Isolation of Two Enteroviruses, Possible "Prime" Strains of Coxsackie A Type 17 Virus.* (32047)

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During an epidemiological study of viral infection in a semi-closed community (Detention Home of the Juvenile Court of Allegheny County) in Pittsburgh, Pa., carried out during 1960-61, 201 enteroviruses, which could not be identified as members of any of the recognized human enteroviruses, were isolated in human amnion (FL line) cells. The 201 viruses fell into two distinct groups whose prototypes were denoted as Hu 504 and Hu 659, respectively. This paper describes the isolation, characterization, and identification of these viruses as possible Coxsackie A Type 17 "prime" strains.

Materials and general methods. The source of prototype viruses and immune sera, the assay of viruses, the technique of neutralization tests, preparation of viruses for electron microscopy, method of preparation of human amnion primary and FL line cell cultures, and primary rhesus monkey kidney cell cultures have been described(1). In addition, the Bryant(2) virus was received from Dr. G. D. Hsiung and the Caldwell virus and immune serum (3) from Dr. P. S. Kamitsuka. Albino mice were obtained from the Charles River Mouse Farms, Inc., North Wilmington, Mass.

Results. Isolation of the viruses. The viruses were all isolated from rectal swabs taken from healthy children. These children were swabbed at the time of admission to the Home and at weekly intervals thereafter.

Identification of the viruses. None of the 201 viruses was pathogenic for rhesus monkey kidney primary cell cultures. Attempts to type out these viruses with the standard reference immune sera prepared in Dr. J. T. Syverton's laboratory (for the National Foundation for Infantile Paralysis, Inc.) in monkeys against Coxsackie A Type 1-21 viruses were without success. Negative results were also obtained in neutralization tests carried out with immune sera prepared against poliovirus Types 1-3, Coxsackievirus B Types 1-6, echovirus Types 1-32, Pett(4), HSO(5), H 136(6), Hu 2080,‡ Hu 39(1), and Bryant(2) viruses. Immune sera prepared against several members of both the Hu 502 and the Hu 659 groups after purification by 5 terminal dilution passages failed to neutralize any of the human enteroviruses listed above. The results of the cross neutralization tests with the representative prototype viruses of the two groups, Hu 504 and Hu 659, are summarized in Table I. The complete cross neutralization tests used in typing out the Hu 504 and Hu 659 viruses were repeated independently with several other members of each group and the results confirmed.

The Hu 504 and Hu 659 viruses were submitted as prototype virus candidates to the Commission on Enteroviruses in 1963. As a "one-way" neutralization of these viruses was noted with Coxsackievirus A, Type 17§ the question of the relatedness of the Hu 504 and Hu 659 viruses to each other and to the Coxsackievirus A, Type 17 was re-examined using the "old" (Syverton) and "new"(8) standard reference sera. As seen from the data presented in Table II, these viruses were indeed neutralized by the "new" but not by the "old" reference serum, and the similar low titer "one-way" type neutralization was noted between the Hu 504 and Hu 659 viruses. These data show that these two viruses share common antigens, but are sufficiently distinct to warrant their being classified as "prime" strains of Coxsackievirus A, Type 17.

General properties of the viruses. Both viruses exhibited the characteristics found

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‡ Unidentified enterovirus isolated in our laboratory.

§ Melnick, J. L., personal communication.

typical of this group of human enteroviruses. Both were found to be resistant to ether treatment. Magnesium ions in 1 Mole per liter concentration fully stabilized both viruses against inactivation by heat at 50°C for 120 minutes.

TABLE I. Cross-Neutralization Tests Conducted in Identification of Hu 504 and Hu 659 Viruses.

1. Prototype immune sera	Viruses Hu 504	Hu 659
Coxsackie A type 1-21, B type 1-6*	Negative	Negative
Coxsackie A type 22, 24†	Not done	Not done
Poliomyelitis type 1-3	Negative	Negative
ECHO type 1-32‡	"	"
Hu 2080, H 136	"	"
HSO, Pett, Hu 39	"	"

2. Prototype viruses	Immune sera Hu 504	Hu 659
Coxsackie A type 2, 3, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 24§	Negative	Negative
Coxsackie A type 1, 4, 5, 6, 19, 22‖	Not done	Not done
Poliomyelitis type 1-3	Negative	Negative
ECHO type 1-32	"	"
Hu 2080, H 136	"	"
HSO, Pett, Hu 39, Bryant¶	"	"

* Dilutions used were, in case of rabbit immune serum, 1:15, and in monkey serum 1:25. National Foundation for Infantile Paralysis, Inc., standard reference Coxsackie A sera.

† Immune serum was not available for testing.

