of the full-term fetuses produced under such conditions showed gross congenital malformations encompassing a wide variety of organ systems, including skeletal, brain, eye, heart, lung, and urogenital defects. The fetuses from zinc-deficient females contained less zinc than did their controls, suggesting that the congenital anomalies resulted from a direct effect of lack of zinc in the fetal tissues.

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Observations on Antigenic Variants of Echovirus Type 11.* (31579)

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Within certain echovirus immunotypes there occur antigenic variants designated "prime" strains. Such strains are neutralized to low titer or not at all by immune sera to the prototype virus strain, but immune serum prepared against a "prime" strain has high neutralizing capacity for both itself and the prototype strain. Thus, "prime" strains have a broader antigenic spectrum than does the prototype strain. Antigenic differences between "prime" and prototype strains are not so apparent in hemagglutination inhibition (HI) and complement fixation (CF) tests as in neutralization tests. To date "prime" strains have been described for echovirus types 1, 3, 4, 5, 6, 9, 29 and 30(1-3).

In the past few years we have isolated a number of strains of echovirus type 11 which are neutralized to low titer or not at all by immune serum to the prototype (Gregory) strain of echovirus type 11, but immune sera prepared against some of these strains neutralize the prototype echovirus type 11 strain. The first such strain was isolated in 1962, and in successive years the majority of echovirus type 11 strains isolated in this laboratory have been found to be more closely related to a representative "prime" strain (Silva) than to the prototype strain.

This report describes the recovery and serologic reactions of antigenic variants of echovirus type 11 and also the antibody responses of patients infected with these viral strains.

Materials and methods. Isolation and identification of viral strains. Virus was recovered from stool suspensions or throat washings in cultures of rhesus monkey kidney (MK) cells and/or human fetal diploid kidney (HFDK) cells by procedures which have been described elsewhere(4). Isolates were tested against immune sera to the polioviruses and group B coxsackieviruses in a colorimetric neutralization system(4), and in neutralization tests against immune sera to the echoviruses and

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TABLE I. Isolation of Antigenic Variants of Echovirus Type 11 from Clinical Materials.

Patient No.	Age	Sex	Date of onset	Clinical diagnosis	Specimen yielding virus	Virus isolated in (cell culture system)
1	2 mo	♂	5/March/62	Pneumonia	Stool	MK,* HeLa
2	4 mo	♂	17/Aug/63	Aseptic meningitis	"	HFDK†
3	1 mo	♂	15/Oct/63	" "	"	MK, HFDK
4	2 yr	♀	6/Dec/63	Pneumonia	"	HFDK
5	3 yr	♂	4/Feb/64	—	"	HFDK
6	3 mo	♀	11/July/64	Aseptic meningitis	"	MK, HFDK
7	3 yr	♀	30/Aug/64	Encephalitis	"	MK, HFDK
8	5 mo	♂	3/Sept/64	Pharyngitis	"	MK, HFDK
					Throat washing	HFDK
9	1 yr	♂	10/Sept/64	Pneumonia	Stool	MK
					Throat washing	MK
10	5 mo	♀	15/Oct/64	Bronchiolitis	Stool	MK, HFDK
					Throat washing	HFDK
11	24 yr	♀	24/Oct/64	Aseptic meningitis	Stool	MK, HFDK
12	1 yr	♂	12/Nov/64	Gastroenteritis	"	MK, HFDK
13	5 yr	♂	18/Nov/64	Meningoencephalitis	"	MK
14	4 mo	♀	16/Dec/64	Pharyngitis	Throat washing	MK
15	37 yr	♂	2/Jan/65	Meningitis	Stool	HFDK
					Throat washing	HFDK
16	2 mo	♀	10/Jan/65	Pneumonitis	Stool	HFDK
17	6 yr	♂	7/Feb/65	Pharyngitis	"	HFDK

* MK = rhesus monkey kidney. † HFDK = human fetal diploid kidney.

group A coxsackieviruses by the "intersecting serum scheme"(5).

The first isolates of echovirus type 11 "prime" strains, which were not identifiable as any of the currently-recognized enteroviruses, were plaque-purified 3 times in monkey kidney cell cultures and immune sera were prepared in monkeys(5) or hamsters(6) using plaque-purified virus. These sera were then cross-checked in neutralization tests against all of the echoviruses, and were found to neutralize echovirus type 11.

Neutralization tests. Cross neutralization tests and antibody assays on patients' sera were conducted in tube cultures of monkey kidney cell cultures by the procedure described elsewhere(4). Two-fold dilutions of serum (inactivated at 56°C for 30 minutes) were tested against approximately 100 TCD_{50} of virus, and endpoints were expressed in terms of the highest serum dilution inhibiting the cytopathic effect (CPE) of the test dose of virus after 7 days' incubation.

Hemagglutination inhibition (HI) tests. Hemagglutination and HI tests were performed by our standard procedure(7); in the first part of these studies they were conducted in 13 × 75 mm glass tubes, but later the microtiter system was utilized.

