / 0/8	2.8 / 0/8	
4. T. K.	4.9 / 0/9	6.0 / 3/8	4.0 / 0/8	2.0 / 0/8	
5. W. G. L.	4.9 / 0/8	6.9 / 8/8	4.9 / 2/10	2.9 / 0/8	
6. J. H.	5.1 / 0/10	6.8 / 7/7	5.8 / 5/9	4.8 / 1/4	2.8 / 0/8
7. C. McL.	6.9 / 0/9	6.9 / 2/8	4.9 / 0/8	2.9 / 0/8	

* $\frac{4.0}{0/7}$ = $\frac{\text{Numerator indicates log 10 } TCD_{50} \text{ of virus inoculated}}{\text{Denominator indicates effect in mice (number paralyzed over number inoculated).}}$

Effect of Multiple Passages in Tissue Culture on Mouse Pathogenicity

In order to study the effect of multiple tissue culture passages of ECHO 9 viruses on mouse pathogenicity we selected seven strains which after a single passage in tissue culture had little or no effect in mice. The subsequent passages in tissue culture were carried out with undiluted culture fluid as inoculum. The culture fluids of the 5th passage were then titrated in mice and in tissue culture. The results are presented in Table V. It can readily be seen that the higher incidence of mouse pathogenicity of the 5th passage culture fluids was

TABLE VI

Enhancement of Mouse Pathogenicity by Two Consecutive Passages of Virus in Newborn Mice

Strain	Material tested	Effect in mice of indicated amount (TCD_{50}) of virus						
		10^7	10^6	10^5	10^4	10^3	10^2	10^1
W. R.	KP 1	8/8*	0/8	0/8	—	0/8	0/8	0/8
	KP 1-MP 2‡	—	9/9	5/8	2/7	1/8	1/16	0/8
J. S.	KP 1	—	8/8	1/8	0/3	0/8	0/8	—
	KP 1-MP 2	—	6/6	6/6	5/7	5/8	1/8	0/8
D. T.	KP 1	8/8	1/8	0/10	—	0/8	0/8	—
	KP 1-MP 2	—	16/16	3/11	1/8	0/8	0/11	0/7

* These mice were inoculated with $10^{7.5}$ TCD_{50}.

‡ KP 1-MP 2, first passage monkey kidney culture fluid submitted to two consecutive passages in newborn mice and suspension of carcass of mice in second passage used for tests.

related to higher concentrations of virus achieved during the tissue culture passages, since the number of TCD_{50} required to produce paralysis remained about the same. This confirmed our earlier results which indicated that mere passage in tissue culture did not favor the selection of mouse pathogenic virus particles.

Enhancement of Mouse Pathogenicity by Consecutive Passages of Virus in Mice

The next question to be considered was why such large doses were needed to produce paralysis in mice. One could assume that among the large number of virus particles in the tissue culture fluid there was only a small proportion of paralytogenic particles, and that only these multiplied in the mice. According to this hypothesis one would expect that virus recovered from paralyzed mice would be paralytogenic in very small doses.

Therefore, the first tissue culture passages of four strains were submitted to two mouse passages, and the 2nd mouse passage was then titrated in mice and tissue culture (see Tables VI and VII). After two mouse passages, the paralytogenic activity of the virus increased 10 to 1000 times. Strain A. B.

(Table VII) which initially required an inoculum of about 10^6 TCD_{50} to paralyze 50 per cent of the mice, required only about 10^3 TCD_{50} after two mouse passages, and after five consecutive passages in mice, which had no effect on the capacity of the virus to grow in tissue culture, the 50 per cent paralyzing dose was reduced to 20 TCD_{50}. On the other hand, five consecutive tissue culture passages failed to alter the paralytogenic activity of this strain.

Thus, while consecutive passages in mice resulted in a progressive increase in mouse pathogenicity of ECHO 9 strains, it was also evident that relatively large doses of ECHO 9 virus were still required to produce paralysis after 2 passages of some strains. Accordingly mere selection of fully pathogenic virus particles preexisting in the tissue culture fluids was not the correct explanation.

