VIROLOGIC AND SEROLOGIC STUDIES ON AN OUTBREAK OF ECHOVIRUS TYPE 11 INFECTION IN A HOSPITAL MATERNITY UNIT

AYAKO HASEGAWA

Central Virus Diagnostic Laboratory, National Institute of Health, 3260 Nakato, Musashimurayama, Tokyo 190-12, Japan

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SUMMARY: An outbreak of echovirus type 11 (E-11) infection occurred among newborn babies in a hospital maternity unit in the summer of 1971. The results of studies are as follows:

1) Forty-one of 188 infants developed febrile illness with stomatitis during one and a half months from July to September. E-11 was isolated from stool specimens of 14 infants and two throat swabs. Antibody response to the virus was shown in all the 19 cases examined. Some of their mothers were suffering from subclinical infection.

2) The isolates were identified as a variant of E-11 which is not neutralized with antiserum against prototype E-11. Antiserum against the current virus neutralized both current and prototype viruses.

3) Sucrose gradient centrifugation of sera from infants revealed that the neutralizing antibody activity resided more predominantly in 19S than in 7S fractions. These antibodies reacted more specifically with the current strain than with the prototype Gregory strain.

INTRODUCTION

Various types of enterovirus are disseminated every summer in communities of the temperate zone, causing outbreaks of different clinical types of illness, particularly among infants and children. In the summer of 1971 unusual prevalence of aseptic meningitis was noted throughout Japan; echovirus type 11 (E-11) was found to have played the main role. It was also noticed that a number of newborn babies and infants under 6 months of age were involved, despite the rule that these age groups are usually exempted from illness because of the passage of immunity from the mothers. In the epidemic areas E-11 was recovered from adults, hence it was suspected that pregnant women might have been infected with E-11 and thus its passage via placenta to babies might have happened.

This report describes the results of studies focused on perinatal E-11 infections breaking out during July and August, 1971, in a maternity unit of K Hospital in Tokyo.
Materials and Methods

Specimens for virus isolation: Stool specimens and throat swabs were collected from 14 infants having fever and stomatitis. They were born between mid-July and the end of September, 1971, at K Hospital in Tokyo. All the specimens were stored at -20°C in a freezer before use.

Patient’s sera: Acute and convalescent phase sera were obtained from the babies described above. At the same time 11 mothers were bled when the acute sera were collected from their babies. As controls, sera were collected from healthy infants who were born at the same hospital during the same period and those of their mothers.

Cell cultures: Primary cynomolgus monkey kidney (MK) and human embryonic lung (HEL) cell cultures were mainly used. MK and HEL cells were grown in lactalbumin-Earle (LE) medium plus 2% bovine serum and Eagle’s MEM plus 10% calf serum, respectively. MK cells were maintained in LE medium without serum and HEL cells in Eagle’s MEM plus 2% calf serum.

Virus isolation and identification: MK and HEL tube cultures were inoculated with 0.2 to 1-ml amounts of properly diluted materials: 10% stool suspensions and throat swabs. All cultures were incubated at 37°C for 7 days. Three blind passages were performed before they were considered negative. Cultures having shown cytopathic effects (CPE) were usually passaged at least once to increase the infectivity titer before identification tests. The isolates were identified by the neutralization test.

Fractionation of 19S and 7S antibodies: A 0.4-ml amount of a serum specimen, diluted 1:4, was layered on 4 ml of 12.5 to 37% (w/v) sucrose-PBS gradient and centrifuged at 35,000 rpm for 16 hr at 5°C in a Spinco SW 39 rotor. After centrifugation, fractions were collected from the bottom of the tubes.

