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BIOLOGICAL AND ANTIGENIC ANALYSES OF NEWLY ISOLATED STRAINS OF ECHOVIRUS TYPE 7[1].

ISAO OISHI, KEIJI OTSU and NOBUHARU KUNITA

Virus Laboratory, Osaka Prefectural Institute of Public Health, Nakamichi, Higashinari-ku, Osaka

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SUMMARY Fifty-eight strains of echovirus type 7 were isolated from 1962 to 1968. Of these, 19 strains were isolated from patients with aseptic meningitis and 39 strains from healthy children.

The biological and antigenic differences between the prototype and the 58 newly isolated strains of echovirus type 7 were tested.

The strains isolated from patients had no hemagglutinating activity (HA) to human group O erythrocytes, but 59% of the strains from healthy children had this activity.

This property of the viruses was stable and was also correlated with their plaque size and virulence in man.

The HA character and plaque size can be used as markers of virulence of echovirus type 7.

Antigenic differences between the prototype and the newly isolated strains tested by cross neutralization and hemagglutination-inhibition tests and the results showed that the HA activity of echovirus type 7 was directly involved in the antigenic differences between them.

It was also concluded that HA negative strains were antigenic variants of echovirus type 7.

INTRODUCTION

It is known that some of newly isolated strains of hemagglutinating enteroviruses do not agglutinate human group O erythrocytes (Bussell et al., 1962; Kern et al., 1966). There are only few reports on the correlation of the biological properties and the virulence of echoviruses (Margalith et al., 1967; Margalith et al., 1968). Margalith et al. reported a correlation between the rct/40 marker and mouse-virulence of newly isolated strains of echovirus type 9 from patients with aseptic meningitis and described genetic characteristics of one strain derived from the naturally occurring strain. However, mouse-virulence of echovirus type 9 did not always seem to be associated with virulence in man.

During the period from 1962 to 1968, fifty-eight strains of echovirus type 7 were isolated

[1] A summary of these results was reported at the 18th Annual Meeting of the Society of Japanese Virologists in September, 1970.

from patients with aseptic meningitis and from healthy children.

About 60% of these, including all the strains isolated from patients, did not agglutinate human group O erythrocytes. This suggests that HA activity related to virulence in man.

Little is known about biological and antigenic variants of echovirus type 7. Therefore, the correlation of HA activity and other characteristics of the isolated strains of echovirus type 7 with their virulence was examined.

The antigenic differences between these strains were also studied by the cross neutralization and hemagglutination-inhibition tests.

MATERIALS AND METHODS

1. *Viruses*

Prototype Wallace stain of echovirus type 7 was supplied by the National Institute of Health, Japan and was passaged 3 or 4 times in primary monkey kidney (PMK) cell cultures in our laboratory. Thirty-nine strains were isolated from healthy children in 1962–1968 and nineteen strains from patients with aseptic meningitis in 1966 and 1967 (Table 1). All these strains were isolated using PMK cell cultures and identified as echovirus type 7 by the neutralization test. Then they were passaged in PMK cells 3 or 4 times.

2. *Cell cultures*

Monolayers of primary cynomolgous monkey kidney (PMK) cell cultures grown in LE medium (Earle's BSS containing 0.5% lactalbumin hydrolysate) supplemented with 5% calf serum were employed for virus isolation, propagation of viruses as antigens for immunological tests and for plaque assays. The cells were maintained in serum-free LE medium after inoculation of viruses.

3. *Plaque assay*

Plaque assays were carried out to observe the morphology of virus plaques and to measure the infectivity (plaque forming units : PFU) on PMK cell monolayers. This was done by the method of Melnick (1964).

4. *Adsorption of virus to erythrocytes*

Three ml of virus ($10^{6.0}$PFU/ml) were mixed with an equal volume of packed human group O erythrocytes and kept at room temperature for 30 min with intermittent shaking. The infectivity and HA activity of the virus suspension was examined after each of the three successive cycles of adsorption.