‡ In the case of ECHO 4, 1:5 diluted immune serum was used.

§ Coxsackie A type 2, 3, 8, and 12 viruses, adapted to primary human amnion cells failed to grow in FL human amnion line cells used in this laboratory. In the case of these 4 viruses, the cross-neutralization tests were done in primary human amnion cell cultures.

‖ Adaptation of these strains to primary human amnion cells was unsuccessful.

¶ Attempts to prepare an immune serum against the Bryant virus have not been successful.

TABLE II. Cross-Neutralization Tests Between the Coxsackie A, Type 17, Hu 504 and Hu 659 Viruses. Monkey immune sera reciprocal of neutralization end point.

Virus*	Coxsackie A type 17†	Coxsackie A type 17‡	Hu 504	Hu 659
Coxsackie A type 17§	ND	ND	<10	<10
Hu 504	<25	160	960	30
Hu 659	<25	320	<10	640

* Approximately 100 $TCID_{50}$ virus used.

† National Foundation for Infantile Paralysis, Inc., reference serum (Dr. Syverton).

‡ Nat. Inst. Health reference serum (Dr. Wenner).

§ Primary human amnion cells adapted strain (Dr. Wenner).

ND = Not done.

Antigenic homogeneity of the viruses isolated within the two groups. The Hu 504 virus was found to be identical in nearly every respect with the other 25 members of this group. In the case of the Hu 659 group, where 175 viruses were isolated, 6 strains, each isolated from a different child at the time of admission, were studied carefully. The biological behavior of these 6 viruses was very similar in FL cell cultures. Differences in the degree of cross-neutralization reaction were noted in the quantitative neutralization test used in typing out the isolated viruses (Table III). The investigation of antigenic homogeneity was greatly hindered by the difficulty of preparing potent immune serum against the selected viruses, even using purified and concentrated virus as antigen. Even in the case of the prototype virus (Hu 659) individual animals immunized against the agent under standard conditions showed varying antibody titers which were low for the most part.

Cytopathogenic effect of the viruses. The CPE observed in infected FL cells (Fig. 1) was indentical with that caused by the other Coxsackie A viruses. The characteristic eosinophilic cytoplasmic inclusion appeared about 7 hours after infection(9). The virus infection did not appear to interfere with the

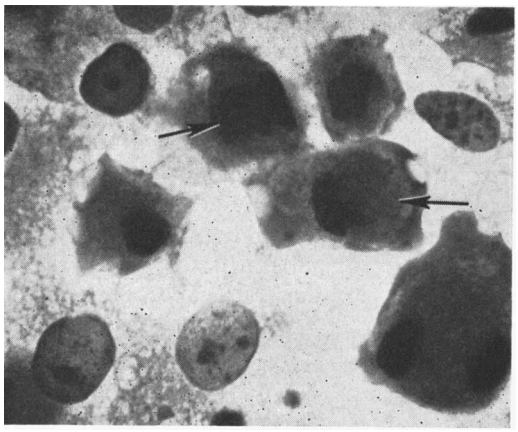

FIG. 1. Human amnion (FL line) cells 12 hours after infection with the Hu 504 virus. Hematoxylin-eosin stained preparation. Magnification × 720. Note the cytoplasmic eosinophilic inclusions.

TABLE III. Cross-Neutralization Between Some Viruses of the Hu 659 Group of Viruses.* Monkey immune sera.

Virus	$TCID_{50}$ used	Hu 659	Hu 843	Hu 1127	Hu 1174
Hu 659	175	640†	640	30	<10
Hu 843	175	640	1280	240	<10
Hu 1127	175	960	1280	640	<10
Hu 1128	300	320	480	240	<10
Hu 1174	175	240	160	<10	240
Hu 1486	175	240	40	20	<10

* The viruses used in this experiment were purified by at least 4 terminal dilutions.
† Reciprocal of neutralization endpoint.

mitotic activity of the nucleus. Cells showing the presence of an eosinophilic cytoplasmic mass, indicating an advanced stage of infection and of virus synthesis in the cytoplasm, were seen in stained preparations in different stages of mitosis. The same was also found in electron micrographs (Fig. 2) of sectioned, infected cells which showed identical morphological changes with those of other enterovirus infected cells. Both viruses multiplied and caused CPE in human amnion cells (FL, AV_3 lines), primary human amnion, HeLa, KB, and primary human kidney culture cells. No CPE was noted when rhesus monkey kidney, squirrel monkey kidney, rabbit kidney, or embryonic mouse and chicken fibroblast cells were inoculated with these viruses.

