The Enteroviruses

COMMITTEE ON THE ENTEROVIRUSES, NATIONAL FOUNDATION FOR INFANTILE PARALYSIS

In 1955 the Committee on the ECHO Viruses issued its first report in which 13 antigenically distinct members of the ECHO group were listed. Since then the name of the committee has been changed to indicate that it deals with poliomyelitis, Coxsackie, and ECHO viruses which are now regarded as members of a single family of human enteroviruses. The Committee on the Enteroviruses in the present report reviews the present status of this field and recent studies of the committee on 19 antigenic ECHO types. With increasing recognition of the pathogenic role of previously unrecognized enteroviruses, the data in this report are of concern to epidemiologists, health officers, public health laboratory workers, and others concerned with communicable disease problems of the community.

The National Foundation for Infantile Paralysis, recognizing that the poliomyelitis, Coxsackie, and ECHO viruses all inhabit the alimentary tract as well as share other properties, has changed the name of its Committee on the ECHO Viruses to the Committee on the Enteroviruses. The functions of the committee are similar to those originally outlined, except that it is now prepared to assist in the characterization and classification of new prototypes of any member of the human enterovirus group.

Since the initial report of the committee in which 13 antigenically distinct members of the ECHO group could be listed,[1] six new antigenic types have been recognized and confirmed. The present report is concerned with a brief review of the present status of the field and with some recent cooperative studies of the committee on 19 antigenic ECHO types.

In addition, the report deals with the preparation, standardization, and recommended use of reference ECHO sera prepared in monkeys. Antisera against the ECHO (and Coxsackie) viruses prepared in rabbits yielded results in neutralization tests similar to those reported here for the monkey antisera except for the lower titers of the rabbit antisera.[2] The rabbit sera are not recommended for complement-fixation tests.

Review

During the past decade a large number of hitherto unknown viruses have been found in the intestinal tract of man by use of infant mice and tissue cultures for their isolation. These viruses include the 19 Group A and five Group B Coxsackie viruses, and the 19 ECHO (enteric cytopathogenic human orphan) viruses. Several Coxsackie and ECHO viruses that are distinct from the established types are now under investigation. The adenoviruses, although predominantly inhabitants of the respiratory tract, may occasionally be found in the intestinal tract. However, their properties are such that they are easily distinguished from the true enteroviruses.

Pathogenicity for infant mice, a criterion originally used to separate the Coxsackie from the ECHO viruses, is presenting new problems of classification. Strains of ECHO 9 and 10 viruses, after passage in tissue culture, have been shown to induce disease and lesions in infant mice like those produced by Coxsackie A and B viruses, respectively.

However, available information is inadequate to warrant the transfer of these types from the ECHO to the Coxsackie group.* It is worth noting that Coxsackie A9 shares with ECHO 9 the property of being readily isolated in monkey kidney cells. In fact, some strains of Coxsackie A9 and Coxsackie B viruses, on primary isolation from the human enteric tract, are not pathogenic for infant mice and, in this way, behave like ECHO viruses. Some of the Coxsackie viruses, notably A7 and A14, and some of the ECHO viruses, have been found to produce neuronal lesions in monkeys; further studies may reveal additional variants among these viruses with affinities for primate neurons. It is because the Coxsackie, ECHO, and polioviruses share these and such other properties as size, resistance to ether, seasonal incidence, and epidemiologic pattern that we believe that these three groups should be considered members of a single family of human enteroviruses.

It appears that the enteroviruses differ from the enterobacteria that establish a lifetime residence in the intestinal tract. The incidence of enteroviruses among healthy persons decreases with increasing age, for they seem to cause only transitory infections. The incidence of clinical disease produced by Coxsackie and ECHO viruses varies with the type and strain of virus in a manner comparable to the behavior of various types and strains of poliovirus.