5. *rct/40 Marker test*

This test was carried out by the plaque method using PMK cell cultures. Cell monolayers were inoculated with 0.2 ml samples of ten-fold serial dilutions of virus using three bottles (2 oz) for each virus dilution and then the monolayers were kept at 40 C in a water bath for five days. The controls were incubated at 35 C in an air incubator. The plaque count of the virus was measured 5 days after inoculation.

6. *Immune sera*

Immune sera for the 5 newly isolated strains and the prototype strain of echovirus type 7 were prepared by inoculation of guinea pigs with virus fluid in medium 199 treated with fluorocarbon.

The schedule of immunization was as follows: animals were inoculated 5 times at 2 day intervals with 5 ml of virus fluid by the intraperitoneal route. After one month, a booster injection was given and the animals were bled seven days later.

7. *Neutralization (NT) test*

The NT test was carried out in tube cultures (12× 95 mm) of PMK cells by the method of Melnick (1964). Serial two-fold dilutions of sera (inactivated at 56 C for 30 min) were mixed with an equal volume of virus (100 $TCID_{50}$/0.1 ml) and incubated at 37 C for 60 min. After incubation, 0.2 ml aliquots of the mixture were inoculated into tubes (4 tubes for each serum dilution) and the tubes were kept at 35 C for 60 min. The NT titer of the antiserum was determined 5 days later as the reciprocal of the highest serum dilution which inhibited development of cytopathogenic effects of the virus in two or more tubes.

8. *Hemagglutination (HA) test*

The method of Melnick (1964) was modified as follows : serial two-fold dilutions of virus were mixed with an equal volume of a 0.7% suspension of human group O erythrocytes in 0.005 M phosphate-buffered saline (PBS, pH 7.4) and after incubation for 2 hours the HA of the mixture was measured. The HA test was also performed with HA negative strains after concentration of the virus with polyvinylpyrrolidone

(PVP) or after treatment with fluorocarbon to unmask the hemagglutinin.

9. *Hemagglutination-inhibition (HI) test*

A modification of the method of Melnick (1964) was used. Four hemagglutination units (HAU) of virus were mixed with an equal volume (0.2 ml) of serial dilutions of antiserum and the mixtures were incubated at room temperature for 60 min. Then two volumes (0.4 ml) of a 0.7% suspension of human erythrocytes were added and the mixture was reincubated at room temperature.

Before the test, antiserum was treated with an equal volume of 25% acid washed Kaolin in PBS and with an equal volume of a 50% suspension of human erythrocytes. After centrifugation, antiserum was inactivated at 56 C for 30 min. The antibody titer is expressed as the reciprocal of the highest dilution of serum causing inhibition of hemagglutination.

RESULTS

1. *Hemagglutination of isolated strains of echovirus type 7*

The newly isolated strains of echovirus type 7 and their HA activities with human erythrocytes are shown in Table 1.

About 40% of them were HA positive (HA(+)), and the rest were HA negative (HA(−)).

Fifty-nine per cent of the strains isolated from healthy children were HA positive. While all the strains from patients with aseptic meningitis were HA negative, irrespective of when or where the virus was isolated.

Even after 20 passages of these strains in PMK cell cultures, their hemagglutination characters were unchanged, and their HA titers did not change on incubation at 35 C, room temperature or 4 C.

2. *Relationship between HA activity and infectivity*

One possible explanation for the HA negative character of HA(−) strains was the very low yield of virus so the relationship between the PFU and HA titers of the isolated strains was examined.

Results on 26 isolated strains and the prototype strain are shown in Fig. 1.

There was little difference between the infectious virus yields of the newly isolated strains, but their HA titers were very different. No HA activity was present in virus samples isolated from patients.

TABLE 1. *Isolated strains of echovirus type 7 and their hemagglutinating activities*

Year	Number of strains	Agglutination of human O erythrocytes	Clinical symptoms	District of isolation
1962	12	11/12[a]	None	Osaka
1966	22	8/22	None	Osaka
	1	0/1	A. M.[b]	Osaka
1967	3	3/3	None	Osaka
	18	0/18	A. M.	Osaka (1)[c] Kobe (2) Tokushima (15)
1968	2	1/2	None	Osaka
Total	58	23/58	—	—

a No. of HA (+) strains / No. of strains tested.
b Aseptic meningitis.
c Numbers in parenthesis indicate numbers of strains isolated.

