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Received December 18, 1958. P.S.E.B.M., 1959, v100

## Echo Virus Type 13. I. Isolation and Characteristics.*† (24650)

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The first 13 ECHO prototype viruses were described in Dec. 1955 by the ECHO Virus Committee(1). Prototype ECHO-13, Hamphill 2-188-20‡, had been isolated in 1954(2) from rectal swab taken Nov. 30, 1953 from a healthy 21-month-old son of American military family stationed at Clark Air Force Base in the Philippines. During 8 weeks preceding collection of above specimen, this infant yielded poliovirus type III and ECHO type 1. Antibody responses occurred to all 3 viruses. Prior to acceptance as prototype ECHO-13, 2-188-20 virus was reciprocally tested against relatively low-titered antisera for the 12 other ECHO prototype viruses by our laboratory and those of Melnick and Sabin. No cross neutralization occurred. The above tests were conducted with 4th passage of 2-188-20 virus in rhesus kidney cell cultures; the $TCID_{50}$ titer was approximately $10^{-4.0}$/0.1 ml. After 3 additional passages the titer rose to $10^{-7.0}$. Aliquots of passage 7 were sent to Dr. Herbert A. Wenner and Microbiological Associates for preparation of hyperimmune monkey and rabbit serum respectively. Composite neutralization tests with these antisera have been reported(3). These data indicated that there was a cross relationship between ECHO viruses 1, 8 and 13 or that passage 7 of 2-188-20 contained either ECHO-1 or 8 and ECHO-13 viruses. A thorough study was therefore undertaken and pure ECHO-13 prototype strain sought. The findings are reported herein. Epidemiologic and clinical studies on ECHO-13 infections are to be published separately.

*Materials and methods.* Monolayer cell cultures of trypsinized rhesus monkey kidney (MKCC) were used in isolation, neutralization tests, preparation of virus pools and plaque studies. Virus pools were grown in medium 199 without serum. Neutralization tests were performed in 0.5% lactalbumin hydrolysate in Earle's balanced salt solution containing 0.5% calf serum. All media contained 400 units of penicillin, 0.2 mg of streptomycin, 20 units of polymyxin and 25 units of mycostatin/ml. Neutralizing antibody (NA) and complement fixation (CF) tests were performed as described previously(4). Results of NA tests were expressed as reciprocal of serum dilution calculated by Reed-Muench method(5) to protect 50% of inoculated cell cultures. Monkeys were hyperimmunized by repeated intramuscular injection of emulsion containing equal volumes of Ar-

* Aided by grant from Nat. Found. for Infantile Paralysis. This work was done under sponsorship of Commission on Viral Infections, Armed Forces Epidemiological Board, and supported in part by Offices of Surgeon General, Dept. of Army and Air Force.

† Technical assistance of Della M. Whitaker is gratefully acknowledged.

‡ 2-188 is number given this child in field study and 20 is number of specimen from which virus was isolated.

TABLE I. Cross Neutralization Tests with 2-188-20 Derivatives, Strain 11-4-1 and ECHO Types 1 and 8.

| | Virus and $TCID_{50}$ | | | | | |
|---|---|---|---|---|---|---|
| Monkey antiserum | ECHO-1 600–32* | ECHO-8 200–64 | 2-188 (1 + 13) 500–20 | 2-188 (As-1) 320–50 | 2-188 (R pl) 200–32 | 11-4 (pl) 1,000–100 |
| ECHO-1 (Wenner) | *3,200–* *27,000* | 320–2,000 | <8–10,000 | <10 | <10 | <10 |
| ECHO-8 (Wenner) | 125–1,300 | *5,500–* *20,000* | <8–630 | " | " | " |
| ECHO-"13" (2-188 (1 + 13))† (Wenner) | 50–2,000 | 10–160 | *640–* *20,000* | 1,280–2,560 | 640 | 40 |
| 2-188 (As-1)‡ | <10 | <10 | <10 | *640–* *2,560* | " | 320 |
| 2-188 (R pl)§ | " | " | " | 20,000–40,000 | *20,000–* *>40,000* | 5,120–40,000 |
| 11-4 (pl)§ | " | " | " | 20,000–>40,000 | 10,000–>40,000 | *10,000–* *>40,000* |

* $TCID_{50}$ = Max and min as determined by control titrations of several tests, yielding range of serum titers shown below as reciprocals of dilutions giving 50% neutralization.

† ECHO-"13" (2-188 (1 + 13)) = Original ECHO-13, passage 7, considered a mixture of ECHO-1 and ECHO-13.

