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Isolation and Characterization of an Echovirus, Possible "Prime" Strain of Echovirus Type 12 (38360)

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During a study of infantile diarrhea in Cairo, Egypt, U.A.R., (1) an echovirus-like enterovirus was isolated from many children which could not be identified using immune sera prepared against the recognized enterovirus types. Attempts to type out two selected prototypes from the many isolates after ten terminal-dilution passages also failed. Antisera, prepared in rabbits against the two viruses, neutralized all the isolates in quantitative neutralization test to about the same extent, indicating the antigenic homogeneity of the group. One of the isolates was therefore studied in detail to reconnoiter the possibility that it might represent a new, as yet unrecognized, human enterovirus. However, potent antiserum prepared in goat against the selected prototype virus (Hu 1943), neutralized the reference strain of echovirus type 12 (Travis) in low titer, indicating that the isolate should be considered a "prime" strain of echovirus type 12 (2, 3).

Materials and Methods. Rectal swabs were obtained either from children with acute diarrheal disease or from matched control children, all residents of Cairo, Egypt, U.A.R. Sera were drawn at the time of visit and about three weeks later, or in the case of children with diarrheal disease, convalescent sera were obtained, when possible, about three weeks after the onset of symptoms. The viruses were isolated in primary human amnion, FL human amnion stable cell line, and primary rhesus monkey cell cultures. (The latter were purchased from the Flow Laboratories, Inc., Rockville, MD). The standard virological methods were used for virus isolation (4). The virus titrations and neutralization tests were carried out for the most part by the micromethod of Takatsy (5). Typing sera were purchased from the Microbiological Associates, Inc., Bethesda, MD. For typing, we used the method of intersecting antibody pool (6). Monospecific immune sera prepared against the recognized human enteroviruses were obtained from the Research Reference Reagent Branch of the National Institute of Allergy and Infectious Diseases. The standard viruses were also obtained from the same source, except for Coxsackie B types 2 and 3, obtained from the National Communicable Disease Center. Antiserum was prepared in goat against one of the prototype isolates, according to the immunization schedule of Hampil *et al.* (7), using virus grown in $LLCMK_2$ cells in large rotating bottles, concentrated and purified by the phase separation method of Albertsson (8). Some of the non-classified enteroviruses isolated in this laboratory were also tested, such as H 136 (9), Hu 2080 (10). The HSO virus was received from Dr. Matumoto (11).

Results. Isolation of virus. The echovirus 12′ was recovered from rectal swabs obtained from nine out of 101 children with acute diarrheal disease (9%) and from 26 out of 150 control children (17%). The virus was successfully reisolated later from the original material stored at 16° from 19 out of the 26 cases, indicating its human origin.

Identification of the virus. Attempts to type out the prototype virus (carried through ten terminal dilution passages) using the standard reference enterovirus antisera were unsuccessful, as shown in Table I. Hyperimmune goat antiserum, prepared against the prototype virus failed to react with any of the enteroviruses

TABLE I. Cross-Neutralization test Conducted in Identification of Echovirus Type 12′ Virus (Hu 1943).

1. Prototype immune sera	Echovirus type 12′
Coxsackie A, types 1-21,[a] 24[b]	Negative
Coxsackie B, types 1-6	Negative
Poliovirus types 1-3	Negative
Echovirus types 1-9, 11-33	Negative
Hu 2080, H 136, HSO	Negative
2. Prototype virus	**Echovirus type 12′ immune serum**
Coxsackie A, types 1-21, 24	Not-tested
Coxsackie B, types 1-6	Not tested
Poliovirus types 1-3	Negative
Echovirus types 1-9, 11, 13-32	Negative
Echovirus type 12	128[c]
Hu 2080, H 136, HSO	Negative

[a] Coxsackie virus group A, type 22, was not tested.
[b] Including viruses Hu 39 and Pett (12).
[c] Reciprocal of neutralization endpoint, using about 100 $TCID_{50}$ virus.

tested, except echovirus type 12. Table II shows the results of cross-neutralization tests using the reference type 12 (Travis) and the prototype virus (HU 1943) against both the Reference Reagent serum and the commercially obtained antiserum prepared against echovirus type 12 (Travis), and the goat antiserum prepared against the prototype virus (Hu 1943). These data indicate a "one-way" neutralization of the standard virus in significantly lower titer than the homologous virus, indicative of "prime" strain relationship. The uniform extent of neutralization of the isolates in quantitative neutralization tests used in typing out the isolates suggests that these represented a homogeneous antigenic composition.