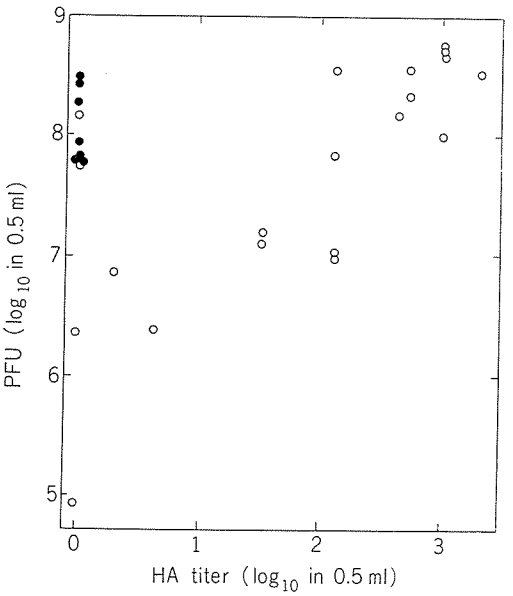

FIGURE 1. *Relationship between infectivity and hemagglutinating activity of some strains of echovirus type 7.*
Strains isolated from patients (●), *and healthy children* (○).

3. *HA activity after concentration of viruses*

To comfirm the above results, HA activity was measured after concentration of the virus samples since if a virus possesses low HA activity, results may change from HA(−) to HA (+) on concentration of the virus sample. Table 2 shows results on the HA activity of virus samples after 100-fold concentration.

TABLE 2. *Infectivity and HA activity after 100-fold concentration of 2 strains of echovirus type 7*

Virus Strain	Original		After concentration	
	PFU	HAU	PFU	HAU
Wallace	$2.8\times10^{8.0}$	1,000	$7.5\times10^{9.0}$	10,000
Liq-74	$9.5\times10^{7.0}$	<10	$3.4\times10^{9.0}$	<10

PFU and HAU in 0.5 ml.

After concentration, the PFU of both strains increased, but the Liq-74 strain, isolated from a patient, still had no HA activity.

4. *Treatment of viruses with fluorocarbon*

It is known that some substances in culture fluid inhibit or affect the HA of enteroviruses (Philipson et al., 1962: Schmidt et al., 1964) and these can be partially removed by treatment of the virus with fluorocarbon (Schmidt et al., 1962).

Accordingly an HA(+) and an HA(−) strain were tested after treatment with fluorocarbon. The HA(−) strain still remained HA(−) while the HA titer of the HA(+) strain increased two to four fold.

5. *Adsorption test*

The differences in the HA characters of the

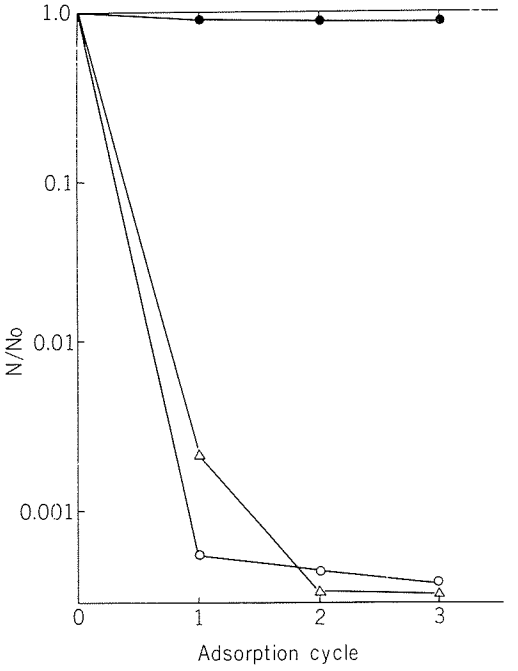

FIGURE 2. *Adsorptions of three strains of echovirus type 7 to human group O erythrocytes.*
N/No: Fraction of residual titer (No: initial titer, N: titer after adsorption). The strains used were Liq-74 (●——●), 16-38 (△——△) *and Wallace* (○——○).

viruses appeared to be caused by differences of their efficiencies of adsorption to human erythrocytes. To examine this possibility, adsorption tests were carried out with two HA(+) and one HA(−) strain.