Serologic tests: (a) Neutralization test (NT) using tube cultures: Serial dilutions of serum, inactivated at 56°C for 30 min, were mixed with equal quantities of a virus suspension containing about 100 TCD₅₀ per 0.1 ml. After incubation at 37°C for 3 hr, 0.2 ml of the mixture was inoculated into each of two HEL cell-culture tubes. The cultures were incubated at 37°C; CPE was read and recorded 4 days postinoculation. (b) Plaque reduction neutralization test (modified by Tagaya, 1964): Serial twofold dilutions of immune sera or fractionated patients’ sera were mixed with about 100 PFU of virus per 0.1 ml. The mixture was incubated for 2 hr at 37°C and 0.2 ml of the serum-virus mixture was inoculated onto HEL cell monolayers in 60-mm plastic dishes (Falcon). After an adsorption period of one hour at 37°C, the monolayers were covered with 0.9% agarose in 199 medium enriched with 2% calf serum and 0.15% sodium bicarbonate. A second overlay medium containing neutral red in a final dilution of 1:60,000 was added to the culture 2 to 4 days postinoculation. The highest dilution of serum giving 80% reduction of plaque count vs. control count was considered as the end point of neutralization. (c) Hemagglutination-inhibition (HI) tests (Philipson and Bengtsson, 1962): Hemagglutination (HA) and HI tests were performed by the
HA antigens were prepared from the prototype Gregory strain and the current strain (YC-71-106) in human embryonic kidney cells. The diluent used was phosphate buffer saline, pH 7.4, to which kaolin-treated normal rabbit serum was added to 0.1%. Human type-O erythrocytes were used within a week after collection. Serial twofold dilutions in volumes of 25 µl were made in microplates U-type (Cooke Engineering Co., Alexandria, Va.), and then the same volume of 4 units of E-11 HA antigen was added to each cup. After incubation at room temperature for one hour, 50 µl of a 0.4% erythrocyte suspension was added to each well. The microplates were shaken and held at room temperature for 90 to 120 min. (d) Titration of hemolysin added as a marker of 19S and 7S antibodies: To each of 25 µl amounts of twofold serial dilutions of fractionated specimens, were added 50 µl of complement (2 CH₅O) and 25 µl of a 1.7% sheep erythrocyte suspension. The microplates were then sealed with Pressure-Sensitive Film (Falcon Plastics, Dickinson & Co. Becton Division) and incubated at 37°C for one hour with occasional shaking. Then the plates were centrifuged at 1,500 rpm for 5 min in an I.E.C. centrifuge with the aid of special adaptors (Cooke Engineering Co., Alexandria, Va.). The hemolysin titer was the dilution giving 50% hemolysis.

Preparation of immune sera: Anti E-11 prototype strain monkey serum was kindly supplied by the Department of Enteroviruses of this Institute. Three immune sera to the current strains, YC-71-106, YC-71-321 and YC-71-589, were prepared in guinea pigs weighing about 500 g by injection with each antigen three times at intervals of 3 to 4 weeks. The sera were collected a week after the last injection. The first injection was made intramuscularly; the second and the third were subcutaneously. The antigen used was MK cell-culture fluid centrifuged at 10,000 rpm for 30 min and then treated with fluorocarbon, whose infectivity was about 10⁸ TCD₅₀ per ml.

**Results**

**An Outbreak of Echovirus Type 11 Infection in a Maternity Unit**

Between 21 July and 9 October 1971, 41 newborn babies at K Hospital in Tokyo developed symptoms: 31 cases had pyrexia; other four with stomatitis; and the other six with fever and stomatitis. Since 188 babies were born during the above period, the attack rate was about 22%. The ages of newborns at the onset of disease ranged from 3 to 21 days after birth. The incidence of infection was the highest in August, the number of patients being 32.

Fecal and throat-swab specimens were obtained for virus isolation from 14 of those 41 infants. CPE agents were recovered from stool of the 14 babies and from two throat swabs. They were not neutralized with intersecting pools of anti-enterovirus sera, but the immune guinea-pig sera against the YC-71-106 strain neutralized both the homologous strain and the prototype Gregory strain; their neutralizing antibody titers were 1:1280 and 1:640, respectively. Therefore, those 16 CPE agents were identified as E-11 together with the following results of serologic tests with paired patients’ sera shown in Table I.
TABLE I
Sero logic results with infected infants and their mothers

<table>
<thead>
<tr>
<th></th>
<th>Neutralizing Antibody</th>
<th>HI Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Rise*</td>
<td>4-fold or more Rise</td>
</tr>
<tr>
<td>Current virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(YC-71-106)</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Prototype virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Gregory)</td>
<td>6</td>
<td>13</td>
</tr>
</tbody>
</table>

* Nineteen paired sera were assayed for neutralizing antibody, and 15 paired sera for HI antibody.