TABLE VII

Comparative Effect on Mouse Pathogenicity of Multiple Passages of Strain "A. B." in Monkey Kidney Tissue Culture and in Newborn Mice

Material tested	Effect in mice of indicated amount (TCD_{50}) of virus							
	10^7	10^6	10^5	10^4	10^3	10^2	10^1	10^0
KP 1	8/8	5/8	1/8	—	0/8	0/8	0/9	—
KP 5	7/7	15/20	3/7	0/8	0/7	0/8	0/8	—
KP 1-MP 2	—	6/6	19/19	12/15	12/16	0/14	0/15	0/5
KP 1-MP 5	—	8/8	8/8	8/8	7/7	8/8	2/8	0/8

Legends same as in Table VI.

Inapparent Multiplication of ECHO 9 Virus Strains in Mice

Another approach to our problem was suggested by studies on mice that were ill or had died of intercurrent disease, mostly diarrhea without any sign of paresis, as a rule later than 12 days after inoculation of original human material or first passage culture fluids (Table VIII). It is evident that multiplication of ECHO 9 viruses occurred in mice inoculated with doses as low as 3 TCD_{50}, regardless of whether the virus had previously been passaged in mice, or in tissue culture, or not at all. It appeared highly improbable that there was any causal relationship between the illnesses and deaths and the observed multiplication of ECHO 9 virus. Subsequently, it was shown that after inoculation of certain doses and strains of ECHO 9 virus in newborn mice multiplication occurred without production of paralysis. Furthermore, histologic examination of two mice 9 days after inoculation of original human stool suspension revealed limited lesions in the striated muscle, although no signs of paresis were observed.

In one instance, blind passage was carried out in newborn mice with material derived from a mouse that was found dead 13 days after inoculation of stool

suspension containing 10^2 TCD_{50} of ECHO 9 virus. All the mice in the litter inoculated with the original stool suspension exhibited diarrhea after the 12th day but none had weakness or paralysis. Histologic examination of two mice that died 14 and 17 days after inoculation revealed only rare minute foci in the striated muscles suggestive of regional involvement of a few isolated

TABLE VIII

Evidence of Multiplication in Newborn Mice of Non-Paralyzing Doses of ECHO 9 Virus Present in Original Human Material, Tissue Culture, or Paralyzed Mice

Material inoculated	Strain	TCD_{50} inoculated per mouse Log 10	Day of sacrifice (S) or death (D) of individual mouse*	TCD_{50} of virus recovered per gm of carcass Log 10
Original human stool or CSF	M. G.	2.0	D 13	5.7
	F. M. B.	2.1	D 20	2.2
	M. T.	1.0	D 8	4.2
	"	1.0	D 12	<1.0
	J. P.	1.0	S 9	2.2
	M. S.	0.5	D 17	5.7
	"	0.5	D 18	1.7
	"	0.5	D 19	2.2
	R. W.	0.5	D 18	<1.0
	J. H.	0.5	D 20	<1.0
	A. H.	0.1	D 18	<1.0
	C. M.	?‡	D 18	<1.0
Monkey kidney tissue culture passage 1	D. T.	6.0	D 18	3.7
	J. H.	5.1	S 19	2.2
	W. G. L.	4.9	S 11	6.2
	M. T.	4.2	D 20	<1.0
KP 1-MP 2	W. R.	2.3	D 18	4.2
	J. S.	2.3	D 14	6.2

* The dead or sacrificed mice had diarrheal disease and did not exhibit paralysis.

‡ Virus was present but not enough CSF available for titration.

muscle fibers. The mouse that was used for the second passage contained $10^{5.7}$ TCD_{50} of virus per gm. and $10^{3.7}$ TCD_{50} was inoculated in each mouse. None exhibited weakness or paralysis and 2 mice were sacrificed for the third passage. Of the 14 newborn mice in the third passage, 7 developed transitory weakness 9 to 11 days after inoculation. Two days after onset of weakness $10^{7.1}$ TCD_{50} of ECHO 9 virus per gm. of mouse carcass was found.

This experience shows that during blind passages of certain strains in mice enough virus with increased paralytogenic activity may ultimately be accumulated and selected to permit isolation of virus without the use of tissue culture.