After each cycle of adsorption, the fraction of virus remaining was determined by calculating the PFU ratio. Results are shown in Fig. 2.

The two HA(+) strains were readily adsorbed to erythrocytes by one adsorption procedure, while only one per cent of the HA(−) strain was adsorbed even after three successive adsorptions.

6. *Correlation between biological characteristics*

The rct/40 marker test was carried out to observe the relation of the virulence of the isolated strains to their other biological characteristics. Results are shown in Table 3.

No relationship was found between the rct/40 marker and the source of the isolated strains, but both the HA activity and plaque size of the strains were closely related to their virulence or pathogenicity judging from their source of isolation.

Namely, without exception, the HA(−) strains from both patients and healthy children formed small plaques while HA(+) strains

TABLE 3. *Biological characteristics of isolated strains of echovirus type 7*

Virus Strain	Plaque size (mm)	Hemagglutinating activity	rct/40	Clinical symptoms
Wallace	6.5–9.0 (7.6)[a]	+	t^-	None
16–38	7.5–10.0 (8.6)	+	$t^\pm$	None
H-T	3.5–4.5 (4.1)	−	t^-	Aseptic Meningitis
Liq-74	2.0–3.5 (2.7)	−	t^-	Aseptic Meningitis

a Numbers in parenthesis indicate the geometric mean diameter of plaques of each strain in PMK cells.

TABLE 4. *Cross neutralization tests with isolated and prototype strains of echovirus type 7*

Virus Antigen	NT antibody titers of immune serum to				
	Wallace[c] A	16–38[c] C	18–02[c] A	H-T[d] A	Liq-74[d] B
Wallace	8,192	256	256	16	8
16–38[a]	128	8,192	4,096	8	8
18–02[a]	1,024	4,096	16,384	16	32
H-T[b]	128	32	16	8,192	1,024
Liq-74[b]	64	64	32	2,048	16,384
K-72-S[b]	128	64	16	512	64
16–34[a]	128	32	32	2,048	4,096

a Strains isolated from healthy children.
b Strains isolated from patients.
c Immune sera prepared against HA (+) strains.
d Immune sera prepared against HA (−) strains.

from healthy children and the Wallace strain formed large ones.

7. *Cross neutralization test*

Five antisera and seven antigens of echovirus type 7 were employed in the cross NT test and their cross patterns are shown in Table 4.

Anti-Wallace strain serum neutralized all the isolated strains tested, but none of the sera against newly isolated strains neutralized the prototype strain appreciably. There were close antigenic relationships between the HA (+) strains and between the HA(−) strains, respectively. However, there was a weak relationship between the antigenicities of HA(+) and HA(−) strains. The HA(−) strain 16–34, isolated from a healthy child, had a similar antigenicity to the strains from patients.

8. *Cross hemagglutination-inhibition test*

The patterns of the cross HI reaction tested with five HA(+) strains and six antisera are shown in Table 5.

Cross tests showed an apparent antigenic difference between HA(+) and HA(−) strains.

TABLE 5. *Cross hemagglutination-inhibition tests with isolated and prototype strains of echovirus type 7*

Virus Antigen	HI antibody titers of immune serum to					
	Wallace A	16-38[b] C	18-02[b] A	16-04[c] A	H-T[c] A	Liq-74[c] B
Wallace	2,048	128	128	<8	8	<8
16-38[a]	32	4,096	2,048	<8	<8	<8
18-02[a]	64	4,096	8,192	<8	<8	<8
16-129[a]	16	4,096	1,024	<8	<8	<8
10-242[a]	32	2,048	4,096	<8	<8	<8

a Strains isolated from healthy children.
b Immune sera prepared against HA (+) strains.
c Immune sera prepared against HA (−) strains; 16-04, strain from a healthy child and others from patients.