** Eleven mothers were bled when the acute sera were collected from their babies.

Nineteen pairs of acute and convalescent sera were assayed for antibody rise to the isolate, YC-71-106 strain, in HEL cell tube cultures. Four-fold or more rises of neutralizing antibody titers were found with 15 paired sera (79%). In the other 4 patients, acute sera already showed high titers of neutralizing antibody. Similar results were obtained with prototype Gregory strain as antigen. Twelve of these 19 paired sera were from the newborns, from whose feces E-11 was isolated. Seven of 11 mothers’ sera had no neutralizing antibody (less than 1:4) against the current strain, and five of them had no antibody to the Gregory strain either. It is peculiar that about half of mothers had no detectable antibody to either the epidemic or the prototype E-11 strain.

NT was carried out with control sera collected during the same epidemic period from 12 healthy infants and their mothers. Ten of the 12 pairs of infants’ and mothers’ sera had titers less than 1:4, and the other two had 1:8 and 1:64.

In HI tests, four fold or greater HI-antibody rises were shown in 10 of the 15 babies; their antibody titers were higher with the current strain than with the prototype strain.

Antigenicity of the Current Strains

As stated above, CPE agents from this outbreak were identified as an antigenic variant of E-11. Therefore, to find if there was any immunological difference among the isolates, cross neutralization tests by the plaque reduction and the cross hemagglutination-inhibition tests were performed between two isolates; strain YC-71-321 isolated on 19 August and strain YC-71-589 isolated on 9 September with
TABLE II
Cross Neutralization Tests with two isolated Strains and the Prototype Strain of Echovirus Type 11

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Immune Serum to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>prototype*</td>
</tr>
<tr>
<td>prototype</td>
<td>80.0**%</td>
</tr>
<tr>
<td>YC-71-321</td>
<td>27.8</td>
</tr>
<tr>
<td>YC-71-589</td>
<td>29.1</td>
</tr>
</tbody>
</table>

* Immune serum prepared in cynomolgus Monkeys
+ Immune serum prepared in Guinea Pigs

** The plaque reduction percentages of each strain with heterologous antisera were measured at one serum dilution giving 80% plaque reduction of homologous virus which was determined by preliminary tests. The homologous neutralizing end points of each serum were: anti-prototype serum, 1:6800; anti YC-71-321, 1:7000; and anti YC-71-589, 1:6500.

TABLE III
Cross Hemagglutination-Inhibition Tests with two isolated strains and Prototype Strain of Echovirus Type 11

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Hemagglutination-Inhibition Titer of Immune Serum to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prototype (Gregory)</td>
<td>prototype</td>
</tr>
<tr>
<td>current (YC-71-321)</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>32</td>
</tr>
</tbody>
</table>

The prototype Gregory strain as a reference and the corresponding immune sera.

Table II summarizes the results of cross neutralization tests. The immune sera against the YC-71-321 and the YC-71-589 strains neutralized both the homologous and heterologous strains to the same extents. The anti-Gregory immune serum showed lower neutralizing activities to the two isolates tested. As shown in Table III, the results of HI tests also indicate similar antigenic relationship between the two isolates and the prototype Gregory strain.

It is clear from the results that two current strains have broader antigenicity than that of the prototype Gregory. However, no significant antigenic difference was found between the two current strains by either cross neutralization or HI tests.

