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Properties of ECHO Types 22, 23 and 24 Viruses*

By

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With 7 Figures

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During our studies on summer diarrhea in 1956 (1), 7 viruses were isolated from rectal swabs of infants with diarrhea, which could not be identified by antisera prepared with the known ECHO virus types. These 7 strains were considered as possible new ECHO viruses and 6 of them were studied. To exclude the possibility that we were dealing with mixtures of viruses, plaque purification was carried out with all 6 strains. By means of cross-neutralization tests with antisera prepared with the purified virus stocks it could be shown that these 6 strains fell into 3 new ECHO serotypes, designated by the Committee for Enteroviruses of The National Foundation for Infantile Paralysis, Inc., as ECHO 22, 23, and 24 virus (3 strains of ECHO 22, 1 strain of ECHO 23, and 2 strains of ECHO 24 virus). In this communication various properties of these viruses as well as the serologic differentiation from other viruses are described. Although most data refer to the selected prototype strains, reference to the other strains of ECHO 22 and 24 is made when their properties deviated from those of the prototype strains.

Materials and Methods

Origin and cultivation of viruses. The rectal swabs from which the virus strains were isolated were obtained during July and August in 1956. Cultures of trypsinized cynomolgus monkey kidney cells were prepared and used as described previously (1). As maintenance medium we used Earle's salt solution containing 0.5 per cent neutralized lactalbumin hydrolysate (LH), gassed to a pH of approximately 7.5 with carbon dioxide. All viruses and sera were kept frozen at $-20°$ C.

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Titration and neutralization. Ten-fold virus dilutions were prepared in the same solution used as maintenance medium and 0.2 ml. inoculated into 3 or 4 tissue culture tubes per dilution. For neutralization, an equal amount of serum and virus, containing approximately 100 TCD_{50} per 0.1 ml., were mixed, left for 1 hour at room temperature (approximately 24° C), and 0.2 ml. of the mixture was inoculated into each of 3 tissue culture tubes. A simultaneous virus titration was always done. Neutralization tests were read first, when all virus control tubes containing 100 TCD_{50} exhibited a definite cytopathogenic effect (CPE). Titration and neutralization tests were usually read for 8 days. The degree of the CPE is designated as 1, 2, 3, or 4 (approximately 25, 50, 75 and 100 per cent of the cells affected). ECHO and other reference monkey sera were supplied by The National Foundation for Infantile Paralysis Inc.

Plaque technique. — Monolayers of cynomolgus monkey kidney cells were grown in flat 3 ounce prescription bottles, as described by *Hsiung* and *Melnick* (2). Hanks' solution with 0.5 per cent lactalbumin hydrolysate and 2 per cent calf serum was used for the outgrowth period. After 4 days the fluid was changed using the above medium with a total of 0.063 per cent sodium bicarbonate. After a complete monolayer had been formed (usually 7 days after seeding of the bottles), this medium, which had a very low pH, was replaced with serum-free, 0.5 per cent lactalbumin hydrolysate in Earle's solution, that we used as maintenance medium for the tube cultures, and left for one hour or more. This additional change, not mentioned in the description of the original technique (2), is to wash out the old, relatively acid and calf-serum-containing-medium which may interfere with optimum adsorption of virus. Before addition of virus, the medium was poured off and 0.2 ml. of the selected dilution was added. The bottles were incubated for 1 hour at 37° C and tilted back and forth every 15 minutes, to ensure an even distribution of the virus particles. At the end of the adsorption period the cell sheets were washed twice with 5 ml. of prewarmed phosphate-buffered saline when pure progeny was to be obtained. For ordinary plaque counts, the overlay containing nutrient medium and 1.5 per cent agar (2) was added without previous washing. This overlay medium contained 2 per cent calf serum, since the preservation of the cell sheets was found to be as good as with 2 per cent monkey serum, proposed by *Hsiung* and *Melnick* (2).

Complement-fixation technique. — Complement-fixing (CF) antigens were prepared by growing all 3 virus prototypes in bottle cultures of monkey kidney cells. Our regular serum-free LH medium in Earle's solution or Earle's salt solution containing 0.35 per cent glucose and 0.01 per cent cysteine, but no lactalbumin hydrolysate (3), was used as maintenance medium. The latter medium yielded less anticomplementary antigens. Culture bottles, exhibiting maximal CPE were frozen and thawed, the fluid centrifuged 5 minutes at 2000 r. p. m., and the supernatant fluid was used as complement-fixing antigen without further treatment, except that the pH was adjusted to approximately 7.5, when necessary. The antigens were stored at −20° C. Heated adenovirus antigen (type 2) was kindly supplied by Dr. *Robert M. Chanock*. When tested in a box titration with a human reference serum pool, the antigen exhibited a maximal fixation up to a 1:32 dilution. Consequently we used a 1:8 dilution for the test proper, thus providing 4 antigen units.

Monkey sera to be used in the CF test were treated with fresh guinea pig serum (4), to reduce their anticomplementary properties, and heated 30 mi-

nutes at 60° C before use. The CF reaction was carried out according to the method of *Schmidt* and *Lennette* (5) with approximately 2 hemolytic units of complement.

Results

Isolation and adaptation to monkey kidney cultures. — All 3 strains of ECHO 22 and the ECHO 23 strain were characterized by a CPE, which involved predominantly, and often exclusively, the peripheral part of the cell sheet. It was not possible to adapt any of these strains regularly to produce complete destruction of the culture, as can be done with many, although possibly not all other ECHO viruses (6).

On primary isolation, 2 of the ECHO 22 strains (the present prototype "101 Harris", and "36") produced a definite CPE in all 3 inoculated tubes 5—6 days after inoculation, and beginning with the second passage the CPE appeared in 1 to 2 days. The third ECHO 22 strain, "57", required a longer adaptation period. On primary isolation 1 out of 3 tubes became positive 6 days after inoculation. As may be seen in Table 1, two attempts to pass this virus in different batches of tissue cultures failed to produce CPE, and on the third attempt the CPE was not observed until 7 days after inoculation. This "incubation period" became shorter in the third and fourth passage (4 and 2 days respectively), and subsequently was similar to that of strains "101" and "36", although the maximal degree of CPE was less for strain "57" than for the other 2 strains. It is furthermore worth mentioning, that after 2 plaque passages a fresh adaptation to tube cultures was necessary for strains "36" and "57". Thus, the second tube passage material from the second plaque of both strains, although harvested at the usual time, repeatedly failed to yield a CPE and only on the third attempt was a further passage possible (Table 1). Although the inoculated material was not titrated, one can exclude loss of titer during storage as a reason for the failures to pass, as the successful passages were obtained after the failures. A similar behavior was not encountered with strain "101". However, as indicated in Table 1, the maximal degree of CPE was dependent on the particular batch of tissue culture used. Even with the virus stock of strain "101" now in use, the maximal CPE may vary from a partial destruction of the peripheral part of the cell culture with a completely intact center to a complete destruction of the whole culture. No dependence on the age of the culture tubes could be observed with all strains of ECHO 22.