Complement fixation (CF) tests. Cross complement fixation tests were performed by the standard procedure of this laboratory(8). Antigens were prepared in HeLa cell cultures (7), and varying dilutions of each antigen were tested against varying dilutions of immune serum in block titrations.

Immunodiffusion tests. Certain virus strains were examined for immunological relationships in the micro gel double diffusion test which has been used with the coxsackieviruses(6).

Results. Recovery of antigenic variants of echovirus type 11 from clinical materials. Table I gives information on the age and sex of the patients from whom antigenic variants of echovirus type 11 have been isolated together with their clinical syndromes, the type of specimen yielding the virus and the cell culture systems in which virus was recovered.

Virus strains were isolated from infants, children and adults with either central nervous system disease or respiratory disease, and virus was recovered from both stool specimens and throat washings.

Starting in 1963, clinical specimens have been inoculated into both rhesus monkey kid-

TABLE II. Cross-Neutralization Tests with Antigenic Variants of Echovirus Type 11.

Virus strain	Neutralization titer of immune serum to: Gregory*	Hill*	Silva*	Stokes†
Gregory (prototype)	*4096*	32	128	64
Hill	<16	*8192*	1024	128
Silva	< 8	128	*2048*	256
Stokes	< 8	<16	32	*128*
Obershaw	< 8	16	256	128
Brager	<16	<16	64	128
Neklason	64	512	64	512
Diaz	<16	<16	32	128
Campbell	< 8	16	256	128
Sampson	128	1024	1024	1024
Cole	< 8	64	512	128
Affemata	< 8	<16	128	—
Shiver	<16	<16	32	128

* Immune serum prepared in rhesus monkeys.
† Immune serum prepared in hamsters.

ney (MK) and human fetal diploid kidney (HFDK) cell cultures for viral isolation attempts, and in Table I it is seen that approximately equal numbers of isolations were made in each cell culture system. The first isolate (Hill) was recovered in both MK and HeLa cell cultures.

The virus strains listed in Table I were identified as antigenic variants of echovirus type 11 on the basis of the fact that they were neutralized poorly or not at all by a 1:20 dilution of immune serum to the prototype (Gregory) strain of echovirus type 11, but were neutralized by immune serum to the Silva strain (see below).

Cross neutralization tests with antigenic variants of echovirus type 11. Immune sera were prepared with plaque-purified virus of the first 3 variant strains isolated (Hill, Silva and Stokes). Table II presents results of cross neutralization tests with these sera, immune serum to the prototype echovirus type 11 strain, and some of the isolates listed in Table I.

Although immune serum for the prototype strain had a high homologous titer, it neutralized only 2 of the isolates (Neklason and Sampson). Immune serum to the Hill strain had a somewhat broader spectrum of neutralizing activity than did the prototype immune serum, but it failed to neutralize some of the isolates, and had a low titer for others. Immune serum to the Silva strain neutralized all of the strains examined, although its titer for the Stokes, Diaz and Shiver strains was relatively low. This immune serum has been included in our "intersecting serum scheme" for identification of echoviruses. The immune serum prepared against the Stokes strain had a relatively high titer for all of the isolates, and it may be that this strain actually has a broader antigenic spectrum than the Silva strain, but the immune serum was prepared in hamsters rather than monkeys, and thus results are not strictly comparable. The Neklason and Sampson strains would appear to be more closely related to the prototype strain than are the other isolates, and they were also neutralized to relatively high titer by immune sera to the 3 variant strains.

Hemagglutination inhibition tests with antigenic variants of echovirus type 11. The isolates for which hemagglutinins could be demonstrated were examined in cross HI tests with immune sera to the prototype strain, and to the Hill, Silva and Stokes strains.

The results presented in Table III clearly indicate the antigenic relationship of isolates to echovirus type 11. Immune serum to the prototype Gregory strain showed a narrow spectrum of activity in HI tests as well as in neutralization tests; although the serum showed some HI activity for all of the isolates, titers for most of them were markedly lower than the homologous titer. Immune sera to each of the 3 variant strains had HI titers against the prototype echovirus type 11 strain almost as high as their homologous titer, and

TABLE III. Cross-Hemagglutination Inhibition Tests with Antigenic Variants of Echovirus Type 11.

Virus strain	Hemagglutination-inhibiting titer of immune serum to: Gregory*	Hill*	Silva*	Stokes†
Gregory (prototype)	*8192*	2048	1024	512
Hill	256	*8192*	2048	512
Silva	256	—	*4096*	2048
Stokes	64	512	4096	*2048*
Obershaw	64	1024	512	64
Brager	64	—	2048	2048
Neklason	1024	—	2048	2048
Sampson	64	64	2048	2048
Cole	4096	4096	4096	4096

* Immune serum prepared in rhesus monkeys.
† Immune serum prepared in hamsters.

TABLE IV. Cross-Complement Fixation Tests with Antigenic Variants of Echovirus Type 11.