Antibodies in 7S and 19S Fractions

To find whether or not the antibody against E-11 detected in acute phase serum specimens of infants originated from their mothers, the sera from infants and their mothers were fractionated by sucrose gradient centrifugation and determined for HI and neutralizing antibody activities to E-11.
Firstly, HI-antibody titers against E-11 were tested on four kaolin treated and untreated sera to determine for nonspecific hemagglutinin inhibitors. In two sera which had no NT antibody, the HI activity detected in the top fractions (fractions No. 10 and above) was considered to have been due to nonspecific inhibitors, because they were removed completely by the kaolin treatment. On the other hand, two sera which had NT antibody showed two peaks of HI activity even after the kaolin treatment. But the peaks were smaller than those of untreated one, suggesting some adsorption of antibody to kaolin. Therefore, subsequent HI tests were performed on untreated sera; hemolysin was determined as a marker to locate 19S and 7S antibodies. The results obtained are shown in Fig. 1.

HI antibody activity to E-11 was detected in the 19S fractions of convalescent sera (I-B & II-B), but not in acute phase sera of the infants (I-A & II-A). These infants were confirmed to have been infected with E-11 by both virus isolation and NT. Similarly, one mother was infected with E-11 at the same time as the baby was, as convalescent serum from the mother (I-D) had detectable HI antibody. The HI activity found in fractions No. 11 and above in these acute phase sera (I-C, II-A & II-C) may probably have been due to nonspecific inhibitors. It is certain that the acute phase sera from the other two infants (III-A & IV-A) contained 19S antibody and the convalescent sera (III-B & IV-B) both 19S and 7S

<table>
<thead>
<tr>
<th>Patient</th>
<th>Infant’s Date of birth</th>
<th>Date of Onset</th>
<th>Date of Serum Specimen</th>
<th>Days after onset</th>
<th>No. of Serum Specimen</th>
<th>Neutralizing Antibody Titer* against E-11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19S-Antibody current</td>
</tr>
<tr>
<td>Mother</td>
<td>8/20/71 9/11/71</td>
<td></td>
<td>S-222 S-1494</td>
<td>N.D. 50</td>
<td>N.D. 110</td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>8/28/71 9/11/71</td>
<td></td>
<td>S-1415</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>8/21/71 9/11/71</td>
<td></td>
<td>S-1407</td>
<td>N.D.</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>8/24/71 8/30/71 8/30/71</td>
<td>1 1</td>
<td>S-1431</td>
<td>80 52</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>8/30/71 9/11/71</td>
<td></td>
<td>S-1433</td>
<td>N.D.</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>8/12/71 8/16/71 8/20/71 9/11/71 8/20/71 9/11/71</td>
<td>5 27</td>
<td>S-1398 S-1399</td>
<td>N.D. 60</td>
<td>N.D. 18</td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>9/11/71</td>
<td></td>
<td>S-1495</td>
<td>17</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

* The neutralizing titer is the highest dilution of serum which gives a 80% reduction in plaque count.
Fig. 1. HI antibody titers against E-11 in sera of infants and their mothers fractionated by sucrose gradient centrifugation.

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HI titers .... Hemolysin titers of rabbit anti-sheep hemolysin as a marker.
antibodies, although the 7S fractions contained some HI activity due to nonspecific inhibitors.

To determine whether the 7S fractions with low HI antibody titers as mentioned above had neutralizing activities or not, plaque reduction neutralization tests were performed. Three fractions from the peak of 19S and that of 7S positions located in reference to the hemolysin titers were pooled and each pool was diluted 1:10. Serial twofold dilutions of these materials were mixed with equal volumes of virus (100 PFU/0.1 ml), the prototype strain and the current strain (YC-71-321) of E-11. The results obtained are summarized in Table IV. The 7S and 19S antibodies to E-11 were found in convalescent sera from all affected infants. Two acute phase sera (III & IV) contained 19S antibody, and moreover one serum (IV) had 7S antibody. It was considered that the infant must have produced 7S antibody within 7 days after delivery, since his mother had no detectable antibodies. The neonatal immune response was more predominant in 19S than in 7S antibody. There was a distinct difference in antibody titers between the prototype and the epidemic strain of E-11. In the 19S fractions, antibodies against both strains were detected, but titers of the antibody to the prototype strain was lower, being approximately one-fourth to one-fifth of those to the current strain. The 7S fractions, on the other hand, usually contained antibody to the current strain only and had titers lower than 1:10 against the prototype strain. The E-11 infection in mothers was suggested by the fact that two convalescent sera (I & V) from mothers had both 19S and 7S antibodies.