Pathogenicity for suckling mice. Newborn suckling mice showed no signs of illness when injected with high titer pools of two members (Hu 504 and Hu 2220) of the Hu 504 group

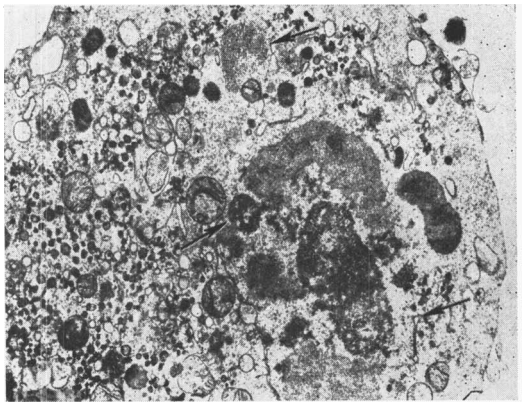

FIG. 2. Electron micrograph of FL cell 8 hours after infection with Hu 659 virus. Note the nucleus in mitosis as evidenced by the chromosome formation, dissolution of the nuclear membrane, and the presence of crystalline masses of virus in the cytoplasm. × 6,667.

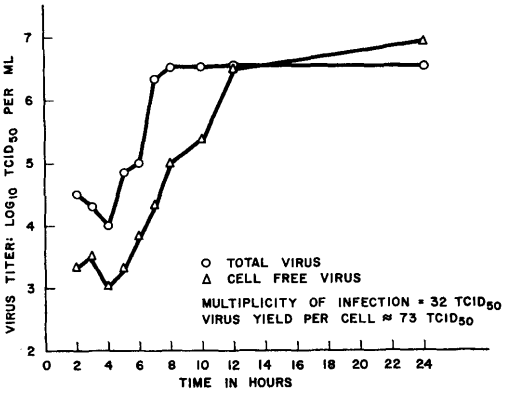

FIG. 3. Multiplication of Hu 504 virus in human amnion (FL line) cells at 37°C.

of viruses by the intracerebral, intraperitoneal or subcutaneous routes. At autopsy an occasional animal showed a few widely scattered microscopic foci of hyaline degeneration and accompanying mild inflammatory reaction in sections of striated muscle stained with hematoxylin and eosin. In contrast, newborn mice injected with Hu 659 virus by various routes occasionally developed paralysis of the extremities, followed in some instances by death. Hematoxylin and eosin stained sections of striated muscles showed lesions (widespread hyalin degeneration and marked inflammatory reaction) typical of Coxsackie Type A infection(10).

Plaque formation. The viruses Hu 504 and Hu 659 formed very small, round, hazy plaques on FL monolayer cell cultures, which became visible on the 4th or 5th day.

Multiplication of the viruses. One step growth curve experiments in FL cells with Hu 504 and Hu 659 revealed growth characteristics identical with the other enteroviruses (Fig. 3). The optimum bicarbonate concentration of the Eagle's medium (with 2%

TABLE IV. Effect of Inhibitors on Multiplication of Hu 504 and Hu 659 Viruses in FL Cells at 37°C in the Standard Maintenance Medium.

Inhibitor	Concentration (μg/ml)	Relative growth* Viruses Hu 504	Hu 659
Guanidine	100	<.01%	.01%
HBB†	100	6.0 %	100.0 %‡
HBB	200	<.01%	.1 %

* Expressed as percentage of titer ($TCID_{50}$) obtained in the inhibitor-free medium (control medium).

† 2-(α-hydroxybenzyl)-benzimidazole.

‡ The extent of CPE was less severe than in the control tubes.

added bovine serum) for growth of these agents was found to be at least 0.69 per liter. Both viruses grew poorly at 0.30 g/liter concentration. The rotation of cell culture tubes did not influence the final titer reached. Both prototype viruses showed maximum multiplication in the temperature range of 35°-37°C. At lower temperatures multiplication fell off rapidly, particularly in the case of Hu 659 which produced less CPE. No multiplication of either virus was noted at temperatures below 30°C or above 38°C, even though the cell cultures were apparently unaffected by these temperatures.

Effect of inhibitors. Guanidine in a concentration of 100 μg/ml completely inhibited the multiplication of both prototype viruses, as in the case of other enteroviruses(11). 2-(α-hydroxybenzyl)-benzimidazole (HBB.) in a concentration of 100 μg/ml partially inhibited the multiplication of the Hu 504 virus. At double the concentration both prototypes showed marked inhibition of multiplication but again Hu 504 was more sensitive (Table IV).

Morphology of the virus particles. The average size of the uniform, round particles was found to be, in the case of Hu 504, 32.6 $\pm$ 8.9 mμ; and in that of Hu 659, 30.5 $\pm$ 4.5 mμ when platinum shadowed individual particles of air dried preparations were measured with the electron microscope.