Why Are Large Doses of Virus Needed to Produce Paralysis in Mice?

Once the inapparent multiplication of some ECHO 9 viruses in mice was established, the hypothesis was considered that for paralysis to occur the virus had to multiply up to a certain level within a certain time. If this "paralyzing level" were not reached within a specified time, because the dose was too small in relation to the rate of multiplication of a particular strain, it appeared possible that the mouse might in the meantime lose its susceptibility. The

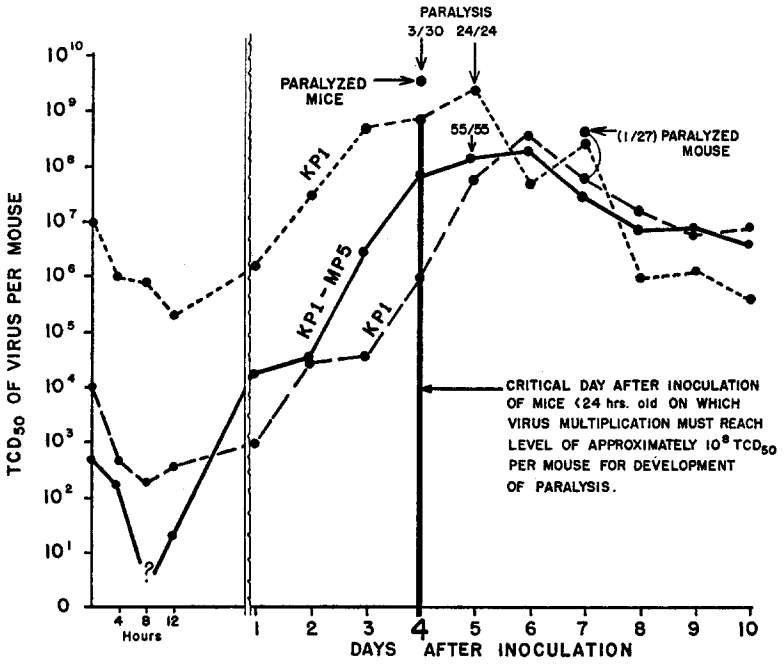

FIG. 1. Rate of growth of ECHO 9 virus (strain "A. B.") in newborn mice after inoculation of large (10^7) paralyzing and smaller (10^4) non-paralyzing doses of tissue culture–passaged virus and of small ($10^{2.8}$) dose of paralyzing mouse-passaged virus.

following observations were in favor of this hypothesis. All paralyzed mice which had been studied thus far at the onset of paralysis had 10^8 TCD_{50} of virus or more per mouse. Among several thousand mice inoculated with various ECHO 9 viruses, almost all exhibited the first signs of paralysis within 5 to 10 days after inoculation, *i.e.*, not beyond the first 11 days of life. Adult mice had been found insusceptible to ECHO 9 virus.

For investigation of this hypothesis we selected the A. B. strain and first of all determined the rate of multiplication of the virus in first passage tissue culture fluid in two groups of mice; one group inoculated with the regularly

paralytogenic dose of 10^7 TCD_{50}, and the other with 10^4 TCD_{50}, a dose that in previous tests was not paralytogenic. Each group comprised about 50 mice, distributed among seven mothers. Three mice from each group were harvested at various intervals after inoculation, indicated in Fig. 1. With few exceptions, when the three mice were handled separately, the combined weight of the three was recorded, and without discarding any part of the body all were ground together in a mortar to give a 20 per cent suspension. The centrifuged supernatant liquids were titrated in tissue culture using 4, mostly 5, tubes per dilution. The virus titers in Fig. 1 represent TCD_{50} per average weight of total mouse.

The data shown in Fig. 1 indicate that after an initial disappearance of 98 per cent of the infective virus, there was a steady, more or less exponential increase of virus in the mice inoculated with the larger dose until a level of $10^{8.7}$ TCD_{50} was reached on the 3rd day after inoculation—2 days before the majority of the mice first exhibited paralysis. There was then a further increase in virus up to a level of $10^{9.4}$ to $10^{9.6}$ at first onset of paralysis and a subsequent progressive drop.