**DISCUSSION**

Antigenic variation among strains of echovirus type 11 was first suggested with so-called “U-virus” strains isolated by Philipson and Wéssel in Sweden in 1956 (Philipson and Rosen, 1959). It was noted afterwards that U-virus was an E-11 virus having broader antigenicity than the prototype strain. A similar antigenic variant of E-11 was recovered from newborn babies during the outbreak reported here. Moreover, antigenic diversity was anticipated between an early isolated strain (YC-71-321) and a late isolated one (YC-71-589) in this outbreak; such antigenic variation of the epidemic strains might represent a progressive “drift” away during transmission from an infant to another. However, such antigenic variation was not found in these two epidemic strains (Tables II and III).

With regard to E-11 infection among newborn babies, there have been several reports (Berkovich and Kibrick, 1964; Lapinleimu, 1971; Cramblett et al., 1973). The data in Table I are similar to those reported previously. E-11, as reported by Tagaya and Moritsugu (1973), has been isolated sporadically since 1956 and the illness due to this agent has often been prevalent in Japan. In 1971, however, adults as well as children were infected with E-11. The reason might reasonably be speculated that they were not immune to either the current virus or the prototype strain.

On immunological response in human fetuses and newborns, many con-
Contradictory results have been reported (Osborn, Dancis and Julia, 1952; Zak and Good, 1959; Koprowski et al., 1956). It is generally agreed that the immune response of newborn babies is weaker than that of adults (Osborn et al., 1952; Zak and Good, 1959). It was demonstrated both in man and in experimental animals that in the primary response, 19S antibody is produced predominantly within one week after the antigenic stimulus and, within a few weeks, there is a gradual shift to 7S. Some papers reported considerable difference in immune response to various virus antigens between newborns and adults (Alfred, 1965; Schludeberg, 1965; Mäkelä, Lapinleimu and Kostiainen, 1968). Mäkelä et al. (1968) reported that in infections with coxsackievirus B-3 or B-5, the immune response of infants was as good as that of their mothers, early 19S antibody was replaced by 7S antibody within 2 months and by the end of the 3rd month very little 19S activity could be found. As shown in Table IV, small amounts of 7S as well as 19S antibodies were present in the serum of one infant as early as 7 days post-partum despite the absence of 7S antibody in his mother, which suggests that the newborns are capable of synthesizing both 19S and 7S antibodies. However, the shift from 19S to 7S was slower in infants than in adults (Figs. I-B & I-D). In fact, in one infant (data are not shown), 19S was detected up to 60 days after birth as a large part of the total antibody.

Infants' sera neutralized the current virus more completely than the prototype strain (Table IV), but immune sera against the current viruses neutralized the prototype as well as the homologous strain as shown in Table II. This difference might be related to the fact that the antibody to E-11 in newborn babies was produced by the primary antigenic stimulus and guinea pigs' immune sera were prepared by at least three successive antigenic stimuli.

It is concluded that the newborns acquired E-11 infection not in utero but after birth, since no antibody to the prototype or the current strains of E-11 was found in their mothers. Despite the serological result (Berkovich and Smithnick, 1968) and the virus isolation from newborns (Hughes et al., 1972; Philip and Larson, 1972) suggesting vertical transmission of echoviruses, the data seem to be far from conclusion. The author attempted to isolate viruses from 38 artificially aborted embryonic specimens taken in the epidemic areas in Tokushima Prefecture, south Japan (Hasegawa and Miyao, 1973), but the results were negative. From these experiences together with the data presented here, there is very little possibility of vertical transmission of E-11. Thus the negative data conform with previous reports stating that intrauterine infection with echoviruses was not found (Sabin, Krumbiegel and Wigand, 1958; Landsman, Grist and Ross, 1964).

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