Virus strain	Complement-fixing titer of immune serum to: Gregory	Hill	Silva
Gregory (prototype)	*256*	256	128
Hill	128	*512*	128
Silva	64	128	*1024*
Stokes	64	128	1024

these sera had relatively high titers for all of the isolates.

Cross complement fixation tests with antigenic variants of echovirus type 11. Complement-fixing antigens for the prototype Gregory strain and for the Hill, Silva and Stokes strains were examined in cross CF tests with immune sera to the Gregory, Hill and Silva strains. Results of these tests, presented in Table IV, further confirm the antigenic relationship between the virus strains; each of the 3 immune sera had fairly high titers for all 4 of the test antigens. (All of the immune sera were shown to have CF titers of $<1:16$ with CF antigens for the other 31 echovirus types.)

Gel double diffusion tests. The immunologic identity of the Hill strain and the prototype echovirus type 11 strain was also evidenced in immunodiffusion tests. Precipitating antigen to the Hill strain was prepared by 200-fold concentration (by high-speed centrifugation) of infected HeLa cell culture fluid. This antigen was tested in a micro gel diffusion test(6) against immune sera to all 32 echovirus types; it gave a strong line of precipitate with the echovirus type 11 (Gregory strain) immune serum, but showed no reaction with any of the other immune sera. When precipitating antigens for the Hill strain and the Gregory strain were placed in adjacent wells and permitted to diffuse toward a well containing immune serum to the prototype echovirus 11 strain, the lines of precipitate formed by each antigen coalesced (a reaction of immunologic identity).

Neutralizing antibody responses of patients infected with antigenic variants of echovirus type 11. Acute- and convalescent-phase serum specimens from certain patients infected with antigenic variants of echovirus type 11 were examined in neutralization tests against both the prototype virus strain and the Silva strain.

Table V shows that antibody responses of these patients to the prototype and "prime" strain of echovirus type 11 were comparable. Titers obtained with the 2 strains were not identical, but they rarely varied by more than 2-fold, and in most instances significant rises in antibody titers were detected with both strains. The fact that the prototype virus strain was neutralized by sera from patients infected with antigenic variants of echovirus type 11 further suggests that the patients' isolates possess a broader antigenic spectrum than does the prototype virus strain (since the isolates themselves were neutralized poorly by immune serum to the prototype strain).

Discussion. Antigenic variation among strains of echovirus type 11 was first suggested by the fact that the so-called "U-virus" strains isolated in Sweden by Philipson and Wesslén(9) in 1956 and 1957 were initially thought, on the basis of neutralization tests with sera to prototype viruses, to be unrelated to currently-recognized echovirus types, but additional studies showed the "U-virus" to be antigenically related to echovirus type 11(10).

The "U-virus" strains were not considered to represent "prime" strains of echovirus type 11(10) since they did not appear to possess

TABLE V. Neutralizing Antibody Titers of Sera from Patients from Whom Antigenic Variants of Echovirus Type 11 Were Isolated.

Patient	Serum, days after onset of illness	Neutralizing antibody titer *vs* Gregory strain	Silva strain
Silva	3	<8	<8
	24	16	32
Obershaw	2	256	—
	49	256	—
Brager	6	16	32
	18	64	128
Nelklason	2	8	<8
	18	32	128
Campbell	3	<8	<8
	34	64	256
Cole	3	16	<8
	23	32	16
Clair	2	<8	<8
	16	32	16
Del Gadillo	17	64	32
	30	128	128

a broader antigenic spectrum than the prototype strain. However, the antigenic variants studied most extensively in this laboratory, *i.e.*, the Silva and Stokes strains, do show broader spectra of neutralizing activity than the prototype strain, and can be considered "prime" strains.

Whether the observed antigenic variation in strains of echovirus type 11 represents a progressive "drift" away from the prototype, such as that which occurs in the case of influenza viruses, can only be established by the examination of large numbers of isolates from successive years and from various geographical locations. To date no such "drifts" in the antigenic composition of enteroviruses have been noted. The echovirus type 11 strains isolated in this laboratory in recent years have been more closely related to the Silva strain than to the prototype, but 2 strains (Neklason and Sampson) still showed a high degree of relationship to the prototype strain.

The observations presented in this report emphasize the need for awareness of the possible occurrence of enterovirus variants with broader antigenic spectra than the prototype virus strain. Also, it is seen that HI, CF and immunodiffusion tests with prototype immune sera can aid in the identification of antigenic variants giving equivocal or negative reactions in neutralization tests.

Summary. Strains of echovirus type 11 isolated in this laboratory since 1962 appear to have a broader antigenic spectrum than the prototype Gregory strain. The isolates were neutralized to low titer or not at all by immune serum to the Gregory strain, but immune serum to a representative isolate (Silva) neutralized both the prototype and homologous strains, as well as the other isolates. Results of cross hemagglutination inhibition and complement fixation tests, as well as immunodiffusion tests, confirmed the relationship of these isolates to echovirus type 11. Sera of patients from whom the antigenic variants were recovered showed significant neutralizing antibody responses to both the prototype echovirus type 11 strain and the Silva "prime" strain.

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