‡ 2-188 (As-1) = 2-188 (1 + 13) after 6 passages in 1:5 dilution of Wenner ECHO-1 antiserum.

§ 2-188 (R pl) = Virus pool from plaque (pl) of reisolation (R) of 2-188-20.
11-4 (pl) = Virus pool from plaque of 11-4-1 strain of ECHO-13.

lacel-Bayol-F adjuvant and unconcentrated or concentrated virus-containing cell culture fluids. Concentrated virus suspensions, when used, were lyophilized and reconstituted to 1/20 of original volume. Prior to lyophilization they were extracted with fluorocarbon by the method of Manson *et al.*(6); these procedures apparently did not alter viral antigenicity. Without concentration, even after repeated injections, a highly potent immune serum was not readily obtained. The plaque technique of Hsiung and Melnick(7) was employed.

*Purification of ECHO-13 virus by neutralization with appropriate antibody.* Undiluted 2-188-20 virus (hereinafter referred to as 2-188(1 + 13) to denote possible mixture of ECHO-1 and 13) was passed 6 times in MKCC in the presence of 1:4 dilution of Wenner ECHO-1 monkey antiserum. The virus thus obtained, referred to as 2-188(As-1) to denote passage with ECHO-1 antiserum, was not neutralized by ECHO-1 and 8 antisera; it was, however, neutralized by Wenner ECHO-13 antiserum (Table I). Similar results (not shown in Table I) were obtained with a virus, 2-188 (As-8), separated in the same manner using ECHO-8 antiserum. Both 2-188(As-1) and (As-8) viruses were then passed 10 times in absence of ECHO-1 or -8 antisera and retested; there was no change in above neutralization pattern.

*Results* of cross-neutralization tests with an antiserum prepared to 2-188(As-1) virus are shown in Table I; the results with 2-188(As-8) antiserum (omitted from Table) were essentially identical. A 1:10 dilution of 2-188-(As-1) antiserum, with a homologous titer of 1:640 to 1:2,560, did not neutralize approximately 100 $TCID_{50}$ of ECHO viruses 1, 8 and 2-188 (1 + 13). By using the 2-188(As-1) antiserum to neutralize the "ECHO-13" component a second virus was found; it was clearly identified in NA tests as an ECHO-1 virus. The ECHO-1 component was also obtained from 2-188(1 + 13) by terminal-dilution and by plaque isolation.

*Establishment of pure ECHO-13 virus. Reisolation of 2-188-20 virus.* Since 2-188 (As-1) and (As-8) strains were "derived" by use of antisera, it was necessary to establish that ECHO-13 occurred in nature as a distinct virus. One approach was to reisolate 2-188-20 from the original rectal swab eluate which had been stored at –20° to –30°C for nearly 4 years after swab had been collected.

A virus was isolated and given 10 passages in MKCC. The 10th passage was tested against appropriate battery of antisera and was neutralized only by 2-188(As-1). 2-188(As-8) and Wenner ECHO-13 antisera. Two monkeys were hyperimmunized with a MKCC pool of a plaque isolate, 2-188(R pl), from the reisolated 2-188-20 virus. The results obtained with this antiserum were similar to those obtained with 2-188(As-1) and 2-188 (As-8) antisera (Table I).

*Isolation of other strains of ECHO-13 and selection of a new prototype strain, Del Carmen* 11-4-1. When we undertook this study concerning relationship of ECHO-1, 8 and 13 viruses, we had identified 3 other ECHO-13 strains(2) using a low-titered rabbit antiserum prepared to passage 4 of the original 2-188-20 virus. However, these strains were neutralized only by a 1:20 or 1:40 dilution of Wenner ECHO-13 antiserum. The Del Carmen 11-4-1 strain (referred to subsequently as 11-4) had been isolated in April, 1955 from rectal swab obtained August 6, 1953 from a healthy 4-month-old female Filipino. An apparently identical virus was isolated from throat swab collected at the same time. A virus pool of a plaque isolate, 11-4(pl), was prepared from the fecal isolate and 2 monkeys hyperimmunized. This antiserum neutralized 2-188(As-1) virus to essentially the same titer as that of the homologous agent (Table I); it did not neutralize ECHO-1 and 8 viruses.

