Isolation and Characterization of Prototype Viruses ECHO-26, ECHO-27, Coxsackie B-6.*† (25446)

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In December 1955, the Committee on ECHO Viruses of the National Foundation described common properties of the first 13 ECHO prototype viruses(1). Subsequently the scope of the Committee was expanded to include Coxsackie and polioviruses and was renamed Committee on Enteroviruses(2). This Committee presently recognizes 3 types of poliovirus, 28 types of ECHO viruses, and 6 types of Coxsackie B. As yet no action has been taken on Coxsackie A group. This report describes the recently accepted enterovirus prototypes, ECHO-26 and 27 and Coxsackie B-6, which we isolated from rectal swabs collected in the Philippine Islands during a longitudinal epidemiological study of enteroviruses at Clark Air Force Base (CAFB), Luzon, in 1953(3).

Materials and methods. 1. *Sources.* Essential details of source of each prototype virus are presented in Table I. All came from normal persons, 2 of whom were children, and 1 an adult; 2 were Americans and 1 was a Filipino. All viruses were isolated from rectal swabs collected in 1953. 2. *Cell culture.* Trypsinized rhesus monkey kidney cell cultures (RhMKCC) were grown in 0.5% lactalbumin hydrolysate in Hank's (LAH) balanced salt solution (BSS) containing 2% calf serum. Prior to use in neutralization tests the cells were changed to 0.5% lactalbumin hydrolysate in Earle's BSS (LAE) containing 0.5% calf serum. For preparation of stock virus pools for immunization of animals and for use in complement fixation (CF) tests, the cells were changed to Eagle's Basal Medium (EBM) in Earle's BSS without serum. Hamster kidney was grown in LAH with 4% calf serum according to method of Diercks and Hammon(4). The transplantable cell lines HeLa (Gey)§, HeLa (Puck S-3).‖ H. Ep. #2,¶ and K.B.** were carried in EBM with 10 to 20% pooled human serum or 10% calf serum. Cytopathogenic tests with these cell lines were conducted in EBM containing 5% inactivated calf serum. 3. *Plaque methods.* RhMKCC grown in 3 oz. bottles, described by Hsiung and Melnick(5), were used in plaque studies. Occasionally, for comparative studies, the final $NaHCO_3$ content was reduced from 3 ml to 1.25 ml of a 7.5% solution/100 ml of overlay. This modified overlay also contained 10 ml of 3% Tris buffer solution (pH 7.4)/100 ml. The final agar concentration was also occasionally reduced from 1.5 to 1%. 4. *Purification of virus.* Each of the 3 viruses was purified by either the plaque picking method (3 serial passages) or, if the agent did not form plaques, by terminal dilution (3 serial pasages). These "purified" agents were used for immunization of animals and as seed virus in all serological tests for typing. 5. *Production of antisera.* "Purified" virus seed grown in RhMKCC in EBM without serum was harvested, extracted once with equal volume of fluorocarbon(6), lyophilized and reconstituted to 1/10 original volume. Two ml of this material and 2 ml of Arlacel-Bayol F adjuvant were emulsified and injected intramuscularly into monkeys. Injections of this same material and same dose were given at 7 to 14 day intervals for 3 to 5 months, length of time being dependent on titer of test bleedings. 6. *Serologic tests.* Neutralizing antibody (NA) tests were performed as described previously, using serial

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§ Cell line obtained from Microbiolcgical Assoc., Washington, D.C.

¶ Cell line obtained from Dr. Jonas Salk, Pittsburgh, Pa.

** Cell line obtained from Dr. Joseph Leighton, Pittsburgh, Pa.

TABLE I. Source, Method of Isolation and Certain General Characteristics of Prototype ECHO-26, ECHO-27 and Coxsackie B-6 Viruses.

	Virus		
	11-3-6* ECHO-26	1-36-4* ECHO-27	1-51-21* Coxsackie B-6
Human source	Coronel	Bacon	Schmitt
Age and sex	1, ♂	10, ♀	25, ♂
Race	Filipino	American	American
Location	Near CAFB,† Luzon	CAFB, Luzon	CAFB, Luzon
Disease	None	None	None
Specimen	Rectal swab	Rectal swab	Rectal swab
Date collected	9/17/53	8/17/53	11/24/53
Isolation(s) RhMKCC	4/ 1/55	12/16/55	10/13/55
Reisolation	Q.N.S.‡	12/20/57	Neg. (12/58)
Additional	None	None	8-Hammon feces 2-Hummeler feces‖
Type of CPE	Polio-like (complete)	Polio-like (complete)	Polio-like (complete)
Titer/0.1 ml¶	10^6	10^8	10^5
Passages	12	13	10
End point	4-6 days	7-9 days	4-6 days
Purification	Plaque§	Terminal dilution	Plaque
Plaque type	Small, hazy	None	Large, clear