General properties of the virus. The echovirus type 12′ strain exhibited the typical characteristics of the echovirus group. The virus grew well in primary rhesus monkey kidney, primary human embryonic kidney, WI-26 and WI-38 human embryonic diploid fibroblast, primary and FL line of human amnion, Flow 1000, 2000, 3000, 5000 human embryonic fibroblasts, Detroit-6 human epithelial, African green monkey primary kidney and DBS-AJ fetal lung, $LLCMK_2$ cell cultures, and less well in Chang's liver and Vero (African green monkey kidney) cells. The virus caused a typical enterovirus-like cytopathic effect (13) as seen by the light microscope in Hematoxylin–Eosin stained preparation (Fig. 1), and in the electron microscope (14) (Fig. 2). The size of the round particles, as measured in lattice in ultrathin sections with the electron microscope, was found to be 33 nm, a size typical of echoviruses. No cytopathic effect was seen in Flow 407 cells derived from human small intestine, guinea pig primary kidney, hamster primary kidney, and BHK-21 cells.

Multiplication of the virus. In one-step growth curve experiments in primary rhesus monkey cell cultures, the infectious virus began to appear between 3–4 hr after the eclipse phase, reaching its maximum between 5–6 hr, a pattern characteristic of enteroviruses (Fig. 3).

Hemagglutination. The hemagglutinating activity of the echovirus type 12′ was tested by the method of Goldfield (15). Unlike the standard type 12 strain, which was used as a control, the echovirus 12′ strain failed to agglutinate human group O red blood cells at any of temperatures 4°, 20°, and 37°. No hemagglutination was noted with cord blood group O cells, demonstrated to be more sensitive to agglutination than the adult red blood cells (16). No hemagglutination was observed with rat, rabbit, dog, and pig red blood cells.

Stability of virus. The virus was not inactivated at pH 1 when kept at room temperature for 30 min, while it was inactivated almost com-

TABLE II. Cross-Neutralization Tests between the Reference and Prime Strains of Echovirus 12 and the Respective Immune Sera.

Virus	Immune serum: Type 12 (Travis) RRRB[a]	Type 12 (Travis) MBA[b]	Type 12′ (Hu 1943)
Type 12 (Travis)	8192[c]	4096	128
Type 12′ (Hu 1943)	< 8	< 8	1024

[a] Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases. Homologous titer 1:5200 against 300 $TCIC_{50}$ virus.
[b] Microbiological Associates, Inc.
[c] Reciprocal of neutralization endpoint, using about 100 $TCID_{50}$ virus.

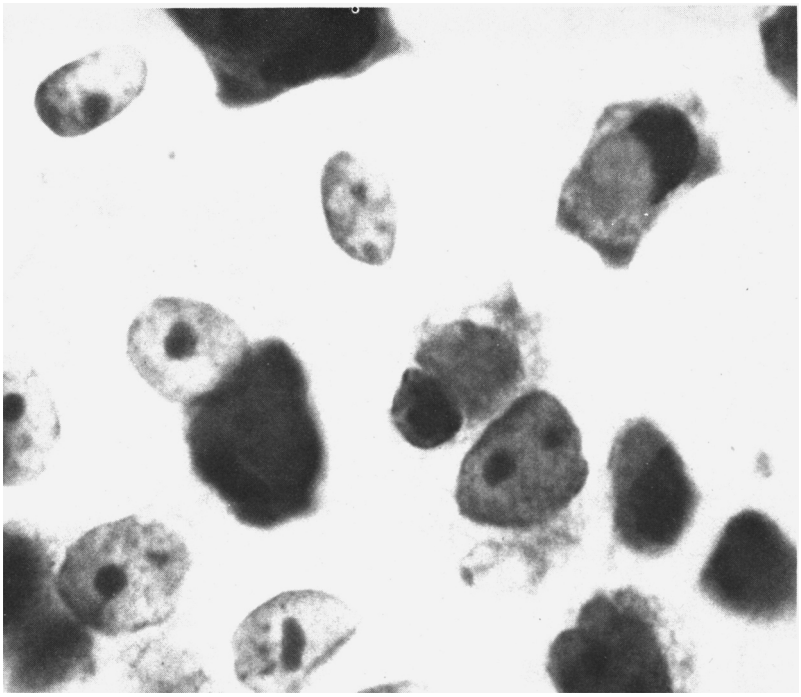

FIG. 1. Cytopathic effect of echovirus type 12′ (Hu 1943) on primary human embryonic kidney cells. Hematoxylin-eosin preparation, magnification approx. × 750.