The "growth curve" obtained in mice inoculated with 10^4 TCD_{50} followed the same course, but at a lower level. After an initial reduction of 98 per cent there was again a steady increase in amount of virus, but by the 4th day the virus had multiplied only up to 10^6 TCD_{50} per mouse. Despite subsequent multiplication to a peak level of $10^{8.6}$ TCD_{50} by the 6th day, only 1 of 27 mice exhibited slight paresis 7 days after inoculation, at which time that mouse yielded $10^{8.6}$ TCD_{50} while the unaffected mice yielded only $10^{7.8}$ TCD_{50}.

Generally, the yield of virus on a certain day in mice inoculated with 10^7 TCD_{50} was not attained until 3 days later in the mice inoculated with 10^4 TCD_{50}. During the logarithmic phase of reproduction the difference in titers between the two groups was constant at about 1000-fold.

In view of these results, it became necessary to determine whether the capacity of small doses of "mouse-adapted" virus to produce paralysis was associated with more extensive and rapid multiplication. In a "growth curve" experiment with $10^{2.8}$ TCD_{50} of the more paralytogenic virus selected by 5 consecutive passages in mice, it was found that up to 4 days after inoculation it had multiplied about 1000 times more than the first tissue culture passage virus (Fig. 1). All 55 mice remaining on the 5th day had extensive paralysis.

These experiments indicated that the 4th day after inoculation of 1-day old mice was a critical day on which virus multiplication had to reach a level of about 10^8 TCD_{50} if paralysis was to develop subsequently. In order to determine whether there actually is a change of susceptibility in suckling mice at this time two litters of mice, 5 to 6 days of age, were inoculated with 10^7 TCD_{50} of first tissue culture passage of the A. B. strain. This was approximately the amount of virus found in 5- to 6-day old mice after inoculation

of 10^4 TCD_{50} of the A. B. strain on the day of birth. For simultaneous control 10^7 TCD_{50} were inoculated into 2 litters of mice less than 24 hours old (see Table IX). All the newborn mice developed extensive paralysis within 4 to 5 days and 8 of 11 died. On the other hand, none of the sixteen 5- to 6-day old mice developed weakness or paralysis and only one mouse had the "suspect" look sometimes seen before appearance of paralysis, but no progression to paresis was observed. The importance of age of a suckling mouse in determining its response to ECHO 9 virus was confirmed in a similar test with "mouse-adapted" virus (Table IX).

These data indicated that speed of multiplication, which may perhaps mean only greater yield per infected cell, determined the paralytogenic activity of a given population of ECHO 9 virus particles in newborn mice. If

TABLE IX

Resistance of 5 to 6 Day Old Mice to Paralytogenic Effect of Large Doses of ECHO 9 Virus, Strain "A. B."

Material inoculated	TCD_{50} inoculated Log 10	Effect in mice of indicated age	
		<1 day	5 to 6 days
KP 1	7.0	11/11	0/16
KP 1-MP 2	6.0	16/16	0/16

most of the virus particles multiply "slowly" the initial inoculum has to be large; if the majority of particles multiply more rapidly a paralytogenic effect can be achieved with a smaller dose.

Behavior of the Prototype (Hill) Strain of ECHO 9 Virus in Newborn Mice

Repeated tests with this strain failed to produce paralysis in newborn mice even with maximal doses of 10^7 TCD_{50} per mouse. Accordingly it was important to determine whether or not inapparent multiplication of this virus could be demonstrated in mice. Each of 18 newborn mice received $10^{5.7}$ TCD_{50} of the Hill strain (0.02 ml. intracerebrally and 0.05 ml. subcutaneously) and 2 mice were sacrificed at 72 hours and at 216 hours (Table X). None of the mice developed paralysis, and the amounts of virus recovered at the indicated times indicated that there was no progressive multiplication. In order to determine whether minimal multiplication may have occurred it was necessary to sacrifice mice at more frequent intervals to detect a possible rise in virus yield after the initial fall. Such an experiment was carried out with an inoculum of $10^{4.5}$ TCD_{50} per mouse, and the results are shown in Table X side by side with those obtained in a similar test with a multiplying strain of ECHO 9 virus (same as data used for Fig. 1). The difference in the two "growth curves" is

clearly evident. To begin with it may be noted that the initial disappearance of about 98 per cent observed with the A. B. strain at 8 hours did not take place with the Hill strain. This indicates that unlike the multiplying A. B. strain, which adsorbed to the susceptible cells and went into the "eclipse" phase, the Hill virus even failed to adsorb. During the subsequent hours the A. B. strain began to multiply, while the Hill strain progressively lost titer presumably owing to deterioration in the mice. Thus it is possible to conclude

