INTERRELATIONSHIPS AMONG ECHO VIRUS TYPES 1, 8, AND 12

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ABSTRACT

BERG, GERALD (Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio), NORMAN A. CLARKE, AND PAUL W. KABLER. Interrelationships among ECHO virus types 1, 8, and 12. J. Bacteriol. **83**:556–560. 1962.—Antigenic relationships among ECHO 1, 8, and 12 viruses were investigated. Three strains of ECHO 1 virus, one of which had been purified, could not be differentiated by neutralization tests. Antiserums produced with these three strains neutralized ECHO 12 virus. The neutralizing material, absent from preimmunization serums, was not absorbed by rhesus kidney cells, was heat-stable, and, therefore, may be viral antibody. Reciprocal neutralization was achieved with all three types. Disagreement of these data with those published by others is discussed.

A recent report suggesting that ECHO virus types 1 and 12 are unrelated serologically (Hammon, Yohn, and Pavia, 1959) conflicts with other published data (Committee on the Enteroviruses, 1957; Kamitsuka et al., 1961).

Data acquired in this laboratory during another study provide further evidence that these viruses are related antigenically. These data are presented herein.

MATERIALS AND METHODS

Viruses. All virus stocks were prepared in rhesus monkey (*Macaca mulatta*) kidney cell cultures. ECHO 1 (R768) was isolated in this laboratory from the rectal swab of a healthy child, and was purified by two terminal dilution passages prior to use in this study. ECHO 1 (OHIO 1), also isolated from a healthy child, was obtained from George Anderson of the Ohio State Department of Health. ECHO 1 (Farouk), ECHO 8 (Bryson), and ECHO 12 (Travis) were obtained from Leon Rosen of the National Institutes of Health.

Antiserums. Antiserums for both the R768 and OHIO 1 strains of virus were produced in this laboratory by a series of intravenous inoculations of undiluted virus into young rabbits. Antiserums for ECHO 1 (Farouk), ECHO 8 (Bryson), and ECHO 12 (Travis) were produced in rabbits for the National Foundation. (These antiserums were produced by Microbiological Associates and kindly supplied to us by S. S. Kalter, Communicable Disease Center, U. S. Public Health Service, Atlanta, Ga.)

Neutralization tests. Neutralization tests were carried out essentially by procedures recommended by the National Foundation's Committee on the Enteroviruses (1957). Viruses and serums were diluted in either Earle's or Hanks' balanced salt solution containing 0.5% lactalbumin hydrolyzate, and 50 units each of penicillin, streptomycin, and nystatin. The pH was adjusted to 7.6 with NaOH. The same diluent was used throughout any test. Equal volumes of viral suspension and serum dilution were mixed in test tubes, incubated at room temperature for approximately 1 hr, and inoculated into monkey kidney cell cultures; 0.1-ml inoculums were employed in some tests, 0.2 in others. Fourfold serum dilutions were used and, usually, four tubes were inoculated with each virus-serum mixture. Concurrent virus titrations were done by inoculating tenfold dilutions of a control virus-diluent mixture into each of four cell cultures. Tests were read for at least 8 days. Because the order of reaction of virus neutralization by antibody is not completely clear (French, Armstrong, and Nagler, 1959), serum antibody titers were calculated by the 50% end point method rather than by the more sophisticated MPN method (Chang et al., 1958), which is valid only with first order or pseudo first order reactions. Neutralization titers were expressed as the reciprocals of the dilutions neutralizing all the virus in 50% of the cultures. For consistency, virus titers were also determined by the 50% end point method.

Cell cultures. Trypsinized kidney cell cultures

TABLE 1. *Neutralization of ECHO 1 (R768), 8, and 12 viruses by ECHO 1 (R768) antiserums**

Test no.	Virus	Virus concn (TCD_{50}/inoculum)	Serum no.	Serum dilution (reciprocal)	Cultures neutralized/cultures inoculated	Neutralization titer (reciprocal)
1	ECHO 1 (R768)	100	10	128	4/4	
				512	4/4	1,024
				2,048	0/4	
			11	128	4/4	
				512	4/4	1,024
				2,048	0/4	
			12	128	4/4	
				512	1/4	384
				2,048	0/4	
2	ECHO 12	75	10	2	2/10	
				8	0/4	1.5
			11	2	8/10	
				8	2/4	6
			12	2	10/10	
				8	2/4	8
3	ECHO 12	50	10	2	1/4	
				8	0/4	1.5
				32	0/4	
			11	2	4/4	
				8	1/4	6
				32	0/4	
			12	2	4/4	
				8	2/4	8
				32	0/4	
4	ECHO 1 (R768)	75	10	128	4/4	
				512	4/4	1,024
				2,048	0/4	
	ECHO 8	100	10	2	3/4	
				8	0/4	3
				32	0/4	
	ECHO 12	100	10	2	1/4	
				8	0/4	1.5
				32	0/4	

* Heated at 56 C for 30 min.