The 2-188(R pl) and 11-4(pl) antisera were prepared simultaneously, using the same 5-month immunization schedule. The final 2 virus injections, which were given essentially 2 and 4 weeks prior to final bleeding, contained 20-fold concentrated virus. Homologous titers of resultant antisera varied from 1:10,000 to >1:40,000 (Table I). Testing of the other pure ECHO-13 strains (only 2-188(As-1) shown in Table) revealed essentially equivalent titers with 11-4(pl) serum. However, 2-188(R pl) serum titers were about 4-fold lower against 11-4(pl) virus and others than against the homologous strain. These differences were readily confirmed. Similarly, the ECHO-13 (Wenner) antiserum prepared from 2-188(1 + 13), although neutralizing approximately 100 $TCID_{50}$ of 2-188 (R pl) and 2-188(As-1) in a 1:640 to a 1:2,560 dilution, only neutralized a similar amount of 11-4(pl) virus in a 1:40 dilution (Table I). As pointed out above, 2 other strains of ECHO-13 were also neutralized by Wenner ECHO-13 antiserum but only at this same low dilution.

Both purified ECHO-13 viruses and their respective antisera were then tested reciprocally with all other enteroviruses known to propagate in MKCC. No significant heterologous neutralization occurred in either direction.

*General characteristics of ECHO-13 virus.* ECHO-13 strains, 2-188(R pl) and 11-4(pl) were non-pathogenic for 1-day-old suckling mice even after serial blind passage. Likewise, they were not pathogenic for 7-day-old chick embryos inoculated by yolk sac route. Both strains failed to produce apparent illness in several rhesus monkeys inoculated by either intracerebral or intramuscular routes; similar results were obtained when 11-4(pl) was inoculated intracerebrally and intraspinally into 2 monkeys. These latter 2 monkeys were sacrificed after 21 days. A chronic meningoencephalitis was observed in sections of brain stem and mid-brain but not in the cord. These findings will be reported later.

Both strains produce small and irregular plaques in MKCC bottle preparations. Cytopathogenic effect (CPE) produced in MKCC roller tubes cannot be differentiated morphologically from that produced by polioviruses and most other enteroviruses. On primary isolation of strain 11-4, CPE first appeared on 7th day. After 2 passages CPE became quite marked by 4th day. Strain 11-4(pl), at 8th passage, produced initial CPE in 24 to 48 hours and proceeded to completion by 5th day with a titer of $10^{-6.7}/0.1$ ml. Strain 2-188(R pl) behaved in similar manner. Replication of ECHO-13 virus with CPE has been observed in H.Ep. #2 and Detroit 504 Fb-L cell lines(8). Variable results have been obtained with HeLa cells, while primary cell cultures of hamster and gerbil kidney§ and monkey heart cells(9) have given negative results.

§ Tests with gerbil kidney cells were performed by G. M. Hodges and M. B. Dobkin of Dr. F. S. Cheever's laboratory in this Department.

Both 2-188(R pl) and 11-4(pl) strains grown in MKCC were tested for ability to hemagglutinate chick and human group O red blood cells at 4°C, 25°C and 37°C. Chick cells were not agglutinated, but human cells were agglutinated equally well at 4°C and 25°C, but not at 37°C. Strain 2-188 (R pl) titered 1:64 while strain 11-4(pl) titered 1:8.

Infected MKCC fluids can be used in CF, NA and hemagglutination inhibition (HAI) tests for viral identification with hyperimmune sera and for detection of antibodies in human sera.

*Discussion.* It has been demonstrated that passage of Hamphill 2-188-20, which was originally distributed as ECHO-13 prototype virus, contained a mixture of ECHO-1 and 13 viruses. Whether the original rectal swab contained both viruses could not be established. After approximately 4 years of storage the rectal swab suspension from which ECHO-13 was originally isolated, was retested; only ECHO-13 virus could be reisolated. The child from whom the isolation was made had been infected with ECHO-1 just prior to or concurrently with ECHO-13 infection as demonstrated by ECHO-1 virus isolation and serological response. However, when the original ECHO-13 isolation was effected many isolations of ECHO-1 were also being made and were being handled extensively in the laboratory. If both viruses were contained in the rectal swab originally, or even if ECHO-1 was added inadvertently during laboratory passage, in retrospect it appears that in early passages only a small amount of ECHO-1 was present. Only in later passages as ECHO-1 began to outgrow ECHO-13 was its presence detectable. Further passages increased ECHO-1 and decreased ECHO-13. The fact that ECHO-1 propagates more rapidly and generally attains a higher titer than does ECHO-13 supports these contentions.