TABLE X

Difference in Growth Curves of Non-Mouse-Pathogenic (Hill) and Mouse-Pathogenic ("A. B.") Variants of ECHO 9 Virus

Time of harvest	Log 10 TCD_{50} of virus per mouse recovered at indicated time after inoculation of indicated strain and dose		
	Hill		"A. B."*
	5.7	4.5	4.0
hrs.			
0		4.3	
4		4.3	2.7
8		4.0	2.3
12		3.6	2.6
18		2.7	
24		2.2	3.0
48		1.8	4.5
72	3.2	1.7	4.6
168		<1.0	7.8
216	<1.0	<1.0	6.8

* First passage monkey tissue culture fluid of strain "A. B." was used. None of the sacrificed mice exhibited paralysis and only 1 of 27 mice remaining at 8 days developed paralysis on 8th day and none thereafter.

that the prototype ECHO 9 strain not only does not multiply in newborn mice but also is not specifically adsorbed by the tissues.

Multiplication in Newborn Mice of ECHO 9 Strains Recovered during Non-Epidemic Periods

Besides the prototype strain the 2nd to 4th tissue culture passage of 7 other ECHO 9 strains recovered from healthy children in Cincinnati and Mexico had been tested in our laboratory for mouse pathogenicity (5). No paresis in any of the mice inoculated could be observed. The inocula of all these strains, however, ranged only from $10^{2.9}$ to $10^{4.7}$ TCD_{50} per mouse.

Subsequently, 3 of these 7 strains as well as the Quigley strain which had

been found non-paralytogenic for mice (4), were tested for inapparent multiplication in mice (Table XI). Three whole mice from each group were pooled and tested 5 to 10 days after inoculation of the indicated amounts of virus. The Daniels and ABV 20 strains apparently did not multiply in mice. The BVA 84 strain exhibited a limited capacity for multiplication, while it remained doubtful whether the Quigley strain multiplied or not.

It should be noted, however, that a strain of virus that was isolated from a healthy child during the winter of 1958 in New York (Sabin and Barnes, unpublished data) was highly paralytogenic for newborn mice inoculated with $10^{6.5}$ TCD_{50}.

TABLE XI

Absent or Limited Multiplication in Newborn Mice of ECHO 9 Strains Recovered during Non-Epidemic Periods

Strain			Inoculum		Log 10 TCD_{50} of virus per mouse sacrificed on indicated day after inoculation	
Source	Place	Name	Tissue culture passage	Log 10 TCD_{50} per mouse	5	10
Healthy children	Cincinnati	Daniels	KP 4	3.1	<1.5	<1.7
	Mexico	ABV 20	KP 3	4.1	<1.5	<1.7
	"	BVA 84	KP 3	4.3	4.6	3.0
Aseptic meningitis	West Virginia	Quigley	Cinci KP 2	5.3	3.1	<1.7

DISCUSSION

The present study revealed the following significant factors about the pathogenicity for newborn mice of various strains of ECHO 9 virus:—

1. Some strains, of which the prototype Hill strain is an example, not only fail to produce paralysis in maximal doses but also cannot multiply in or be adsorbed by the tissues of newborn mice.

2. Many naturally occurring strains can multiply to a varying extent in newborn mice with the production of only rare lesions in isolated muscle fibers but without clinically apparent weakness or paralysis.

3. The virus must be capable of multiplying to a certain high level (10^8 TCD_{50} or more per mouse) within a specified time, which is 4 days after inoculation of mice less than 1 day old, for paralysis to appear. This is due to the fact that at 5 to 6 days of age there is a change in the susceptibility to large doses of virus.