prepared from rhesus or from African Green (*Cercopithecus aethiops sabaeus*) monkeys by the continuous method of Rappaport (1956) were used in all experiments. The cells were grown at 36 C for 5 to 7 days in a growth medium consisting of 96.28% Hanks' balanced salt solution, 0.5% lactalbumin hydrolyzate, 3% calf serum, 0.22% $NaHCO_3$, and 50 units each of penicillin, streptomycin, and nystatin. The pH was adjusted to 7.4 with NaOH. Thereafter, this growth medium was supplemented with or was replaced by a maintenance medium consisting of 99.28% Earle's balanced salt solution, 0.5% lactalbumin hydrolyzate, 0.22% $NaHCO_3$, and 50 units each of penicillin, streptomycin, and nystatin. The pH was adjusted to 7.6 or 8.0 with NaOH. Cell cultures were washed at least three times with maintenance medium, which was always replaced just prior to inoculation.

RESULTS

Neutralization of ECHO virus types 8 and 12 by heat-inactivated antiserums produced with the R768 strain of ECHO 1 virus is demon-

TABLE 2. *Effect of heating serum on the inhibition of ECHO viruses 1 (R768), 8, and 12 by ECHO 1 (R768) antiserum**

Virus	Virus concn (TCD_{50}/inoculum)	Serum dilution (reciprocal)	Cultures neutralized/cultures inoculated	Neutralization titer (reciprocal)
ECHO 1 (R768)	25	128	4/4	2048
		512	4/4	
		2048	2/4	
ECHO 8	500	2	4/4	6
		8	1/4	
		32	0/2	
ECHO 12	25	2	9/9	12
		8	3/4	
ECHO 1 (R768)	25	128	4/4	3072
		512	4/4	
		2048	3/4	
ECHO 8	500	2	4/4	6
		8	1/4	
		32	0/2	
ECHO 12	25	2	9/10	4
		8	1/4	

* Serum 11. The top half of the table gives results obtained with serum heated at 56 C for 30 min. The bottom half gives results with unheated serum.

TABLE 3. *Effect of serum absorption with rhesus kidney cells on the neutralization of ECHO 1 and ECHO 12 viruses by ECHO 1 antiserums*

Virus	Virus concn (TCD_{50}/inoculum)	Antiserum treatment	Serum dilution (reciprocal)	Antiserum*			
				ECHO 1 (R768)†		ECHO 1 (OHIO 1)‡	
				Cultures neutralized/cultures inoculated	Neutralization titer (reciprocal)	Cultures neutralized/cultures inoculated	Neutralization titer (reciprocal)
ECHO 12	100	Absorbed	2	4/4	4	3/4	3
			8	0/4		0/4	
			32	0/4		0/4	
		Unabsorbed	2	4/4	8	4/4	4
			8	2/4		0/4	
			32	0/4		0/4	
ECHO 1 (R768)	500	Absorbed	128	4/4	1024	4/4	512
			512	3/4		2/4	
			2048	1/4		0/4	
		Unabsorbed	128	3/4	512	3/4	192
			512	2/4		0/4	
			2048	1/4		0/4	

* Heated at 56 C for 30 min.
† Serum 11.
‡ Serum 13.

strated in Table 1. With antiserums produced in three rabbits (10, 11, and 12), neutralization titers for ECHO 12 virus ranged from 1.5 to 8. Heat-inactivated, preimmunization serums from the same rabbits (and from rabbits 11 and 13 immunized subsequently with OHIO 1 virus) had no demonstrable neutralizing antibody at the 1:2 dilution level when tested with 50 to 100 TCD_{50} of all five viruses used in this study. (Each serum-virus mixture was tested in four cell cultures.)

To determine whether heat inactivation of serum reduced the quantity of neutralizing substance present, samples of R768 antiserum were heated at 56 C for 30 min and tested together with unheated samples against ECHO viruses 1, 8, and 12 (Table 2). Little or no heat-labile neutralizing material was present in the serum, since titers obtained with the two samples were not significantly different.

Since monkey kidney cell antibody may interfere with the adsorption of virus to host cells (Quersin-Thiry, 1958, 1959; Habel et al., 1958), an experiment was designed to determine whether amounts of such antibody produced during the preparation of antiserums in rabbits were sufficient to account for the low-level neutralization of ECHO 12 virus by ECHO 1 antiserums. Antiserums prepared with ECHO 1 (R768) and ECHO 1 (OHIO 1) viruses were heated at 56 C for 30 min and mixed with twice-washed rhesus kidney cells obtained from primary cultures scraped from the walls of glass bottles with a rubber policeman. The resulting suspensions of cells in serums were heavy, one consisting of 1⅛ ml of R768 antiserum and 1/16 ml of cells (packed volume) and the other of 5 ml of OHIO 1 antiserum and 3/32 ml of cells (packed volume). The suspensions of cells in serums were incubated for 1½ hr at room temperature and, after agitation, overnight at 4 C. Subsequently, cells were removed from the serums by two 10-min cycles of centrifugation at about 192 × *g*.