In the first report of the committee [1] it was stated that whenever an ECHO virus was established as the etiologic agent of a clinically distinct disease it would be removed from the ECHO group. However, subsequent experiences have demonstrated that it is no easy decision to remove viruses from this group, because included within it are the etiologic agents of undifferentiated clinical syndromes, that is to say, syndromes which may be caused by a variety of agents. For example, ever since their first isolation and recognition, a number of different enterovirus types have been shown to be associated with, and responsible for, aseptic meningitis. However, because this is a syndrome rather than a clinically distinct disease, it will have to be designated in a particular patient as aseptic meningitis due to ECHO Type 6, or whatever other virus type happens to be involved.

The list of enteroviruses and the diseases which they cause, as given in Table 1, indicates the scope of the problem. For establishment of etiologic association the virus must have a much higher prevalence among patients with the disease than in healthy individuals of the same age and socioeconomic status living in the same area at the same time as the patients. Antibodies against the virus must develop during the course of the illness. Virologic or serologic evidence must be negative for concurrent infection with other agents that already are known to cause the same clinical syndrome. Etiologic probability is increased if the virus is isolated in significant concentration from body fluids or tissues manifesting the lesion, as from the cerebrospinal fluid in cases of aseptic meningitis, or the heart muscle in cases of myocarditis.

* ECHO-10 probably should be classified elsewhere in view of its large size, its distinctive cytopathogenic effects, the existence of certain mouse pathogenic strains, and the respiratory disease with which the virus has been associated in chimpanzees and children.

This report of the committee was presented before a Joint Session of the Epidemiology and Laboratory Sections of the American Public Health Association at the Eighty-Fifth Annual Meeting in Cleveland, Ohio, November 12, 1957.

Members of the committee are: Joseph L. Melnick, Ph.D., chairman; Gilbert Dalldorf, M.D.; John F. Enders, Ph.D.; W. McD. Hammon, M.D., Dr.P.H.; Albert B. Sabin, M.D.; Jerome T. Syverton, M.D.; and Herbert A. Wenner, M.D.

Table 1—Association of Enteroviruses with Human Disease

Enteroviruses	Associated Disease
Poliovirus	Paralysis (complete to slight muscle weakness) Aseptic meningitis Undifferentiated febrile illness particularly during the summer
Coxsackie viruses, Group A	Herpangina Undifferentiated febrile illness particularly during the summer Aseptic meningitis (Types A7, A9)
Coxsackie viruses, Group B	Aseptic meningitis Pleurodynia Undifferentiated febrile illness with pharyngitis Myocarditis or encephalomyocarditis during neonatal period and early childhood Mild paralysis (?)
ECHO viruses	Aseptic meningitis (Types 2, 3, 4, 5, 6, 9, 14, 16) Summer rash (Types 4, 9, 16*) Summer febrile illness Mild paralysis (?) (Type 6) Summer diarrhea of infants and children (Type 18 and others)

* Neva has found the etiologic agent of Boston exanthem to be antigenically related to ECHO virus Type 16.

Newly Established ECHO Types (No. 14–19)

Since the first report of the committee on the establishment of 13 types, six new types have been identified and confirmed in at least three laboratories of committee members. These are listed in Table 2. The criteria for establishing a new type are the same as previously published,[1] with the exception that the reference antiserum should be prepared from progeny purified by plaque passage, or by terminal dilution passage in series.

Purity of Early Established Strains

Because all new strains will be typed only with antiserum prepared by use of purified strains (purified by plaque passage or by terminal dilution), the 13 strains previously established were purified and used as immunizing antigens in monkeys.* With the exception of antiserum to Type 13 virus (see the following) the antisera thus prepared were found to neutralize the parent virus strain and the purified strain to approximately the same degree.

Reference Monkey Antisera

In the process of establishing each of the ECHO types, reference prototype antisera had been prepared in the laboratories in which prototype strains were isolated and recognized. In order to expedite work in this field large pools of antiserum against the first 13 types now have been prepared in Wenner's laboratory, and the sera standardized as a cooperative venture between the laboratories of Hammon, Melnick, Sabin, and Wenner.