* First 2 numbers identify group and individual, last number represents particular specimen of that individual.
† CAFB = Clark Air Force Base.
‡ Q.N.S. = Quantity not sufficient.
§ Plaques produced under Tris buffer overlay only.
‖ Personal communication from Dr. Klaus Hummeler, Children's Hospital, Phila., Pa.
¶ Pool used for identification studies.

serum dilutions and an estimated 100 $TCID_{50}$ inoculum of virus(7). CF tests were performed in 13 x 100 mm test tubes using 0.2 ml of each reagent. Virus for CF antigens was grown in RhMKCC in EBM without serum and routinely extracted once with fluorocarbon. Antisera were diluted 1:5, inactivated for 30 minutes at 60°C, adsorbed with equal volume of 10% RhMKCC suspension for 1 hour at 37°C, then centrifuged for 30 minutes at 2,000 rpm. Undiluted antigen and dilutions of antiserum plus guinea pig complement were incubated at 37°C for 1 hour; sensitized sheep red blood cells were added and the test incubated another hour at 37°C. The endpoint was taken as highest dilution of antiserum which showed less than 50% hemolysis in presence of 2 full units each of complement and of hemolysin. 7. *Animal pathogenicity tests.* Baby mice, 24 hours old or less, received either 0.01 ml of undiluted virus intracerebrally (i.c.) or 0.03 ml subcutaneously (s.c.). Each virus was tested by both routes. Blind passages in mice were made at least twice, using 20% mouse brain and carcass suspensions. Monkeys received 0.5 ml i.c. and some 0.1 ml intraspinally (i.s.); rabbits were given 0.5 ml i.c. and adult guinea pigs, hamsters, and mice 0.1 ml i.c. Seven to 8-day-old embryonated eggs were inoculated with 0.1 ml of undiluted virus into the yolk sac. 8. *Hemagglutination tests.* These were performed according to method of Goldfield *et al.*(8) at 4, 25 and 37°C, with both chicken and human "O" cells. 9. *Desoxycholate test.* This was a modification of the method of Theiler(9). Equal volumes of 1:500 sodium desoxycholate and undiluted virus were incubated 1 hour at 37°C and then titrated in 10-fold dilutions in RhMKCC. Results were compared with control titrations of virus treated in the same manner but without desoxycholate.

Results. 1. *Isolations.* Dates of isolation, results of reisolation attempts and the isolation of additional strains are presented in Table I. 2. *Common characteristics.* The following characteristics were common for the 3 viruses: (1) They produced a cytopathogenic effect (CPE) in RhMKCC similar to that produced by polioviruses. (2) No CPE was noted in HeLa-Gey, HeLa S-3, H. Ep. #2,

TABLE II. Neutralizing Antibody Tests in RhMKCC with ECHO-26, ECHO-27 and Coxsackie B-6 Viruses against Antisera to Known Enteroviruses.

Antisera	Source laboratory	Homologous N.A. titer	Virus — ECHO-26 $TCID_{50}$*	ECHO-26 Titer†	ECHO-27 $TCID_{50}$	ECHO-27 Titer	Coxsackie B-6 $TCID_{50}$	Coxsackie B-6 Titer
ECHO- 1 to 12	Wenner	1,700–65,000‡	32–100	<10	20–50	<10	10	<10
13	Hammon	40,000	50	"	20	"	"	"
14 to 20	Wenner	600–16,000	"	"	"	"	10–32	"
21	Enders	128	100	"	"	"	320	"
22 to 24	Sabin	1,000–10,000	"	"	"	"	"	"
25	Rosen	2,500	"	"	100	"	100	"
26	Hammon	7,500	"	*7,500*	20	"	50	"
27	"	40,000	"	<10	"	*40,000*	"	"
Coxsackie A-7, 9, 11, 13, 15, 18	Syverton	64–1,024	50	"	"	<10	10	"
B-1 to 5	"	128–1,024	"	"	"	"	10–32	"
Idem	Wenner	400–20,000	"	"	"	"	32	"
B-6	Hammon	15,000	100	"	"	"	320	*15,000*
Poliovirus 1, 2, 3	Wenner	>1,000	50	"	"	"	10	<10

* $TCID_{50}$ of virus used in test as determined by control titration.
† Reciprocal of serum dilution calculated to protect 50% of tubes inoculated.
‡ Homologous N.A. titer of ECHO-4 was 1:30 to 1:128; CF tests carried out in addition (see text).