Similar difficulties were encountered with the ECHO 23 strain ("92" Williamson) (Table 2). On primary isolation, no CPE was observed on the first attempt, but on repetition with the same rectal swab material one out of 3 tubes was found positive 7 days after inoculation. As with ECHO 22, strain "57", the second passage was successful only on repetition. The third passage was done several times, and the results indicated,

that both the time of appearance of the CPE and the maximal degree observed were highly dependent on the particular culture series used. This was likewise found to obtain for the fourth passage, in which the inoculum

Table 1. Variation in effect of 3 strains of ECHO 22 virus on kidney tissue cultures derived from different cynomolgus monkeys

Virus		Maximal degree of CPE obtained in different tissue culture batches		
Strain	Passage No.	1	2	3
57	2	0	0	1
	10*	0	0	1
36	17*	0	0	2
101 (Prototype)	13	1	4	
	14	3	2	4

* Third tube passage from second plaque. Same tissue culture batches were used for virus strain 57 and 36 with corresponding results.

Table 2. Variation in susceptibility for ECHO 23 virus among batches of monkey kidney tissue culture tubes

Virus passage	Culture tubes		Cytopathogenic effect	
	Batch No.	Age days	Day of first appearance	Maximal degree
2	1	6		0
	2	9	7—8	2
3	3	7	7—8	0—1
	4	7	2	2
	5	6	7	1—2
4*	6	11	2	1
	7	6		0
	7	10	5	1

* The inoculum contained about 100 TCD_{50}. The other passages were not titrated.

was titrated later and was found to contain approximately 100 TCD_{50}. It is noteworthy, that with culture batch No. 7 (see Table 2) the tubes used 6 days after seeding did not exhibit any CPE, whereas 10-day old

Table 3. Comparative behavior of ECHO 23 virus in cynomolgus and rhesus kidney cultures of different age

Material titered	Date of test	Cynomologus					Rhesus				
		Series	Age days	Log_{10} TCD_{50}/ml. 3 – 4 days	Log_{10} TCD_{50}/ml. 7 – 8 days	CPE	Series	Age days	Log_{10} TCD_{50}/ml. 3 – 4 days	Log_{10} TCD_{50}/ml. 7 – 8 days	CPE
cynomolg. passage 11	11- 3-58	348	9	6.0	6.0	extensive	349	7	<1.0	5.5	slight
	11-25-58	351	7	5.2	6.7	extensive	349	19	4.5	6.7	extensive
cynomolg. passage 15	12- 1-58	351	13	7.2	7.7	extensive	349	25	7.0	7.5	extensive
cynomolg. passage 11 + rhesus passage 1	11-20-58	350	9	5.5	6.2	extensive	349	14	5.5	6.2	extensive

tubes of the same series showed a definite, albeit slight and late-appearing CPE. This possible influence of the age of the culture tubes on the degree of the CPE could not be borne in subsequent tests with cynomolgus kidney cells, in which material of later virus passages was used.

The adapted ECHO 23 virus regularly produced a definite CPE in culture tubes, although its extent varied, and occasionally in repeat titrations up to 100 times lower infectivity titers were obtained, indicating that small amounts of virus were not able to produce a CPE in a particular batch of culture tubes. A loss in titer could be ruled out in these cases, as the same stock of virus yielded the expected titer on later occasions.

Many investigators to whom we sent our prototype cultures reported either difficulties in passage or very low titers, and they usually used rhesus kidney cultures instead of cynomolgus. When we carried out simultaneous titrations in cynomolgus and rhesus cells of different ages (Table 3) we found that in 7-day old rhesus cultures the CPE was delayed and much less marked than in cynomolgus cultures. However, when 14-day or older rhesus cells, appropriately maintained by changes of medium every 4 to 5 days, were used, they proved to be as sensitive as cynomolgus. Moreover, virus grown in rhesus cells yielded equally high titers. After a series of rapid passages, the prototype ECHO 23 strain yielded fluids with titers of $10^{7.7}$ TCD_{50} per ml. when grown in bottles with large numbers of cynomolgus cells and small amounts of medium. Using 14-day old rhesus cells for growth of virus it was also possible to obtain fluids with titers of $10^{7.2}$ TCD_{50} per ml. For production of virus stocks, cultures seeded with undiluted virus were frozen when about 50 to 75 per cent of the cell sheet exhibited CPE, which usually occurred 3 to 4 days after inoculation.

No problems of adaptation were encountered with the 2 strains of ECHO 24 virus. On first isolation, the CPE appeared in both inoculated tubes 9 days or 5 to 7 days after inoculation for strain "93" (de Camp; prototype) and "89" respectively. On passage, the CPE usually started on the first day for strain "93" and on the second for strain "89". The CPE involved the whole culture, but did not always lead to complete destruction of the cell sheet, not even with the stocks in current use, after the viruses have been passaged 14 and 17 times respectively.

Behavior of adapted virus strains in culture tubes. — The time of first appearance and maximal CPE observed with different amounts of virus are listed in the first columns of Table 4. We always harvested the viruses at the indicated time of maximal CPE. The infectivity titers of the plaque purified virus stocks, usually obtained in tube cultures, are also listed. Strain "36" of ECHO 22 virus yielded about the same titer as the prototype "101" strain, and strain "57" a 10-fold lower titer.