To prepare antisera each virus was used to immunize 13 to 20 rhesus monkeys. Absence of antibodies from the prevaccinal sera was indicated by tests of 1:5 to 1:20 serum dilutions against approximately 100 TCD_{50} of each of the 13 ECHO viruses. The immunization schedule consisted of three biweekly inoculations by the intramuscular route of

* The experiments with prototype strains for Types 1, 2, 3, 4, 5, 6, 6′, 14, 15, and 16 were carried out by Melnick and Emmons; for Types 7, 8, 9, 10, and 11 by Wigand and Sabin; and for types 12 and 13 by Hammon.

Table 2—Description of Newly Established ECHO Viruses

Type	Prototype Strain	Geographic Origin	Illness in Person Yielding Virus	Investigators
14	Tow	Rhode Island	Aseptic meningitis	Melnick
15	Charleston 96–51	West Virginia	None	Ormsbee and Melnick
16	Harrington	Massachusetts	Aseptic meningitis	Kibrick and Enders
17	CHHE-29	Mexico City	None	Ramos-Alvarez and Sabin
18	Metcalf	Cincinnati	Diarrhea	Ramos-Alvarez and Sabin
19	Burke	Cincinnati	Diarrhea	Ramos-Alvarez and Sabin

1.0 ml of active, undiluted virus grown in monkey kidney cultures and suspended in an equal volume of adjuvant mixture consisting of four parts Bayol and one part Arlacel. This was followed by a trial bleeding two weeks after the third inoculation. If the antibody levels were satisfactory (e.g., 1:1,000 or greater), the animals were bled. Fifty to 100 ml of blood was obtained from each animal. Animals usually survived the bleeding procedure; then they were revaccinated and rebled at biweekly intervals. Following such treatment animals received two or three additional boosters of viral antigen and were bled as many as three to five times. Sera of each group were pooled and titrated in the four cooperating laboratories for homotypic and heterotypic antibodies against the 13 ECHO viruses. The sera were finally dispensed in 0.5 ml amounts into ampules and dried. Following desiccation the titer of homotypic antibody was redetermined. Before use the contents of each vial should be dissolved in 2.5 ml of Hanks' salt solution to give an initial dilution of 1:5.

Neutralization Tests

These tests were carried out in monkey kidney cultures grown in M-H medium * and, before use, changed to M-E medium or to Medium No. 199. The recommended procedure follows. Virus and serum dilutions are made with Hanks' salt solution. Virus in 0.5 ml amount, calculated to contain 100 TCD_{50} per 0.1 ml, is added to 0.5 ml of each serum dilution and mixed. After incubation for one hour at room temperature 0.2 ml of the mixture is added to each of four monkey kidney tubes. When the virus-serum mixtures have been inoculated, control virus (a challenge dose containing the calculated 100 TCD_{50} per 0.1 ml) is diluted 10, 100, and 1,000 times, and 0.1 ml of each dilution inoculated into each of four cultures. The cultures are incubated at 37° C; results are recorded three to four and seven to eight days later. The earlier readings are made to detect any subsequent breakthrough of virus, which may indicate antigenic relatives. At the time of the last reading the control titration indicates the amount of virus which was present in the challenge dose; this should be approximately 100 TCD_{50}. Serum titer should be calculated from the 50 per cent end point per 0.1 ml against the challenge dose of virus. Twenty units (see the following) are defined as

* M-H contains 0.5 per cent lactalbumin enzymatic hydrolysate, 2 per cent calf serum, Hanks' balanced salt solution, and antibiotics. M-E is used for maintenance; it is similar to M-H, except that it contains Earle's salt solution and only 1 per cent calf serum. M-H and M-E media were used by Melnick and by Hammon. Sabin used a maintenance medium as above, but without serum, and gassed with CO_2 to a pH of about 7.5. Wenner used both M-E and Medium No. 199 for maintenance.