Hemagglutination. The hemagglutinating activity of the virus was tested by the method of Goldfield(12) at 37°C, room, and 4°C temperatures. No hemagglutination was observed with the Hu 504 virus using chick, rhesus monkey, rabbit, guinea pig, rat, sheep, human type O, bovine, and mouse erythrocytes. Another member of this group virus agglutinated rabbit red blood cells in low titer: 1:16 at 4°C and 1:4 at room temperature; no agglutination was observed at 37°C. A low (1:16) titer was found with the Hu 659 virus and mouse red blood cells tested at 37°C. No reaction was noted with the RBC of other species.

Stability of the prototype viruses. Heat inactivation experiments with Hu 504 and Hu 659 viruses yielded similar results in that both were somewhat more sensitive to heat inactivation than other enteroviruses. They were resistant to the action of hydrogen and hydroxyl ions within a pH range of 1.5 to 11.0. Both agents were readily inactivated by ultraviolet light (source: General Electric germicide lamp delivering 32 ergs/sq cm with an energy maximum at 2560 A wave length) with a single hit inactivation curve (Fig. 4). The rate of inactivation was approximately one log $TCID_{50}$/minute in both cases.

Prevalence of the viruses in Western Pennsylvania. Virus Hu 504 was isolated from a child on admission on one occasion in 1960. In 1961, the virus was recovered from another child on admission and subsequently on 24 occasions from 10 children who had been present in the Home for varying periods of time, indicating that one child was the source of infection of most or all of the others. In the sera of normal adults resident in Western Pennsylvania, no antibody against the Hu

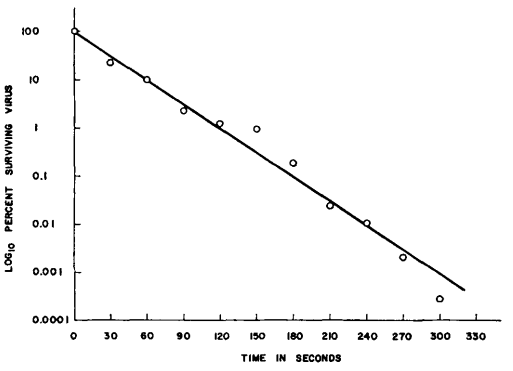

FIG. 4. Inactivation of Hu 504 virus by ultraviolet light.

504 virus was found in the 343 sera drawn in 1961 or in the 228 sera drawn in 1963.

Hu 659 virus was recovered from 6 children at the time of admission and in the case of numerous other children from weekly screening specimens before there had been significant opportunity to acquire an infection within the Home. Thus it is possible that some of the negatives admission specimens represented failures in technique. Of the 347 sera drawn in 1961 none showed antibody, and of the 228 sera drawn in 1963 only one, and that in low titer (1:16).

Discussion. The isolation and characterization of two distinct groups of enteroviruses (prototype strains Hu 504 and Hu 659) from healthy children from Pittsburgh, Pa. in 1960-61 are reported. Attempts to identify these viruses in cross neutralization tests using the standard reference sera available at that time were unsuccessful. These viruses, when submitted to the Commission on Enteroviruses as possible prototype candidate strains, were found to be related by a "one-way" neutralization to the Coxsackie A, Type 17 virus when tested against the "new" standard reference serum prepared by Dr. H. A. Wenner for the National Institutes of Health. Retesting of these agents against the "old" standard reference serum gave negative results once more. Further investigation is required to clarify the reasons for this lack of cross neutralization with the "old" reference serum as well as for the "one way" neutralization pattern with the "new" reference serum.

The two prototype agents (Hu 504 and Hu 659) show sufficient differences in antigenic composition and mouse pathogenicity to justify their tentative classification as "prime strains" of Coxsackie A, Type 17, group. Murphy(13) isolated 15 viral agents from children suffering from acute diarrhea in Australia and showed that all were immunologically identical with Hu 659, but apparently unrelated to other enteroviruses, including Hu 504. Although his agent ("Mill" virus, which is non-pathogenic for suckling mice) and Hu 504 and Hu 659 apparently fall in the heterogeneous Coxsackie A Type 17 group, their final classification must await decision by the Enterovirus Commission.