Table 4. Properties of ECHO 22, 23, and 24 viruses in tubes and plaque bottles of monkey kidney tissue culture

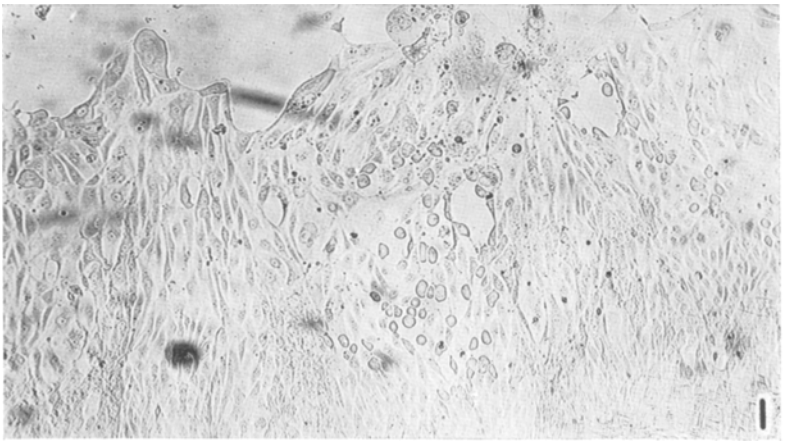
Virus	Tubes*					Plaques							Infectious titer*		
	Day of CPE with inocula of				Maximal degree and type of CPE	Day of first appearance and maximal number after inoculation		Plaque shape	Adsorption to cells	Size (mm) at indicated days after appearance			In tubes (TCD_{50} per ml.)	In bottles (PFU per ml.)	Ratio $\frac{PFU}{TCD_{50}}$
	$10^{4.5}$ TCD_{50}		10^{2} TCD_{50}												
	First	Max.	First	Max.		First	Max.			1	3	5			
ECHO 22 (Harris)	1	3	2	5	2 to 4 (predominantly peripheral)	4—5	8—9	round, regular	rapid	<1–2	3—5	7—11	$10^{7.0}$	$10^{8.5}$	30
ECHO 23 (Williamson)	2	4—5	3	5	1 to 3 (mostly peripheral)	5	9—10	round, regular	rapid	1	2—4	3—8	$10^{5.2}$	$10^{6.2}$	10
ECHO 24 (93 de Camp; prototype)	1	3	2	5	3 to 4	5—6	9—11	irregular	very slow	1	1.5—3	3—5	$10^{5.7}$	$10^{6.7}$	10
ECHO 24 (89)	2	4	3—4	7—8	2 to 4	5—6	9+	irregular	slow	1	2—3	2—4	$10^{5.4}$	$10^{5.3}$	1

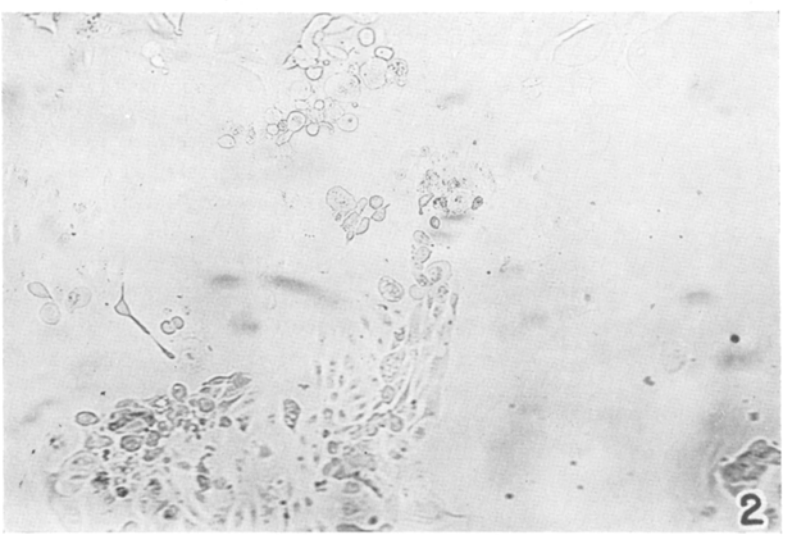
* Data concerning the behavior in tubes refer to purified virus stocks.
Properties of virus plaques are identical with unpurified and purified viruses.
Ratio figures PFU/TCD_{50} refer to purified viruses.

Description of the CPE. — The type of CPE is indistinguishable for the 3 strains of ECHO 22 and for ECHO 23 virus. In the beginning, a few groups of round or ovoid cells with clear cytoplasm and refractile edges are seen at the periphery of the cell sheet, sometimes also at the butt of the tube (Fig. 1, 2). One day later, the whole edge of the culture is often affected, and, to a smaller or larger extent, similar groups are formed in the center of the culture or on the edges of cell islands, which have become isolated by contraction. Somewhat later the affected cells fall off the glass and, if the CPE was slight, the culture may appear perfectly healthy, which emphasizes the importance of examining the tubes at frequent intervals. ECHO 24 virus produces the same type of CPE as other ECHO viruses. The changes do not usually affect cell groups, but rather single cells (Fig. 3), and 50 per cent or more of the culture is usually destroyed.

Examination of cultures stained with hematoxylin and eosin under low magnification did not reveal any significant differences in the CPE among the 3 new ECHO virus types or from other ECHO viruses (Fig. 4, 5). The majority of the affected cells was found to be somewhat smaller than normal cells, but some were definitely enlarged (Fig. 5). The nuclei were pycnotic and shrunken, often somewhat eccentrically located. No intranuclear inclusions could be seen. The cytoplasm, especially in larger cells, was often eosinophilic or contained granular eosinophilic masses, which sometimes resembled inclusion bodies (several visible in Fig. 5). *Shaver, Barron* and *Karzon* recently reported (Bacteriological Proceedings, 1960) that ECHO 22 and 23 viruses produced a distinctive cytopathic effect consisting of an emptying of nuclear material including nucleoli, with a condensation of chromatin material on the nuclear membrane. This effect was best seen in coverslip preparations stained with hematoxylin and eosin.

Properties of virus plaques. — During the process of plaque purification we studied the properties of plaques formed by these viruses on monkey kidney monolayers. The 3 ECHO 22 strains (2 of them shown in Fig. 6, designated as D 36 and D 101) and the ECHO 23 virus produced the same kind of round plaques with fairly regular edges, appearing 4 to 5 days after inoculation and reaching their maximal number 8 to 9 days after inoculation (Table 4). The plaques of ECHO 23 virus (Fig. 7) and of strain "57" of ECHO 22 (not shown) grew somewhat slower than those of ECHO 22, strains "101" and "36". At the beginning, the plaques of all these strains were not clear and white, but had an irregular structure. The presence of pink or grayish areas inside the plaque indicated incomplete cell destruction. Sometimes, but not in all tests, with ECHO 23 virus undestroyed spots persisted (Fig. 7). Usually, however, the plaques cleared