Table 3—Neutralizing Antibody Titers of ECHO Reference Monkey Antisera

Prototype ECHO Virus	Laboratory	TCD_{50} in Tests	Type of Antiserum 1	2	3	4	5	6	7
1	Hammon	320– 32	4,000	0*					
	Melnick	600– 32	4,000–27,000						
	Sabin	500–200	3,200– 4,000						
	Wenner	100– 32	6,400–25,000						
2	Melnick	100– 63		1,700–11,000					
	Wenner	1,000– 32		<4,000– 8,000					
3	Melnick	63			10,000–50,000				
	Wenner	150			54,000				
4	Melnick	200– 32				30–75 †			
	Wenner	10				<128			
5	Melnick	100– 32					11,000–38,000		
	Wenner	50					65,000		
6	Melnick	200– 32						20,000–100,000	
	Wenner	66						28,000	
6′	Melnick	500– 50						50– 1,300	

* The open spaces indicate absence of heterotypic antibody at serum dilutions of 1:8 or less.
† 2000 by plaque reduction method.

6″	Melnick	320– 32		1,200– 4,500	
7	Sabin	64			16,000
	Wenner	250– 34			14,000–58,000
8	Melnick	130	2,000		
	Sabin	200– 64	320		
	Wenner	180– 16	640– 3,300		
9	Melnick	600–320			
	Sabin	600– 32			
	Wenner	250– 53			
10	Sabin	32			
	Wenner	16			
11	Melnick	200–100			
	Sabin	200– 32			
	Wenner	1,000– 66			
12	Hammon	100– 50			
	Melnick	600– 32			
	Sabin	32– 10			
	Wenner	100– 16			
13	Hammon	320– 20	<8– 8,000		
	Melnick	200–130	5,000– 7,000		
	Sabin	500– 20	100–10,000		
	Wenner	133– 16	7,200		

Table 3—Neutralizing Antibody Titers of ECHO Reference Monkey Antisera—Continued

Prototype ECHO Virus	Laboratory	TCD_{50} in Tests	Type of Antiserum					
			8	9	10	11	12	13
1	Hammon	320– 32	>128				>128	400–2,000
	Melnick	600– 32	125– 1,300				125– 1,300	200–4,000
	Sabin	500–200	320				16	50– 200
	Wenner	100– 32	200– 450				<16– >256	1,024
2	Melnick	100– 63						
	Wenner	1,000– 32						
3	Melnick	63						
	Wenner	150						
4	Melnick	200– 32						
	Wenner	10						
5	Melnick	100– 32						
	Wenner	50						
6	Melnick	200– 32						
	Wenner	66						
6′	Melnick	500– 50						
6″	Melnick	320– 32						

7	Sabin	64				16		
	Wenner	250– 34						
8	Melnick	130	20,000				50	80
	Sabin	200– 64	10,000				<5	10
	Wenner	180– 16	5,500–32,000				16– 20	40– 160
9	Melnick	600–320		15,625–35,000				
	Sabin	600– 32		15,000–35,000				
	Wenner	250– 53		27,000–64,000				
10	Sabin	32			16,000			
	Wenner	16			27,000–65,000			
11	Melnick	200–100				3,200– 7,000		
	Sabin	200– 32				3,200– 7,000		
	Wenner	1,000– 66				3,400–16,000		
12	Hammon	100– 50					20,000	
	Melnick	600– 32					5,000–50,000	
	Sabin	32– 10					5,000–50,000	
	Wenner	100– 16					16,000–32,000	<4– 16
13	Hammon	320– 20	<8– >128				>128– 1,000	640–16,000
	Melnick	200–130	160– 630				4,000	6,000–20,000
	Sabin	500– 20	16– 500				500	1,000–16,000
	Wenner	133– 16	<4– >64				400–>2,000	<4,000–16,000

a 20-fold concentration of that dilution giving 50 per cent neutralization of 100 TCD_{50} of virus.

To emphasize the variations that may be found in using these sera the range of titers found in the cooperating laboratories is indicated in Table 3. As can be seen, the antibodies are of high titer except in the Type 4 antiserum. It should be noted that when this same antiserum was tested in Melnick's laboratory by the method of plaque reduction, its titer was 1:2,000.