K. B. and primary hamster kidney and cat kidney cell cultures. (3) They were not inactivated by desoxycholate. (4) Chicken and human "O" red blood cells were not agglutinated at 4, 25 and 37°C. (5) The 3 viruses passed gradocol membrane filters of 36 mμ porosity but were retained completely (Coxsackie B-6 and ECHO-27) or almost completely (ECHO-26) by a 28 mμ porosity filter.†† (6) No significant immunologic relationship to any other human enterovirus presently known to propagate in RhMKCC was established by reciprocal testing in NA tests (Tables II, III). Further exclusion of identity with ECHO-4 virus was demonstrated in reciprocal CF testing. (7) A human antiserum known to yield a group reaction in CF tests with adenoviruses did not react with these viruses. (8) Serologic rises and persistence of specific NA's at 1:8 to 1:32 serum dilution were demonstrated for each of the 3 viruses in human sera collected in the Philippines (Table IV).

3. *Specific characteristics.* (a) *Coxsackie B-6 virus (1-51-21)* produced paralysis, death and typical Coxsackie B pathology in suckling mice upon direct passage from RhMKCC. Dr. Klaus Hummeler of Children's Hospital of Philadelphia reports that he has recently isolated 2 strains directly in suckling mice from feces of children with aseptic meningitis (personal communication). These were identified by sera sent to him by this laboratory. Coxsackie B-6 killed embryonated eggs inoculated by the yolk sac in an irregular manner; however, the virus could not be passed serially in eggs. Chick embryo pathogenicity

TABLE III. Neutralizing Antibody Tests in RhMKCC with Antisera to ECHO-26, ECHO-27 and Coxsackie B-6 Viruses against Known Enteroviruses.

Virus	$TCID_{50}$ range	Monkey antisera ECHO-26	ECHO-27	Coxsackie B-6
ECHO-26	20	*7,500*	<5	<10
-27	100	<5	*40,000*	"
Coxsackie B-6	320	"	<5	*15,000*
ECHO-1 to 25	3.2–500	<10	<10	<10
Coxsackie A-7	10	<5	<5	*10**
A-9	50	"	"	<5
B-1	50–500	"	"	"
B-2	100		*20*†	
	200	<5		<5
B-3, 4, 5	16–160	"	<5	<10
Poliovirus 1, 2, 3	50–320	"	"	<5

* 20 units of this serum represent a 1:750 dilution—result with low dilution used not significant for typing.
† 20 units of this serum represent a 1:2,000 dilution—result with low dilution used not significant for typing.

†† Tests kindly performed by Dr. Albert Sabin, Cincinnati, O. and results made available to us.

TABLE IV. Neutralizing Antibody Tests in RhMKCC with Human Sera and Gamma Globulin against ECHO-26, ECHO-27 and Coxsackie B-6 Viruses.

Serum source	Virus 11-3-6 (ECHO-26)	1-36-4 (ECHO-27)	1-51-21 (Coxsackie B-6)
Original host	No serum	<4*–64	No serum
Other:			
With titer rise	<4–8		<4–128†
With persisting titer (1:8 or >) in serial bleedings of individuals possessing antibody	3	2	3
Gamma globulin: 1 lot American	<25	>50	<25
" Japanese	"	"	"

* Reciprocal of serum dilution calculated as 50% endpoint.

† Dr. Klaus Hummeler has obtained serologic rises in 2 patients with aseptic meningitis from whom were isolated agents identified as Coxsackie B-6 with antiserum supplied by this laboratory.

is compatible with results occasionally obtained with other Coxsackie viruses(10). Coxsackie B-6 virus did not produce CPE in several human cell lines, HeLa, K.B. and H. Ep. #2, known to support growth of certain strains of Coxsackie B-1 through 5(10). This characteristic, therefore, differentiates it from the first 5 Coxsackie B viruses. Three of 8 strains of Coxsackie B-6 isolated were neutralized by very low dilutions of Coxsackie B-1 antiserum while 5, including 1-51-21, were not. No antisera prepared against the new strains neutralized Coxsackie B-1 virus. Furthermore, a 1-51-21 CF antigen, which reacted with homologous antiserum at a dilution of 1:128, did not react with antisera to Coxsackie B-1 to 5, or to ECHO viruses 1-9, 11-20, 24-27, to all 3 polioviruses, and to Coxsackie A-9.‡‡