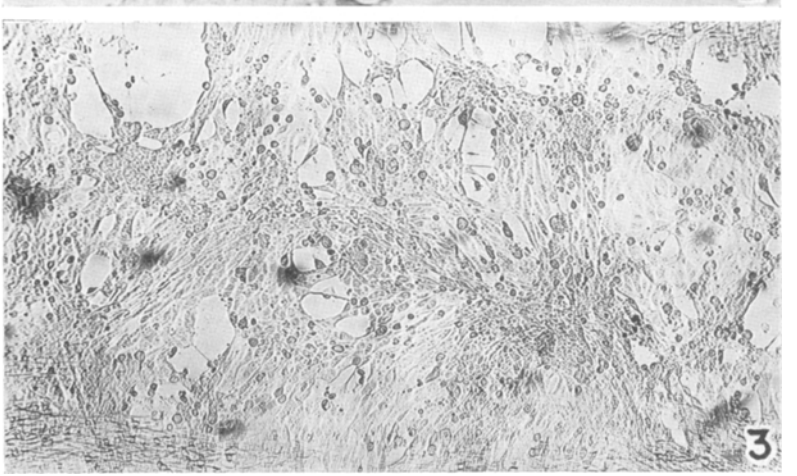
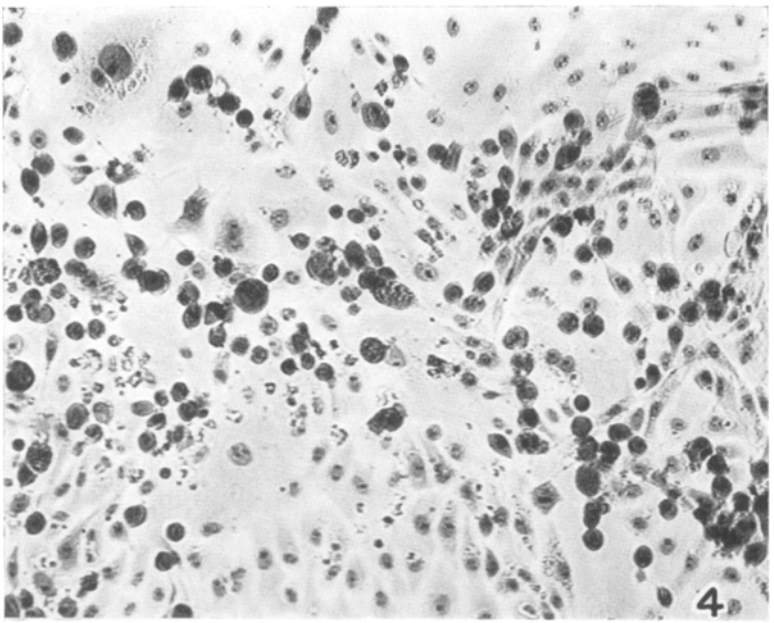


Fig. 1. ECHO 22 virus — group of affected cells on edge of monkey kidney culture. Unstained. 32×. — Fig. 2. ECHO 23 virus — group of affected cells on edge of monkey kidney culture. Unstained. 32×. — Fig. 3. ECHO 24 virus — isolated round refractile cells. 32×.



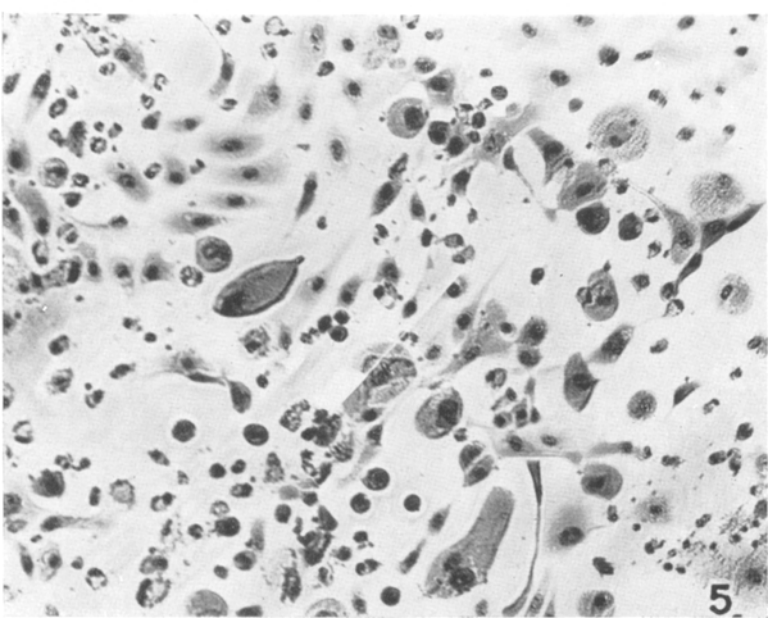


Fig. 4. ECHO 22, culture stained with hematoxylin-eosin. Pycnotic or shrunken nuclei, cytoplasm filled with eosinophilic material in some cells. 160×.

Fig. 5. ECHO 24, stained culture. As in Fig. 4, eosinophilic masses in some of the enlarged cells simulating cytoplasmic inclusions bodies. 160×.

Fig. 6. Plaque morphology of 2 ECHO 22 strains (D 36, D 101) and one ECHO 24 strain (D 93), 9 days after inoculation.

up after further incubation, which indicated that the cell destruction caused by these viruses under agar was more complete than in tube cultures. It could be shown, that this was not due to differences in the composition of the medium, but rather to the different conditions caused by the agar overlay. For when the nutrient medium used for the overlay medium, but mixed with an equal volume of distilled water instead of agar, was used in tube cultures, the CPE was not more complete with ECHO 22 and 23 prototype viruses than with the regular maintenance medium.

Fig. 7. Plaque morphology of ECHO 23 virus, 10 days after inoculation. The dark spots inside the plaques appeared only in this test. On other occasions the plaques resembled those formed by ECHO 22 virus (Fig. 6).

The plaques produced by both ECHO 24 strains differed from the ECHO 22 and 23 plaques. They appeared 5—6 days after inoculation, were irregular in outline and grew more slowly (Fig. 6, D 93, and Table 4). Both strains formed similar plaques.

It is noteworthy, that the titers obtained in plaque bottles were considerably higher than in tubes. The ratio PFU/TCD_{50}, determined by repeated titrations of the same virus stock in tubes as well as in plaque bottles, was 20, 16, and 100 for the 3 unpurified ECHO 22 strains "101", "36", and "57" respectively, and 30 for the plaque-purified strain "101". For ECHO 23 this ratio was 30 before and 10 after plaque purification. The 2 ECHO 24 strains differed in this respect. The prototype strain "93" yielded higher titers in plaque bottles with a ratio of 30 for the unpurified and 10 for the purified stock. On the other hand, the strain "89" exhibited consistently a ratio of 1 (only unpurified virus tested). Therefore, this strain, which had a similar titer in tube cultures as strain "93", had a considerably lower titer in terms of PFU (Table 4).