Strains of Broader Antigenicity (Prime Strains)

Within certain ECHO virus types there exist strains which are not uniform in antigenicity. Those strains showing broad antigenicity have been called prime strains. Although antiserum to a prime strain neutralizes other strains within its type, the prime strain itself may be poorly neutralized by antisera prepared against other strains of the same type. Examples are illustrated in Table 3 by the titer of Type 6 serum against 6′ and 6″ viruses; this ECHO Type 6 serum must be used at a 1:100 dilution to avoid missing such strains. Naturally occurring strains belonging to Types 5, 7, 9, 10, 11, and 13 are known, which also exhibit only partial crossing with their respective prototypes. Accordingly, antisera to these viruses should also be used at a dilution no greater than 1:100.

Status of ECHO 13 Virus

After the isolation of the Hamphill virus by Hammon it was passed several times through monkey kidney cultures, when it was tested in at least two different laboratories against the relatively low-titering antisera then available for the other 12 types. No crossing was observed. When antiserum against the Hamphill strain, also of relatively low titer, was tested against the other viruses in Hammon's, Melnick's, and Sabin's laboratories, no crossing was observed. On this basis Hamphill virus was regarded as a new type.[1] After several more passages in monkey kidney cultures, during which its titer increased somewhat, it was sent to Wenner for preparation of hyperimmune monkey serum and to Microbiological Associates for hyperimmune rabbit serum. When tested in four laboratories the high-titer monkey serum neutralized Type 1 virus in serum dilutions ranging from 1:50 to 1:4,000, and neutralized Type 8 virus in serum dilutions from 1:10 to 1:160 (see Table 3). The Type 1 antiserum neutralized Type 13 virus irregularly; at times it failed to neutralize at 1:8, while at other times neutralization occurred in dilutions as high as 1:10,000. Occasionally Type 8 serum exhibited some significant neutralization of Type 13 virus. Results with rabbit serum prepared by Microbiological Associates were similar in their general pattern.[2]

Subsequent study in Hammon's laboratory strongly indicates that the Type 13 passage virus used for serum production and distributed to other persons was a mixture of Types 1 and 13 viruses. A strain reisolated from the original rectal swab and two new isolates from other persons in the Philippines (11–4 and 11–5), as well as strains separated from the mixture, are now available. From these three isolates a "prime" relationship is evident, as seen in certain other ECHO types, i.e., strains exist which have a broader antigenicity than the original Type 13 virus.

Viruses 11–4 and 11–5 are neutralized only by dilutions of 1:20 or less of the Type 13 reference monkey antiserum listed in the table. Antiserum to either of these strains, prepared by Hammon, neutralize the Type 13 component and the reisolated Hamphill strain (ECHO 13 prototype) to high

titer, i.e., essentially to that of the homologous strain. One of the new isolates, when completely tested and if found satisfactory, will be used for production of a new antiserum to Type 13.

The Type 13 reference monkey serum is being made available at this time because none has yet been prepared in quantity from purified virus and because the presently available reference serum may aid in identification of new Type 13 isolates. It should be used, however, in a dilution not greater than 1:20 if strains like 11–4 and 11–5 are to be detected. This low dilution of the Type 13 serum also will undoubtedly neutralize Type 1 and probably Type 8 isolates, so that any agent neutralized by Type 13 serum must be tested subsequently against serial dilutions of Types 1, 8, and 13 sera to determine its true identity.

Cross Relations Between ECHO Types 1, 8, and 12

As shown in Table 3, Types 1 and 8 viruses are partly neutralized reciprocally by high titer Types 1 and 8 antisera. This cross-neutralization of Types 1 and 8 cannot be explained on a basis similar to that of Types 1 and 13 (i.e., a mixture), because studies in Melnick's laboratory have shown that plaque-purified Type 1 and 8 viruses, and Type 8 virus passed in the presence of Type 1 antiserum, still manifest the same partial cross-neutralization. As 20 units of Type 1 antiserum neutralize 100 TCD_{50} of certain Type 8 strains, any agent neutralized by both Type 1 and Type 8 antisera should be tested with serial dilutions of each antiserum to determine which agent it resembles more closely.

The relationship between Types 8 and 12, in one direction only (Type 12 antiserum against Type 8 virus), is too minor to be of significance in typing. Types 1 and 12 also cross in one direction only (Type 12 antiserum against Type 1 virus), but the degree of crossing is only slightly greater than with Type 8. The very high titer of Type 12 antiserum, however, should preclude confusion in typing. Crossing between Type 12 and a suitably purified Type 13 virus has not been investigated completely, but present results suggest little or no relationship.