4. If the dose is too small or the rate of virus multiplication is too slow to achieve the necessary muscle involvement within the specified time, no weak-

ness or paralysis appears. Conversely paralysis occurs if the initial dose is large, such as can be achieved by preliminary propagation in tissue culture, and if the virus possesses the ability to multiply in the mouse.

5. Mere propagation in tissue culture did not alter the paralytogenic activity of the virus beyond increasing the dose that can be inoculated. On the other hand, passage in mice resulted in a gradual enrichment of virus particles capable of more rapid multiplication in mice and in concurrent greater paralytogenic activity of smaller doses.

It has been suggested (17) that virulence for newborn mice may be a special property of ECHO 9 strains derived from patients. However, the Quigley strain derived from a patient with aseptic meningitis yielded data which indicated that it was incapable of multiplication in mice, while a strain that we recovered from a healthy child in the winter of 1958 was entirely comparable to the paralytogenic, epidemic strains. It seems to us more important to stress the fact that even the epidemic strains of ECHO 9 virus vary greatly in their capacity for multiplication in newborn mice, and that with very few exceptions only very large amounts of virus, such as are not usually encountered in human specimens, are capable of producing paralysis. Dr. J. H. S. Gear (personal communication) observed paralysis in one of 7 newborn mice inoculated with a fecal suspension from a boy with aseptic meningitis. Serial passage in mice was readily accomplished and the agent was identified as ECHO 9. In the light of our studies Gear's unique finding may be interpreted as indicating that the human specimen probably had an unusually high concentration of virus, for in the absence of quantitative data one cannot estimate the virulence of the virus for mice.

There has been considerable discussion (17–21) as to whether the capacity of some strains of ECHO 9 virus to multiply in the muscles of newborn mice and with very large doses to produce paralysis in them, warrants a reclassification of ECHO 9 with the Coxsackie A viruses. Instead of attempting to classify viruses on the basis of a single character, especially one so variable as pathogenicity, that may or may not be present in all members of the species, it seems to us better to recognize that in a family of related viruses the predominant characteristic of one group may be present to a limited degree among variants of other groups. Accordingly the creation of the family of enteroviruses (20) to include the ECHO, Coxsackie, and polioviruses appears to us a more appropriate solution of the problem. Otherwise we shall have to reclassify every Coxsackie and ECHO virus, with some capacity to produce neuronal lesions in monkeys, as new types of poliovirus.

SUMMARY

While some strains of ECHO 9 virus were found to be completely incapable of multiplying in newborn mice or even of being adsorbed by their tissues (*e.g.*, the prototype Hill strain), other naturally occurring strains readily

multiplied even after inoculation of as little as 3 TCD_{50} of virus. With the multiplying strains, the infection remained clinically inapparent except after inoculation of very large doses, usually in the range of 10^5 to $10^{7.5}$ TCD_{50}. Investigation of the question why such large doses were required to produce paralysis indicated that for paralysis to occur virus multiplication had to reach a level of 10^8 TCD_{50} or more within 4 days after inoculation of mice less than 1 day old. The reason for this was found in the fact that at 5 to 6 days of age the mice lost their susceptibility to paralysis even when multiplication was capable of progressing to the indicated high level. Thus, speed of multiplication and extent of muscle involvement before the 5th day of life were the determining factors. Passage in tissue culture had no effect except to yield a larger dose for inoculation, while serial propagation in mice resulted in a gradual enrichment of virus particles capable of more rapid multiplication in mice and in a concurrent greater paralytogenic activity of smaller doses.