Hsiung and *Melnick* (7) mentioned, that they found 2 to 10 times higher titers in plaque bottles than in tube cultures with some ECHO viruses, without presenting further data. We found ratios between 3 and

8 with 3 epidemic ECHO 9 virus strains (8). The ratios observed with ECHO 22, 23, and with one strain of ECHO 24 exceed these figures and suggest, that these viruses may be more readily isolated in plaque bottles than in tube cultures. This becomes even more likely in view of the problems encountered in the tube isolation and passage, previously noted for the ECHO 22 and 23 viruses.

Attempts to obtain single plaques in bottles, from which the inoculum was washed off after 1 hour adsorption, showed, that all 3 strains of ECHO 22 virus as well as ECHO 23 virus yielded virtually the same number of plaques in washed as in unwashed bottles. As the adsorption appeared to be much slower for ECHO 24 virus in preliminary tests, 2 hours' adsorption at 37° C were allowed. Under these conditions about 1/10 the number of plaques was obtained in the washed bottles, as compared with the unwashed, with strain "89", and no plaques were obtained even with more than 100 PFU per bottle of strain "93"; the same behavior was found with this strain after plaque purification.

Plaque purification. — For the first plaque purification preference was given to washed bottles containing only single plaques which were harvested and tested in culture tubes for virus content. Occasionally harvests were used from bottles with more than one plaque, but only if they were more than 20 mm apart, measured from one plaque edge to the other. Virus-containing plaque harvests were again passed in plaque bottles and another plaque, meeting the same criteria as before, was harvested. For the second purification the bottles were not washed after adsorption. A virus stock with a good titer, suitable for immunization of rabbits, was then prepared from the progeny of the second plaque.

Only with strain "93" of ECHO 24 virus was it necessary to follow a different procedure. In view of the slow adsorption it was necessary, to harvest the first plaque from an unwashed bottle. Furthermore, repeated attempts to obtain a direct subplaque from the plaque harvest failed. Finally a second plaque was obtained from a tube passage of the first plaque, and the progeny therefrom was used for animal inoculation.

Behavior in other host systems. — A number of newborn mice (less than 24 hours old) was inoculated with tissue culture passage material before and after plaque purification of all 6 strains, 0.01 ml. being inoculated intracerebrally and 0.03 ml. subcutaneously into each mouse. The number of PFU inoculated and other data are listed in Table 5. No specific deaths or illnesses were observed in any of these mice. One blind passage into newborn mice was carried out with five strains: 4 mice of the first passage were killed 5 days after inoculation and a pooled 20 per cent carcass suspension (without skin, viscera, and distal extremities) was used as further inoculum. From these suspensions either no virus or only traces

Table 5. Lack of pathogenicity of ECHO 22, 23, and 24 viruses for newborn mice

Virus		Virus passage No.	Number of PFU inoculated into each mouse	Result*	Results of blind Passage	
Type	Strain				T. C.**	Mice*
ECHO 22	101	6	$10^{4.8}$	0/10		
		9	$10^{6.0}$	6/10 a.	10^{2}	0/10
		17 (P)†	$10^{7.0}$	0/8		
	36	9	$10^{5.2}$	2/19 b.		
		12	$10^{5.6}$	0/6	$<10^{2}$	0/9
		20 (P)	$10^{6.0}$	0/9		
	57	5	$10^{5.3}$	1/9 c.		
		11 (P)	$10^{5.5}$	5/10 d.		
ECHO 23	92	6	$10^{4.5}$	0/9		
		10 (P)	$10^{4.6}$	0/8		
				0/13	$<10^{2}$	0/6
ECHO 24	93	8	$10^{6.0}$	3/11 e.	$10^{2.2}$	9/10 f.
		13 (P)	$10^{5.4}$	0/8		
	89	11	$10^{4.0}$	0/5	$<10^{1.5}$	1/10 g.
		17 (P)	$10^{3.7}$	0/9		

* Numerator: number of mice affected (dead, sick, or found missing); denominator: number of mice inoculated. Inoculum 0.01 ml. intracerebrally and 0.03 ml. subcutaneously.

** Number of PFU per gram mouse recovered in tissue culture from the carcass of 4 healthy mice harvested and pooled 5 days after inoculation.

† (P) = plaque-purified material.

a. 6 mice missing 2 to 5 days after inoculation.
b. one mouse dead and one missing 9 and 11 days after inoculation.
c. one mouse dead 9 days after inoculation.
d. 5 mice missing 2 to 6 days after inoculation.
e. 3 mice dead 11, 13, 13 days after inoculation.
f. 9 mice either missing or sick without characteristic symptoms between 6 and 11 days after inoculation. No virus recovered in tissue culture.
g. 1 mouse dead 9 days after inoculation.

of virus could be demonstrated in tissue culture. No effect was seen in mice during the second passage, with the exception of ECHO 24, strain "93", in which 9 mice died of nonspecific causes 6 to 11 days after inoculation. No virus could be recovered from the 2 tested mice. The 3 proto-

type strains were tested in kidney cultures prepared from 3-week old rabbits and no CPE occurred. In ERK cell cultures (Westwood's stable embryonic rabbit kidney line, obtained from Dr. *Wilson Smith* of London) ECHO 24 produced a definite CPE, which was complete in 3 days and in the second passage almost complete at the first day after inoculation. ECHO 22 and 23 failed to produce a CPE. The maintenance medium for these cultures, which contained 5 per cent calf serum, did not by itself prevent the CPE produced by these viruses, when used in monkey kidney tubes. It should be noted that tests carried out by one of us (A. B. S.) showed that the ERK cells were antigenically identical with human and not rabbit cells.

Neuropathogenicity for cynomolgus monkeys. — To test the possibility, that one of these strains may be able to produce poliomyelitis-like or other lesions in the central nervous system of monkeys, 3 cynomolgus monkeys were inoculated with each of the 3 prototype viruses. Each monkey received an inoculum of 0.1 ml. into the gray matter of the lumbar cord and 0.5 ml. into the right thalamus. The spinal and cerebral inocula in terms of PFU were $10^{7.6}$ and $10^{8.3}$ for ECHO 22, $10^{5.2}$ and $10^{5.9}$ for ECHO 23, and $10^{5.7}$ and $10^{6.4}$ for ECHO 24 virus. Temperatures were not taken, but all monkeys were found otherwise well during the 30 days of observation. After that time the central nervous system was examined histologically. In all 3 monkeys inoculated with ECHO 22 and 2 of those inoculated with ECHO 23 and ECHO 24 virus it could be shown that the inoculum had indeed entered the gray matter of the lumbar cord. No abnormalities except the reaction due to the inoculation trauma could be found in any of the 3 monkeys inoculated with ECHO 22 and ECHO 24 virus and in 2 monkeys inoculated with ECHO 23 virus. The third monkey inoculated with ECHO 23 virus, the one in which the inoculum could not be located in the lumbar cord, showed multiple scattered glial foci, perivascular infiltration and focal meningeal infiltration in the cerebral cortex, not obviously connected with the site of the cerebral inoculation. Neutralizing antibodies were formed in all 9 monkeys to a varying extent. No preexisting antibodies were found except in one monkey inoculated with ECHO 24 virus, which had a neutralizing titer of 1:10 in its pre-inoculation serum, rising to 1:640 in the post-inoculation serum.