The results of concurrent neutralization tests with absorbed and unabsorbed antiserums are presented in Table 3. Absorption with monkey kidney cells did not significantly reduce the neutralizing capacity of either ECHO 1 antiserum against ECHO 1 or ECHO 12 virus.

Table 4 demonstrates the results of cross-

TABLE 4. *Cross neutralization relationships among ECHO viruses 1, 8, and 12**

Antiserum	Virus			
	ECHO 1 (Farouk)	ECHO 1 (R768)	ECHO 8	ECHO 12
ECHO 1 (Farouk)	384	1536	4	3
ECHO 1 (R768)	256	384	<1.5†	1.5
ECHO 1 (OHIO 1)	112	192	4	2
ECHO 8	8	32	1024	2
ECHO 12	3	6	12	1024
Virus concn (TCD_{50}/inoculum)	88	10	10	50

Results are expressed as the reciprocals of the neutralization titers. The antiserums were inactivated by heating at 56 C for 30 min. ECHO 1 (R768) antiserum was from serum 11; ECHO 1 (Ohio 1) antiserum was from serum 13.

† Neutralizatioe occurred in other tests (Table 1).

TABLE 5. *Neutralization of ECHO 8 and 12 viruses by antiserums prepared with different strains of ECHO 1 virus*

Test	Antiserum	Neutralization titer (reciprocal)	
		ECHO 8	ECHO 12
1	OHIO 1*	<3	4
	Farouk	3	6
	Virus concn (TCD_{50}/inoculum)	25	50
2	R768†	8	3
	OHIO 1*	<3	8
	Virus concn (TCD_{50}/inoculum)	75	10

* Serum 15.

† Serum 10.

neutralization tests with ECHO viruses 1, 8, and 12. The R768 and Farouk strains of ECHO 1 virus could not be differentiated from each other. The OHIO 1 strain, which appears to be differentiable from the Farouk strain in this test, could not be distinguished in other tests. ECHO 12 antiserum neutralized ECHO viruses 1 and 8, and ECHO 8 antiserum neutralized ECHO viruses 1 and 12. Even relatively low-titered homologous antiserums were capable of neutralizing the heterologous virus types.

Table 5 presents additional data demonstrating the neutralization of ECHO viruses 8 and 12 with antiserums prepared with the R768 and Farouk strains of ECHO 1 virus. While the OHIO 1 strain of ECHO 1 virus did not neutralize ECHO 8 virus in these tests, it did so in other tests (Table 4). OHIO 1 antiserum did neutralize ECHO 12 virus.

DISCUSSION

The data presented in this paper demonstrate a relationship among ECHO viruses 1, 8, and 12. These data are not in agreement with data obtained in several other laboratories. The Committee on the Enteroviruses (1957), Hammon et al. (1959), and Kamitsuka et al. (1961) failed to demonstrate neutralization of ECHO 12 virus by ECHO 1 antiserum, but such neutralization is demonstrated by our data.

Both monkeys and rabbits were used for the preparation of antiserums. The Committee on the Enteroviruses (1957) and Kamitsuka et al. (1961) reported results with monkey antiserums, and the Committee report indicated that similar results had been obtained with rabbit antiserums. Hammon et al. (1959) used monkey antiserums. We used rabbit antiserums. The serum source does not seem to explain the disagreement in the data.

It is improbable that the material used by us to prepare antiserums for ECHO 1 virus contained some ECHO 12 virus, since the R768 strain was purified by terminal dilution passages before use for immunization. Furthermore, neutralization of ECHO 12 virus was accomplished with antiserum prepared in this laboratory with nonpurified OHIO 1 virus and with anti-Farouk serum prepared elsewhere. These three virus strains were neutralized by high dilutions of the homologous antiserums.

Since the ECHO 1 preimmunization serums tested contained no demonstrable ECHO 12 neutralizing substance, it seems reasonable to assume that this substance was generated by the immunization procedure. Inasmuch as this material in two different ECHO 1 antiserums was not absorbed by rhesus kidney cells, it seems reasonable to assume that it may be viral antibody. The stability of this material to heat adds further support to such an assumption.

We were also able to demonstrate inhibition of ECHO 12 virus by ECHO 8 antiserum. However, we do not know whether this inhibitor was antibody.

Considering the consistency of our results, it is difficult to explain why no antigenic relationship between ECHO 1 and 12 viruses was detected by Hammon et al. (1959) and only a one-way cross by the Committee on the Enteroviruses (1957) and by Kamitsuka et al. (1961). It is possible that the use of undiluted serums in our work accounted, in large part, for the differences in results. It may be that the reluctance of many investigators to test serums at low dilutions because of possible nonspecific activity, the nature of which is often ill defined, may have resulted in the neglect of other heterologous reactions. It is probable that assay by the much more sensitive plaque technic would allow the demonstration of antigenic relationships not previously encountered.

Greater emphasis on precise serological relationships in the classification of viruses might do much to allow an orphan which has found its disease to claim its birthright.

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