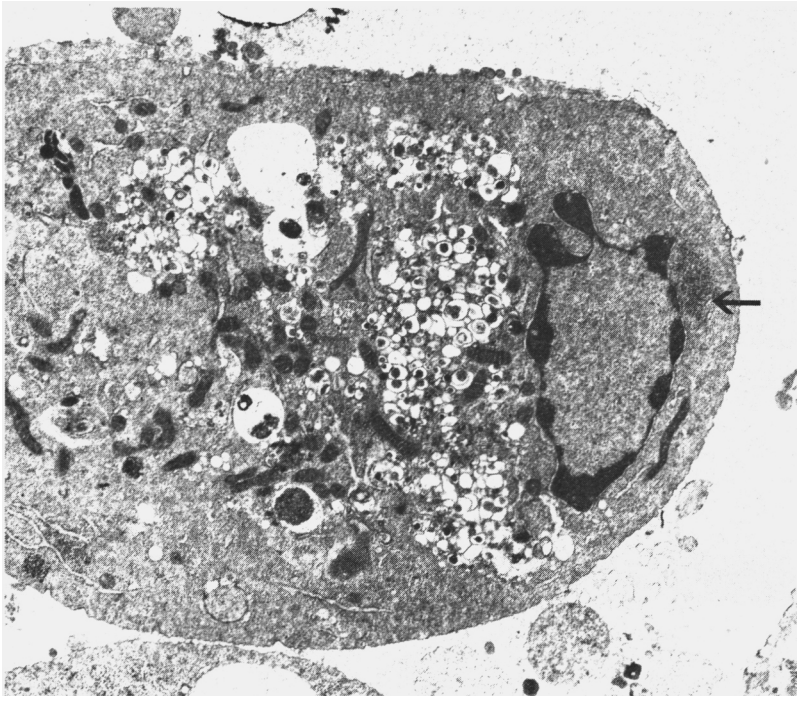

FIG. 2. Electron micrograph of echovirus type 12′ (Hu 1943)-infected primary human embryonic kidney cell. Note the characteristic cytoplasmic vesicles, the sickle-shaped, picnotic nucleus and cytoplasmic masses of virus particles (arrow). Magnification approx. × 6,500.

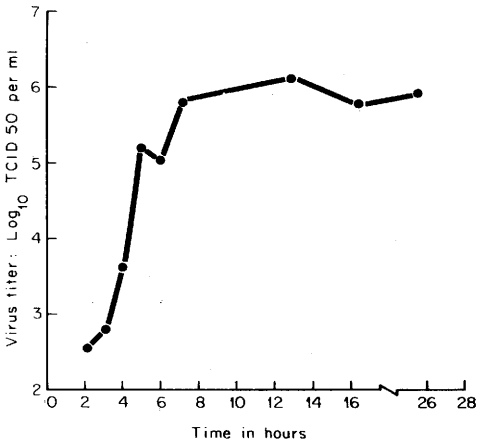

FIG. 3. "One step" growth curve of echovirus 12′ (Hu 1943) in primary human embryonic kidney cells at 37°.

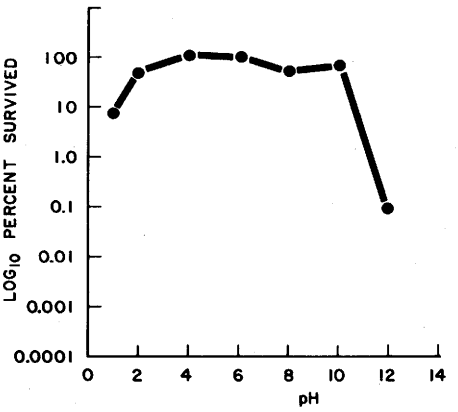

FIG. 4. Stability of echovirus 12′ (Hu 1943) at different pH.

pletely at pH 12 (Fig. 4), as is characteristic for enteroviruses. The virus was quite heat labile, as it was readily inactivated even at low temperatures (Fig. 5).