DISCUSSION

The relationship between the biological characteristics and the virulence of 58 isolated strains of echovirus type 7 was studied, and the antigenic variations of these strains were analysed. The 58 isolated strains were divided into two groups on the basis of their HA activity with human group O erythrocytes.

About 60% of the strains isolated from healthy children had HA activity, while 40% of the strains from healthy children and all the strains from patients with aseptic meningitis had no HA activity. This indicates that there were two natural groups of echovirus type 7, HA(+) and HA(−), during the years 1962–1968.

It has already been reported that newly isolated strains of enterovirus such as echovirus type 6 (Bussell et al., 1962) and some enteroviruses (Kern et al., 1966), do not show HA activity even though their prototype strains do. But in previous reports, only the HA character of the newly isolated viruses was described and neither their HA characters after long-term passage in vitro nor other characteristics of the viruses were examined.

In this study on echovirus type 7, the correlation of HA activity with other biological properties of the viruses was studied in detail. All the isolated strains of echovirus type 7 tested were passaged in PMK cells to minimize any

change of their HA activity, since the HA activity of hemagglutinating echoviruses often changes from positive to negative on passage from a primary cell culture to an established cell line (Maisel et al., 1961 ; Podoplekin 1964). A similar phenomenon was observed in the case of rubella virus by Suganuma (1970). The HA activity of the viruses tested did not change after multiple in vitro passages in PMK cells.

We obtained good yields of infectious virus with HA(−) strains in PMK cells, so the HA negative character is not due to a low virus yield. In comfirmation of this, the HA activity of HA(−) strains after their 100-fold concentration was still negative.

Treatment of the virus with fluorocarbon to remove HA inhibitors in the culture fluid did not change the HA activity of HA(−) strains. These results indicate that the HA(−) character of viruses is stable and is not due to the presence of hemagglutinin in a cryptic form. Thus it is concluded that the difference between the HA activities of HA(+) and HA(−) strains is due to the presence or absence of hemagglutinin in the virus particles.

This stable HA negative character of isolated strains is correlated with their virulence in man, as shown in Table 1, and provides an interesting clue to the problem of the virulence of echovirus.

Other biological properties of some of the isolated strains were also examined (Table 3). The HA activity was closely related with the plaque size of the virus on PMK cell monolayers : the strains isolated from patients formed small plaques, while the HA(+) strains from healthy children and the prototype strain formed large ones. The HA(−) strains from healthy children also formed small plaques.

However, the results of the rct/40 marker test did not always correspond with HA activity. In the case of echovirus type 9, both t^- and t^+ strains were isolated from patients with aseptic meningitis and the t^+ character of the isolated strains was correlated with the virulence of these strains in mice (Margalith et al., 1967). The mouse-virulence of this virus did not always seem to be correlated with its virulence in man.

The HA activity, plaque size and source of the virus, which seemed to be closely related with the virulence, correlated well with results of the cross NT and HI tests (Tables 4 and 5), and the cross HI test particularly showed marked antigenic differences between HA (+) and HA(−) strains.

The newly isolated strains tested which were HA(−) seemed to be antigenically different from the prototype strain, for they were weekly inhibited by antiserum to the Wallace strain in the NT and HI tests. A particularly striking antigenic difference was obtained by the HI test (Table 5). HA(−) strains must be antigenic variants of echovirus type 7.

It is still uncertain whether the strains from patients had any hemagglutinin before infection of man or whether they became HA(−) after infection.

The HA(−) strains from healthy children seem to be virulent ones judging from their plaque size and antigenic properties, and these children probably received inapparent infection with these strains.

This conclusion is important in relation to epidemiological studies on echovirus.

Further studies on the characteristics of these viruses, including their genetic properties, are in progress.

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