To examine further the possibility that ECHO 23 virus might be able to produce encephalitis in monkeys, 5 other cynomolgus monkeys were inoculated only intracerebrally with $10^{6.4}$ PFU of ECHO 23 virus. The animals showed no clinical abnormalities and had normal body temperatures up to the 23rd day after inoculation. Four monkeys stayed well until the 32nd day, when they were sacrificed. They did not show any lesions in the CNS besides the reaction to the inoculation trauma. The fifth mon-

key had elevated temperatures from the 24th day and was sacrificed on the 30th day. On autopsy, pneumonia of the right middle lobe was found. No virus was recovered from either cerebral cortex or spinal cord of this monkey and histologically no abnormalities were found in the CNS. Consequently, the encephalitic foci found in the single monkey in the previous test may or may not have been caused by the virus.

Table 6. Demonstration af antigenic homogeneity of original strains by neutralization of original purified ECHO 22, 23, and 24 viruses by homologous rabbit antiserum prepared with purified virus

Virus			TCD_{50}	50 per cent neutralization endpoint per 0.1 ml. by homologous rabbit serum before and after immunization	
Type	Titer (TCD_{50}/ml.)	Material used		Pre serum	Post serum
ECHO 22	$10^{6.7}$	original	10	4	10,000
	$10^{7.0}$	purified	32	<2	10,000+
ECHO 23	$10^{4.4}$	original	70	3	1,600
	$10^{5.2}$	purified	100	2	1,600
ECHO 24	$10^{6.2}$	original	50	<1	160
	$10^{5.7}$	purified	50	<1	160

Antigenic homogeneity of isolated viruses. — The main purpose of the plaque purification was to exclude the possibility that the failure of established ECHO prototype antisera to neutralize a given strain might be due to the presence of more than one virus in the strain. Antisera were prepared in rabbits with the purified virus stock of each of the 6 strains by repeated intravenous inoculations. The pre- and postimmunization rabbit sera were then tested quantitatively for neutralization against the original and purified homologous virus. The results with the prototype strains are recorded in Table 6; those with the other 3 strains showed a similar pattern. Whereas the pre-inoculation sera showed only a very slight or no effect, the post-immunization sera exhibited virtually the same neutralization titer against the original and purified virus, used in identical or similar virus doses. It is noteworthy that potent rabbit antisera were readily obtained with all 3 strains of ECHO 22 and with ECHO 23 virus, but less potent sera with ECHO 24 virus. The neutralization titers observed in early and late readings were in most instances identical for the 3 ECHO 22 strains and for the prototype ECHO 24 strain. On the other hand, a marked "break-through" occurred almost

invariably with strain "89" of ECHO 24 virus, which is the only strain with a PFU: TCD_{50} ratio of 1, and with ECHO 23 virus in some tests but not in others.

Serologic differentiation from other viruses. — In initial screening tests the 6 viruses being studied were not neutralized by antisera prepared against known prototype enteroviruses. The selected prototypes for

Table 7. Failure of ECHO 22, 23, and 24 viruses to be neutralized by ECHO antisera types 1 to 21, and neutralization by human gamma globulin

Antiserum ECHO type	Homologous neutralization titer per 0.1 ml.	Designation	Neutralization titer per 0.1 ml. vs.		
			ECHO 22 virus (100)*	ECHO 23 virus (500)	ECHO 24 virus (200)
1, 2, 5, 7, 8, 9, 10, 12	4,000—25,000	Reference monkey sera	<20	<20	<20
11, 14, 15, 16, 17, 18, 19, 6′, 6″	1,000—32,000	Reference monkey sera	<10	<10	<10
3	25,000	Reference monkey sera	<100	<100	<100
4	50	Reference monkey sera	<5	<5	<5
6	25,000	Reference monkey sera	10	<20	<20
13	20,000	Monkey serum**	<20	<20	<20
20 (JV-1)	5,000	Rabbit serum**	<25	<25	<25
21 (Farina)	128+	Rabbit serum**	<5	<5†	<5†
Human gamma globulin pool			800	100	<5

* Figures in parenthesis: number of TCD_{50}.

** The antisera for ECHO types 13 (purified), 20, and 21 were kindly supplied by Drs. *Hammon, Rosen,* and *Kibrick* respectively.

† Slight delay by 1:5 diluted serum.

ECHO 22, 23 and 24 were tested with higher concentrations of the ECHO reference monkey sera, types 1 to 12, 14 to 19, ECHO 13 monkey serum, prepared with purified ECHO 13 virus, and with rabbit sera for 2 newly recognized ECHO viruses, types 20 and 21 [Table 7 — see ref. (9) for the homologous titers of ECHO reference monkey sera, types 1 to 12]. ECHO 22 was neutralized by ECHO 6 serum in a 1:10 dilution (homologous titer

1:25,000). A slight delay of ECHO 23 and ECHO 24 virus by ECHO 21 rabbit serum was observed, which requires further study with a more potent ECHO 21 serum. In all other instances no neutralization was observed. Also no neutralization occurred in tests with 40 or more antibody units of antisera prepared against poliovirus types 1, 2 and 3 (monkey reference sera), Coxsackie A 9, B 1 through B 5 (rabbit sera), and Coxsackie A 7 and A 14 (monkey sera).

Table 8. Failure of ECHO 22, 23, and 24 rabbit antisera to neutralize ECHO 1 to 21 viruses — lack of antigenic crossing between ECHO 22, 23, and 24 viruses

ECHO virus type	TCD_{50}	Neutralization titer with indicated antiserum		
		ECHO 22 (101)	ECHO 23 (92)	ECHO 24 (93)
1 to 9 11 to 21	3 to 320	<25	<16	<10
10 (Reovirus I)	200	<25	12*	<10
22	100	5,000	<16	<10
23	100	<25	1,600	<10
24	50	<25	<16	1,600

* Titer of pre-inoculation serum <5.