Presence of ECHO-13 was demonstrated with certainty only when the ECHO-1 component was suppressed with neutralizing antibody. The only objection to separation of viruses by this latter method is hypothetical, in that antibody in some unknown manner might alter the antigenic properties of the propagating virus, as seen *in vivo* in mice actively immunized with influenza A virus (10). Although this possibility was not investigated extensively, the antigenic properties of "antiserum-derived" ECHO-13 viruses, 2-188(As-1) and (As-8), were not observed to differ from subsequently reisolated 2-188 (R pl) strain.

Two plaque purified strains of ECHO-13, 2-188(R pl) and 11-4(pl), have been studied to determine which would be the better prototype virus. Both fulfill requirements of an ECHO virus as described by ECHO Virus Committee(3). Their antigenic potentials when used to hyperimmunize monkeys are essentially equal. However, results with test bleeding from each of 2 monkeys immunized with each virus taken every 2 or 3 weeks during immunization, suggested that 11-4(pl) antisera were broader than 2-188(R pl) antisera. Even after the 2-188(R pl) monkeys were given concentrated virus, their antisera still neutralized other ECHO-13 strains to a slightly lesser degree than the homologous strain. It should be mentioned parenthetically that perhaps this immunization technic should be employed to prepare hyperimmune antisera to those enteroviruses, such as Coxsackie B's, which exhibit considerable variation in neutralization capacities(11). Because of apparently broader antigenic pattern of 11-4(pl) and the fact that it propagates in cell culture more rapidly and generally with a higher infectivity titer, $10^{-6.7}$ compared to $10^{-5.5}$ for 2-188(R pl), 11-4(pl) was recommended to and accepted by the Enterovirus Committee as the new ECHO-13 prototype. On the basis of our total work to date there is no convincing evidence that either strain serves to demonstrate presence of antibody in an unknown serum better than the other. However, since 11-4(pl) has a higher infectivity titer and, therefore, can be used in higher dilution, it should serve as the virus of choice for ECHO-13 NA tests.

Although no grouping of ECHO viruses has been accepted by the Enterovirus Committee, ECHO-13, as determined by plaque morphology, falls into the proposed group A of Hsiung and Melnick which contains types 1, 2, 3, 4, 5, 6, 6', 9, 11, 13 and 14(12). Fur-

thermore, ability of ECHO-13 to hemagglutinate human group O erythrocytes at 4°C and 25°C, but not at 37°C, is characteristic of ECHO types 3, 6 and 11(13); as noted above these latter 3 virus types are included in Hsiung and Melnick's proposed group A.

*Summary.* 1) The originally distributed ECHO-13 prototype virus, Hamphill 2-188-20 passage 7, is a mixture of ECHO virus types 1 and 13. ECHO-1 was found in higher titer than ECHO-13 during passage and only the former was isolated at terminal dilution. It was necessary to neutralize ECHO-1 virus with antiserum to demonstrate the presence of ECHO-13 in this mixture and this apparently did not alter its antigenic properties. ECHO-13 virus was successfully reisolated in pure form from the original rectal swab eluate after 4 years storage. 2) Plaque purified strains of reisolated 2-188-20 virus and a Del Carmen 11-4-1 virus isolate were examined as possible ECHO-13 prototypes. The plaque purified 11-4-1 strain produced a broader spectrum antiserum in monkeys in early test bleedings and produced a higher infectivity titer in MKCC. It has been accepted as prototype ECHO-13 by the Committee on Enteroviruses. 3) The biologic properties of ECHO-13 virus in certain animals, chick embryos, cell culture and cell culture fluids are described. A clinically inapparent meningoencephalitis occurred in inoculated monkeys.

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Received December 19, 1958. P.S.E.B.M., 1959, v100.

## Identification of a Prolonged Post-synaptic Potential of Cerebral Cortex.* (24651)

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Our prior observations upon cerebral cortex using d.c. recording(1,2) indicate that a long-lasting surface negativity (150-300 msec. duration) develops in the wake of the evoked dendritic potential of negative polarity described by others(3,4). Chang(4) likewise observed that a negative wave could follow the dendritic potential evoked by a single stimulus. Following one surface stimulus the slow negativity we are to describe was observed to be partially submerged by a simultaneously occurring slow positivity (positive after-effect), the signs of the 2 potentials giving the appearance of summing algebraically. However, with serial stimulation (6-20/sec.) the slow positivity disappears uncovering the slow negativity which then increases in amplitude through summation. With such serial stimulation the slow negative responses to the successive shocks also fuse to provide a smooth d.c. change which can outlast a one sec. stimulus

* Aided by grant of U.S.P.H.S. and the Allen P. and Josephine B. Green Fn.