BIBLIOGRAPHY

1. Committee on the ECHO viruses, Enteric cytopathogenic human orphan (ECHO) viruses, *Science*, 1955, **122**, 1187.
2. Ramos-Alvarez, M., and Sabin, A. B., Characteristics of poliomyelitis and other enteric viruses recovered in tissue culture from healthy, American children, *Proc. Soc. Exp. Biol. and Med.*, 1954, **87**, 655.
3. Ramos-Alvarez, M., and Sabin, A. B., Intestinal viral flora of healthy children demonstrable by monkey kidney tissue culture, *Am. J. Publ. Health*, 1956, **16**, 295.
4. McLean, D. M., and Melnick, J. L., Association of mouse pathogenic strain of ECHO virus Type 9 with aseptic meningitis, *Proc. Soc. Exp. Biol. and Med.* 1957, **94**, 656.
5. Wigand, R., personal communication.
6. Johnsson, T., A new clinical entity?, *Lancet*, 1957, **272**, 590.
7. Nihoul, E., Quersin-Thiry, L., and Weynants, A., ECHO virus Type 9 as the agent responsible for an important outbreak of aseptic meningitis in Belgium, *Am. J. Hyg.*, 1957, **66**, 102.
8. Tyrrell, D. A. J., and Snell, B., Recovery of a virus from cases of an epidemic exanthem associated with meningitis, *Lancet*, 1956, **271**, 1028.
9. Godtfredsen, A., and von Magnus, H., Isolation of ECHO virus Type 9 from cerebrospinal fluids, *Dan. Med. Bull.*, 1957, **4**, 233.
10. Archetti, I., Felici, A., Russi, F., and Fua, C., Researches on the etiologic agent of the Marche meningoneuraxitis during the epidemic outbreak of the summer and autumn of 1955, *Sc. Med. Ital.*, 1956, **5**, 321.
11. Laforest, R. A., McNaughton, G. A., Beale, A. J., Clarke, M., Davis, N., Sultanian, I., and Rhodes, A. J., Outbreak of aseptic meningitis (meningoencephalitis) with rubelliform rash: Toronto 1956, *Canad. Med. Assn. J.*, 1957, **77**, 1.
12. Boissard, G. P. B., Stokes, L. J., Macrae, A. D., and MacCallum, F. O., Isolation of viruses related to ECHO virus Type 9 from outbreaks of aseptic meningitis, *Lancet*, 1957, **272**, 500.

13. Brohl, I., Helmstaedt, K., Lennartz, H., and Maass, G., Über eine Epidemie von Coxsackie-Meningitis, *Z. ärztl. Fortbild.*, 1957, **51**, 499.
14. Baumann, Th., Barben, M., Marti, R., Hassler, A., and Krech, U., Erkrankungen durch ECHO Virus Typ 9. Eine epidemiologische, klinische und virologisch-serologische Studie, *Schweiz. med. Woch.*, 1957, **87**, 307.
15. Sabin, A. B., Krumbiegel, E. R., and Wigand, R., ECHO Type 9 virus disease. Virologically controlled clinical and epidemiologic observations during 1957 epidemic in Milwaukee with notes on concurrent similar diseases associated with Coxsackie and other ECHO viruses, *Am. J. Dis. Child.*, 1958, **96**, 197.
16. Eggers, H. J., and Sabin, A. B., Factors determining mouse pathogenicity of ECHO 9 viruses, *Fed. Proc.*, 1958, **17**, 510.
17. Archetti, I., Dubes, G. R., and Wenner, H. A., A comparative study of the prototype Hill strain of ECHO virus, Type 9, and several Coxsackie-like viruses related to it antigenically, *Arch. ges. Virusforschung*, 1959, **9**, 73.
18. Tyrrell, D. A. J., Clarke, S. K. R., Heath, R. B., Curran, R. C., Beswick, T. S. L., and Wolman, L., Studies of a Coxsackie virus antigenically related to ECHO 9 virus and associated with an epidemic of aseptic meningitis with exanthem, *Brit. J. Exp. Path.*, 1958, **39**, 178.
19. Quersin-Thiry, L., Nihoul, E., and Dekking, F., ECHO virus Type 9 (new member of Coxsackie group Type A?) as a cause of epidemic meningitis, *Science*, 1957, **125**, 744.
20. Committee on the Enteroviruses, The enteroviruses, *Am. J. Pub. Health*, 1957, **47**, 1556.
21. Melnick, J. L., and Sabin, A. B., The ECHO virus group, *in* Viral and Rickettsial Infections of Man, (T. M. Rivers and F. L. Horsfall, Jr., editors), New York, J. B. Lippincott Co., 3rd edition, 1959, 547.