As ECHO 4 virus is not readily neutralized in tube cultures (9), an antigenic relative of ECHO 4 virus could be missed by the tube neutralization method. Therefore, ECHO 24 virus, which produces a CPE similar to that of ECHO 4 virus, was tested by means of the plaque reduction method with ECHO 4 monkey serum, which has a homologous titer of 1:2000, when used with this technique (9). In the virus control bottles about 84 plaques per bottle were counted, while in 3 bottles inoculated with virus + 1:10 dilution of ECHO 4 serum, the count was 20, 22, and 24 plaques; in 3 other bottles inoculated with virus + 1:10 dilution of pooled normal monkey serum, 21, 28 and 29 plaques appeared. It is apparent that both monkey sera had reduced the number of plaques to approximately the same extent. Therefore, a relation to ECHO 4 virus is excluded. We cannot decide, whether the plaque reduction observed is specific or non-specific. The same pool of normal monkey serum had no neutralizing effect on ECHO 24 virus, when tested undiluted in tube cultures.

The results of the reverse tests, i. e. the effect of ECHO 22, 23, and 24 rabbit antisera on other ECHO viruses, are shown in Table 8. With only one exception no neutralization occurred. This exception was a slight

neutralization of ECHO 10 virus (now reovirus, type 1) by ECHO 23 serum, not present in the pre-immunization serum. It should also be noted, that the 3 new ECHO virus types do not show any cross-neutralization in the dilutions tested (see also Table 11). Dr. *J. L. Melnick* also tested these rabbit antisera against ECHO 1 to 19 prototype viruses, Dr. *W. McD. Hammon* against ECHO 1, 12 and 13 viruses, also with negative results. These antisera were also found to have no effect on 6 to 320 TCD_{50} of poliovirus types 1, 2, and 3, and on Coxsackie A 9, B 1 through B 5 prototype viruses.

Table 9. Antigenic variation among 3 ECHO 22 strains

Virus		Neutralization titer per 0.1 ml. with indicated antiserum		
Strain	TCD_{50}	101	36	57
101 (prototype)	32	10,000+	1,600	320
	100	3,200	1,100	150
36	320	1,600	10,000	160
	100	2,500	5,600	112
57	25	10,000+	3,200	10,000
	32	5,600	2,500	5,000

Results are from 2 tests carried out on different occasions.

Neutralization by human gamma globulin and human sera. — One of the requirements for the establishment of a new ECHO virus type is the neutralization by human gamma globulin or individual human sera (10), to establish its occurrence in human beings. As is shown in Table 7, ECHO 22 and 23 were neutralized by human gamma globulin with titers of 1:800 and 1:100 respectively. On the other hand, only one of 3 tubes was protected, when inoculated with 200 TCD_{50} of ECHO 24 virus and gamma globulin, diluted 1:5, which is the highest concentration that could be used without extensive toxic damage to the cells. As this neutralization can hardly be considered as significant, we tested 20 sera of healthy adult persons, who originated from different parts of the U. S. and from lower socio-economic groups. Three of these had neutralizing antibodies with a titer of 1:10 or more, 5 others showed a partial neutralization by undiluted serum only, and the remaining 12 showed no antibodies. This result indicated that this virus occurs in human beings. To be absolutely sure, that it was derived from the human material and not picked up from the monkey kidney culture, both strains were reisolated successfully from the original rectal swabs and the virus identified by ECHO 24 serum.

Antigenic relation between different strains of ECHO 22 and ECHO 24 virus. — Results of cross-neutralization tests with rabbit sera prepared against the 3 strains of ECHO 22 virus after plaque purification are shown in Table 9. The neutralization titers obtained in 2 different tests are presented. The antisera prepared with strain "101" and "36" show a 2- to 6-fold lower neutralization titer with the heterologous than with the homologus strain in both directions. If these differences were significant, they would indicate a slight bilateral antigenic difference between these 2 strains. On the other hand, the serum prepared against strain "57",

Table 10. Difference in neutralizability and tendency to "break through" exhibited by 2 ECHO 24 virus strains

Virus			Neutralization titer per 0.1 ml. with indicated antiserum	
Strain	Titer (TCD_{50}/ml.)	TCD_{50}	93	89
93 (prototype)	$10^{5.7}$	50	160	1,600
		200	150	1,280
89	$10^{5.4}$	50	50/16*	100/16
		320	n. t.	180/10

* Numerator: results of early reading; denominator: results of late reading.

Results are from 2 tests carried out on different occasions.

with a homologous titer of 1:5000, exhibited 16- to 50-fold lower neutralization titers, when tested against strains "101" and "36". Since the "57" virus was well neutralized by both "101" and "36" antisera, one may conclude that strain "57" is antigenically narrower than the 2 other strains, and that "57" antiserum may also fail to identify other ECHO 22 strains, when used in a dilution containing 20 antibody units.

The cross-reactions of the 2 ECHO 24 strains exhibited quite a different pattern (Table 10 — results of 2 different tests). Strain "89" showed consistently lower neutralization titers than strain "93" with its own as well as with "93" antiserum. This is evident in early readings, and becomes even more pronounced in late readings, as strain "89" usually breaks through extensively. It appears, that these 2 strains are not different antigenically, but that strain "89" is less readily neutralized and has a greater tendency to break through in neutralization (11).

Lack of relationship to adenoviruses. — On the basis of the CPE produced in monkey kidney cells, as observed in unstained and stained cultures, it appeared very unlikey, that these viruses might be adenoviruses.

Table 11. Lack of antigenic cross reaction in complement-fixation between ECHO 22, 23, and 24, and adenovirus. Antigenic cross reactions and reactions with normal monkey sera of ECHO 22, 23, and 24 in complement-fixation. No cross reaction in neutralization

Antigen	Complement-fixation titer of indicated serum						
	C. 1249 (ECHO 22)		C. 1286 (ECHO 23)		C. 1255 (ECHO 24)		Human adeno-virus serum pool
	Pre	Post	Pre	Post	Pre	Post	
Adenovirus (1:8)	<4	<4	<4	<4	<4	<4	128;128
ECHO 22 ($10^{7.7}$ TCD_{50}/ml.)	8	64	<8	64	8	16	<8
ECHO 23 ($10^{3.7}$ TCD_{50}/ml.)	64 (8)*	64 (16)	16 (<8)	64 (32)	128 (32)	128 (64)	<8
ECHO 24 ($10^{6.7}$ TCD_{50}/ml.)	8	16	<8	32	8	32	<8
M. H. unclassified Coxsackie ($10^{5.2}$ TCD_{50}/ml.)	n. t.	n. t.	n. t.	n. t.	<8	<8	n. t.
ECHO 7 ($10^{8.7}$ TCD_{50}/ml.)	<4	4	n. t.	n. t.	n. t.	n. t.	n. t.