Sera listed in Table 3, as well as exhibiting the reactions shown, failed to react with ECHO viruses Types 14 through 19. With the exception of ECHO Types 3 and 6 antisera, these sera, at a dilution of 1:16 or less, also failed to neutralize the three types of poliovirus, 10 adenoviruses, Coxsackie Types B-1 to B-5 and A-9. In Wenner's laboratory the ECHO Type 3 serum pool had a titer of 1:40 against Coxsackie Type A-9, and the ECHO Type 6 serum had a titer of 1:64 against Coxsackie Type B-3. A number of the sera contain antibodies to significant titers (up to 1:256) against one or more of Hull's simian viruses, particularly against SV_4, SV_{18}, and SV_{19}. These antibodies were present prior to vaccination.

Recommended Use of Sera for Typing Unknown Strains

The neutralization procedure used above should be employed, the sera being used so that approximately 20 units are present per 0.1 ml of each antiserum dilution, except for Types 5, 6, 7, 9, 10, 11, and 13; these latter sera should be used at a stronger concentration, namely, at a dilution of 1:100 or lower (see above). Type 4 serum should be used at a dilution of 1:5. In view of the variations encountered in the titers obtained at different times in the same laboratory or in different laboratories (see Table 3), it is recommended that the antisera for the types not listed above be regarded as having the following titers for the purpose of calculating the 20 units: Type 1–4,000; Type

Table 4—Complement-Fixing Antibody Titers in ECHO Reference Antisera

ECHO Virus Type	Serum Titer Versus Homologous Antigen: Hammon's Laboratory	Wenner's Laboratory	NIH Laboratory	Serum Titer * Versus Heterologous Antigens
1	2,048	2,048	4,096	128–256 (Type 8); 128–8,192 (Type 13)
2		1,024	1,024	
3	128	1,024	1,024	
4			1,024	
5		2,048	4,096	
6	4,096	2,048	4,096	
7	>128	256	2,048	
8	2,048	1,024	8,192	32–256 (Type 1); 128–512 (Type 13)
9		512	1,024	
10	512	1,024	2,048	
11	1,024	512	4,096	
12	512	64	512	32–256 (Type 1); 512 (Type 13)
13	512	1,024	8,192	128–1,024 (Type 1); 32–64 (Type 8)

* Only titers of 64 or greater are listed. Reactive heterologous antigens are listed in parentheses.

2–4,000; Type 3–25,000; Type 8–10,000; and Type 12–16,000.

Complement-Fixing (CF) Antibodies

The monkey reference sera contain CF antibodies of high titer, when tested against virus antigens prepared from infected monkey kidney cultures, or from strains adapted to HeLa cultures. The CF titers of the reference sera as determined at the University of Pittsburgh (by Sather and Hammon) at the University of Kansas,[3] and at the National Institutes of Health (by Halonen) are listed in Table 4.

REFERENCES

1. Committee on the ECHO Viruses. Enteric Cytopathogenic Human Orphan (ECHO) Viruses. Science 122:1187–1188 (Dec.), 1955.
2. National Foundation for Infantile Paralysis. Statement concerning ECHO virus rabbit antisera prepared by Microbiological Associates, 1957.
3. Archetti, I.; Weston, J.; and Wenner, H. A. Adaptation of ECHO Viruses in HeLa Cells; Their Use in Complement Fixation. Proc. Soc. Exper. Biol. & Med. 95:265–270 (June), 1957.

Certification Examinations, Board of Preventive Medicine

The American Board of Preventive Medicine has announced the date, place, and deadline date for applications for certification examinations in the coming months.

Aviation Medicine, March 20–22, 1958, Washington, D. C.; deadline date, December 30, 1957
Occupational Medicine, April 18–20, 1958, Atlantic City; deadline date, January 30, 1958
Public Health, April, 1958—on a regional basis—deadline date, January 30, 1958