(b) *ECHO-26 virus* (*11-3-6*) produced no clinical illness in mice, hamsters, rabbits, guinea pigs or monkeys by routes previously described. Sections of the cord and brain of the monkey sacrificed 27 days after inoculation, revealed no significant pathology. An interesting characteristic of this virus is that it produces plaques under 1% agar overlay with low $NaHCO_3$ content but not under agar containing high $NaHCO_3$ (See Materials and methods). Under the former conditions very small hazy plaques with ragged edges are formed.

‡‡ A portion of these tests was kindly performed by Dr. Leon Rosen, Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S. and results made available to us.

(c) *ECHO-27 virus* (*1-36-4*) was not pathogenic for suckling baby mice and embryonated eggs. It did, however, produce questionable clinical signs of central nervous system involvement in one monkey inoculated i.c. and i.s. The monkey, which had been irritable and tremulous prior to inoculation, had a single convulsion 4 days after inoculation. The animal survived but was sacrificed on 27th day. Sections of brain and cord revealed no significant pathology except for a mononuclear inflammatory reaction involving a portion of the choroid plexus of the lateral and fourth ventricles. A 1:20 dilution of ECHO-27 antiserum with homologous titer of 1:40,000 neutralized 100 $TCID_{50}$ of Coxsackie B-2 virus (Table III). Such a reaction is not interpreted to indicate identity or even close antigenic relationship. Furthermore, prototype ECHO-27 virus, in addition to being nonpathogenic for suckling baby mice as mentioned previously, does not produce plaques in RhMKCC and does not produce CPE in human cell lines. These 3 characteristics further differentiate it from recognized strains of Coxsackie B-2.

Summary. Herein are described the antigenic specificity and biologic properties of 3 new prototype human enteroviruses, ECHO's 26 and 27 and Coxsackie B-6. All 3 fulfill the necessary criteria descriptive of their respective enterovirus group and have been assigned numbers by the Committee on Enteroviruses. All 3 were isolated from rectal swabs taken from healthy persons living in the Philippines in 1953.

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Perphenazine and Reserpine as Antiemetics for Staphylococcal Enterotoxin.* (25447)

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Vomiting is one of the most characteristic reactions in staphylococcal food poisoning. The only laboratory procedure now available for detection of the etiological agent, staphylococcal enterotoxin, is the provocation of emesis in monkeys or cats following suitable challenge(1). Recently a number of antiemetics have been reported effective against various types of emetic agents or which control vomiting in various disease states. No specific study seems to have been made on use of these agents as antiemetics against staphylococcal enterotoxin induced emesis. The present communication reports on effectiveness of 2 tranquilizing drugs in inhibiting emesis in monkeys fed enterotoxin. Antiemetics effective in dogs against one or more of the emetics whose site of action is the chemoreceptor trigger zone(2) were tested: chlorpromazine(3,4), perphenazine(5,6), and cyclizine lactate(7). Reserpine was included, although reports on its ability to protect against apomorphine induced vomiting in dogs are contradictory(7,8). Non-specific protection by depression of the medullary reticular emetic center was evaluated by determining change in sensitivity of monkeys to orally administered copper sulfate which evokes vomiting by peripheral action on the gastro-intestinal tract(9).

Materials and methods. Monkey feeding tests were essentially that previously described(10). *Macaca mulatta* were selected into groups of approximately equal sensitivities to enterotoxin. The paired groups whose response to the toxin was to be compared were alternated, wherever possible, as control and drug treated groups to compensate for variations in reactivities to enterotoxin. Complete control of this variable was impossible due to development of tolerance to the emetic. Each drug was tested in different sets of animals. Enterotoxins were partially purified preparations derived from S-6 and 196E strains of *Staphylococcus* and are antigenically different. Dosage was increased with each feeding of same animals to overcome development of immunity. For intravenous challenge an S-6 enterotoxin of high purity (90% purity) was used. Monkeys were injected intramuscularly with perphenazine (Trilafon) immediately before and with chlorpromazine (Thorazine) and cyclizine lactate (Marezine) 30 min before oral administration of enterotoxin. Since these animals seldom vomit within 1 hr of feeding enterotoxin, seda-

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