Virus		Neutralization titer per 0.1 ml. with indicated monkey serum					
Type	TCD_{50}	C. 1249 (ECHO 22)		C. 1286 (ECHO 23)		C. 1255 (ECHO 24)	
		Pre	Post	Pre	Post	Pre	Post
ECHO 22	64	<2	640	<5	<5	<5	<5
ECHO 23	200	<5	<5	<5	>5000	<5	<5
ECHO 24	200	<1	<5	<5	<5	<1	40

* Figures in parenthesis: Results obtained with 3 units of complement.

The failure of ECHO 22 and 23 to produce a CPE in ERK cell cultures is also against such a possibility. A relationship to adenoviruses could further be excluded by means of complement-fixation. The different batches of antigen prepared with each of the 3 prototype viruses failed to react in a 1:8 dilution of a human serum pool with 1.5 to 2 units of complement. The same serum exhibited a CF titer of 1:128 against

4 units of adenovirus antigen with 2 units of complement (Table 11). Furthermore, pre- and post-immunization sera of rabbits and monkeys inoculated with ECHO 22, 23, and 24 virus failed to show a CF reaction in a 1:4 dilution with adenovirus antigen.

Complement-fixation of ECHO 22, 23, and 24 antigens with monkey sera. — Antigens which were only slightly anticomplementary, were prepared using a maintenance medium without lactalbumin hydrolysate. In preliminary box titrations with post-inoculation monkey sera, the ECHO 22 and 24 antigens proved to contain at most 1 optimal unit of antigen in 0.2 ml. of undiluted fluid. The ECHO 23 antigen was not tested by this means because of shortage of material.

With 2 units of complement the ECHO 22 and 24 antigens yielded serum CF titers of 8 with the pre-immunization sera of monkeys C 1249 and C 1255, and <8 with C 1286, while the ECHO 23 antigen yielded titers of 64, 16 and 128 respectively. With 3 units of complement, negative results were obtained with the ECHO 22 and 24 antigens, while the pre-immunization sera of C 1249 and C 1255 still yielded titers of 8 and 32 respectively (Table 11). The question of a possible relationship between ECHO 23 virus and some simian viruses raised by these observations needs further investigation. The postimmunization monkey sera all showed a distinct rise in CF titer with the homotypic antigens but not with control antigens prepared with other viruses grown in same monkey kidney cells and maintenance medium (see Table 11). The fact that monkey C 1286, inoculated with ECHO 23 virus, developed CF antibodies also for the ECHO 22 and 24 antigens but not neutralizing antibodies for these viruses, indicates the need of further studies on the possible existence of a common CF antigen among these three viruses.

Discussion

Although all 6 investigated strains now classified as ECHO 22, 23 or 24 viruses were isolated from infants with diarrhea, whereas none was found in the healthy children who served as controls, their full significance for human disease is unknown. It is noteworthy that other strains have already been encountered for all 3 virus types. A virus isolated by Dr. *Victoria Drouhet* of Paris from the stool of a patient with aseptic meningitis was identified by us as ECHO 22 virus. Dr. *C. J. Jhala* informed us that during the summer of 1960 he isolated ECHO 23 virus from three consecutive stool specimens of a patient with aseptic meningitis in Indianapolis. Dr. *Leon Rosen* (National Institutes of Health, Bethesda, Md.) informed us that he isolated several strains of ECHO 23 and ECHO 24. Major *Edward L. Buescher* of the Walter Reed Army Medical Center in Washington, informed us of the isolation of 3 strains of ECHO 22 virus from the

throat washings of three 11 to 16 month old children with febrile respiratory disease in Hawaii. All 3 children developed antibody for ECHO 22 virus during the course of their illness. Dr. *A. L. Barron* of the University of Buffalo School of Medicine informed us of the isolation of 6 strains of ECHO 22 virus — 4 strains from healthy children, 1 strain from an infant with diarrhea, and 1 from an infant with diarrhea and jaundice. The scarcity of the CPE produced by ECHO 23 viruses and some strains of ECHO 22, and the difficulty in passage of unadapted material in monkey kidney culture suggest, that these viruses are recoverable with difficulty and may frequently be missed. The higher virus titers obtained in plaque bottles further suggest, that these may be more efficient for primary isolation than tube cultures, particularly for ECHO 22 and 23 viruses.

The 3 selected prototype viruses correspond in all studied properties to the criteria of ECHO viruses (10). They grow in monkey kidney cultures, they are not pathogenic for newborn mice or monkeys, they have no relationship to the adenovirus group, and they are neutralized by human gamma globulin (ECHO 22, 23) or human sera (ECHO 24). Moreover, ultrafiltration tests (*Barnes* and *Sabin*, to be published) showed that all 3 viruses are in the same range of small size as the polioviruses and other enteroviruses. No serologic relationship to other ECHO viruses or with one another was found in neutralization tests. The question of a relationship by complement-fixation remains to be elucidated. At least one of the 3 ECHO 22 strains was found to be antigenically different from the other 2, which is in line with the antigenic heterogeneity found among strains of other types of enteroviruses (9).

Summary

The viruses now classified as ECHO 22, 23, and 24 were isolated in monkey kidney tissue culture from rectal swabs of infants with diarrhea. The cytopathogenic effect of ECHO 22 and 23 viruses is often confined to the peripheral part of the culture. Difficulties were encountered with passage and adaptation of these viruses. ECHO 24 virus affects the whole culture. On monkey kidney monolayers ECHO 22 and 23 viruses produce round and regular plaques, and ECHO 24 irregular plaques. All viruses, except one strain of ECHO 24, yielded considerably higher titers under agar in plaque bottles than in tube cultures with fluid medium. The individual strains were shown to be antigenically homogeneous by means of plaque purification and testing of rabbit antisera prepared with purified virus against both original and purified virus. They were not pathogenic for newborn mice and exhibited no definite neuropathogenicity for cynomolgus monkeys after intraspinal and intracerebral inoculation. In neutralization tests they showed no cross-reactions with ECHO virus types 1 to 21, polioviruses 1 to 3, Coxsackie A 9, B 1 through B 5 virus,

and among each other. No serologic or other relation to adenovirus was found. ECHO 22 and 23 viruses were neutralized by human gamma globulin, while ECHO 24 virus was not, but was neutralized by individual human sera. Antigenic differences were shown among 3 strains of ECHO 22 virus.

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