In view of the many isolations of both prototype viruses (Hu 504 and Hu 659) the absence of antibodies in the sera of the population of Western Pennsylvania was rather unexpected. It is in agreement with the findings of low neutralization antibody titers in the sera of children from whom the Mill virus was isolated by Murphy. He was also unable to find the presence of antibodies against this virus in the sera taken from other children. No antibodies were found by him in the sample of gamma globulin of presumably Australian origin. We were also unable to find antibodies against the Hu 504 and Hu 659 virus in gamma globulin of American manufacture. There was no opportunity to obtain sera from children showing viral infection, and so the appearance of antibodies in relation to the virus infection could not be studied. No relationship of these 2 viruses to human illness could be established.

Summary. Two enterovirus-like agents were isolated in human amnion (FL line) cells from rectal swabs taken from healthy children during a longterm epidemiological study of viral infection in a semi-closed community during 1960-61. Of the first type (Hu 504), 26 representatives were isolated, of the second (Hu 659), 175. Both viruses were shown to exhibit the properties typical of human Coxsackie A type enteroviruses. They were shown to exhibit the properties typical of human Coxsackie A type enteroviruses. They were shown to be prime strains of Coxsackie A Type 17 virus, with no apparent (Hu 504) or erratic (Hu 659) pathogenicity for suckling mice. Serological studies in a normal population group from the Pittsburgh area failed to show the presence of antibodies against either of these viruses. No conclusion can be drawn concerning the pathogenicity of these viruses for humans. The Mill virus, isolated by Murphy in Australia in 1963 from children suffering with acute diarrhea, appears to be identical with the Hu 659 virus.

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1. Abraham, A., Proc. Soc. Exp. Biol. & Med., 1963, v112, 743.
2. Hsiung, G. D., Ann. N. Y. Acad. Sci., 1962, v101, 413.
3. Wenner, H. A., ibid., 1962, v101, 398.
4. Kasel, J. A., Cramblett, H. G., Utz, J. P., ibid., 1958, v99, 703.
5. Matumoto, M., Mutai, M., Aoyama, Y., Oikawa, T., Japanese J. Exp. Biol. & Med., 1960, v30, 213.
6. Abraham, A., Cheever, F. S., Proc. Soc. Exp. Biol. & Med., 1963, v113, 300.
7. Yohn, D. S., Hammon, W. McD., ibid., 1960, v105, 55.
8. Kamitsuka, P. S., Lou, T. Y., Fabiyi, A., Wenner, H. A., Am. J. Epid., 1965, v82, 27.
9. Barsky, G., Virology, 1962, v18, 159.
10. Gifford, R., Dalldorf, G., Am. J. Path., 1957, v27, 1047.
11. Picornavirus Group, International Enterovirus Study Group, Virology, 1963, v19, 114.
12. Goldfield, M., Shrihongse, S., Fox, J. F., Proc. Soc. Exp. Biol. & Med., 1957, v96, 788.
13. Murphy, A. M., J. Hyg. Camb., 1964, v62, 425.

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Studies of Insulin Effect on Permeability of Dermal Connective Tissue. (32048)

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Our previous studies have shown that numerous metabolites and other compounds affect the permeability of dermal connective tissue(1-4). The changes in permeability appeared to be due to alterations in the physicochemical properties of hyaluronic acid, which is the main constituent of the ground substance of soft connective tissue(1-3). Since insulin has been reported to be an important agent regulating the permeability of cell membrane towards glucose(5) and proteins(6), it suggested the investigation of the effect of this hormone on the permeability of soft connective tissue, *i.e.*, dermis.

The studies were performed on 3- to 4-month-old albino rats of both sexes. They concerned two types of experiments: the effect of local application of insulin (U-40 Regular Iletin, Insulin Injection, U.S.P., Eli Lilly & Co.) and its systemic effect by intraperitoneal injection.

Materials and methods. The permeability was measured by one of our previously described methods(1,2) based on the rate of dermal diffusion of dye alone or combined with test substance. The procedure consists of intradermal injection of 0.05 ml of a 0.4% solution of Evans blue in physiological saline (pH 7.1) with or without test substance. Three injections on each animal are made for the control (generally dye alone) and 3 for the test compound. Thus, each animal serves as its own control. The contours of the blue spots formed are traced onto semi-transparent paper at 30-, 60-, 120-, and 180-minute intervals after injection. The areas of the spots traced on the semi-transparent paper are cut out, weighed, and calculated as mm^2. The area of each spot is entered as an individual result in the calculation of the average value and standard deviation. Reproducibility of results and tests obtained by this method were discussed elsewhere(1,2,4).

Results and discussion. Two concentrations of insulin, 1.6 and 5 units per ml were used in local applications injected intradermally along with the dye (Table I). In the study of the systemic effect of the hormone, the rats were treated for 3 weeks with daily intraperitoneal administration of 1 unit insulin. Then the effects of dye alone, ascorbic acid and ascorbic acid combined with thiourea

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