Prevalence of antibodies against echovirus 12′ among children and adults from Cairo, Egypt, U.A.R., and among the adult population of Philadelphia. In the Cairo population antibodies were found in the blood of three out of 14 children of less than 6 months of age (21%), in 14 out of 68 children of less than 3 years of age (21%), and in 55 out of 72 in the juvenile population of 11–20 years of age (76%); while adults over the age of 20 years showed a comparable occurrence of antibodies, with 65 out of 94 positive (70%), indicating widespread early infection with this virus at an early age, as is characteristic of enterovirus infections in Egypt, U.A.R. (17). A considerable number of these sera contained neutralizing antibodies against the virus in high titer (1:512 when tested against 100 TCID 50 virus). In contrast, only six out of the 500 sera obtained from adults in the Philadelphia area showed the presence of neutralizing antibodies, usually in low titer, against the virus, indicating a low prevalence of infection with this virus in this area.

Discussion. An untypable echovirus, isolated from children, residents of Cairo, Egypt, U.A.R., was shown to be a possible "prime" strain of echovirus type 12, possessing a broader antigenic spectrum than the prototype echovirus 12 (Travis). The virus was not neutralized by either the reference or the commercial immune serum prepared against the reference echovirus 12, while the goat antiserum prepared against the isolated virus neutralized the standard strain in significantly lower titer than the homologous virus, indicating a "prime" strain relationship. The many isolates recovered from 35 children manifested a very similar antigenic structure,

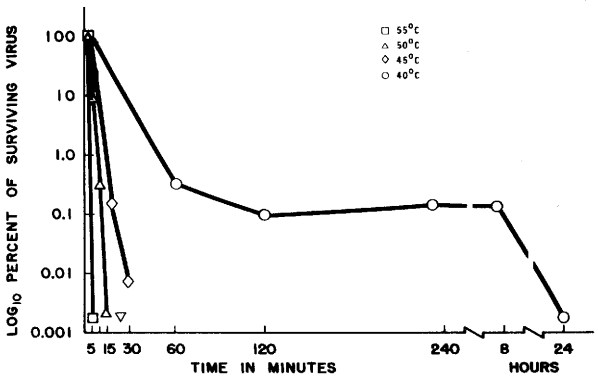

FIG. 5. Heat inactivation of echovirus 12′ (Hu 1943).

showing a like extent of neutralization to that of the homologous prototype virus. Unlike the reference strain of echovirus 12, the 12′ strain did not hemagglutinate human type O red blood cells. As differences in hemagglutinating property are known to exist between strains of the serotype, as for example among intratypic variants of echovirus type 6 (3, 19), this may not be a critical difference.

The recognition of antigenic variants between echovirus type 12 strains is not surprising in view of recognized antigenic differences among other echovirus strains. Prime strains have already been described for echovirus types 1, 3, 4, 5, 6, 9, 11, 29, and 30 (2, 3, 17, 18).

There can be no doubt about the human origin of the isolated virus. It was recovered repeatedly from 35 children, simultaneously in several cell lines (three specimens were obtained from each child and at least three different cell cultures were used to isolate the virus). In addition, the virus was successfully re-isolated several years later from 19 out of the 26 specimens kept at −60°. Human infection was documented by demonstrating a significant rise in antibodies in the acute and convalescent sera of several children resident in Cairo, Egypt, U.A.R. Antibodies were also commonly found (often in high titer) in the sera of young adults (70%) resident in the same city. Antibodies were also found in a small percentage of sera of adult residents of the City of Philadelphia, PA.

There was no correlation between the finding of acute diarrheal disease and the isolation of this virus in the stool; quite the contrary, the virus was more commonly isolated (9% vs 17%), respectively, in the stool of control children.

Summary. An echovirus, isolated from children, residents of Cairo, Egypt, U.A.R., was characterized and was shown to be a possible "prime" strain of echovirus type 12.

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