An epidemic of meningitis caused by ECHO virus type 4, was reported in many areas of Japan during the summer and early autumn of 1964. (1) During May to August of 1965, aseptic meningitis was prevalent chiefly in the northern parts of Kyushu. It was revealed that this was an epidemic of ECHO virus type 4.

The present paper reports the results of study of this epidemic. The study is divided into the following parts:

1. Laboratory data from aseptic meningitis cases.
2. Distribution of cases according to age, sex, geography and season.
3. Antigenic and plaque characteristics of isolated ECHO virus type 4 strains.

MATERIALS AND METHODS

Virus

ECHO virus type 4 Pesascek strain and DuToit strain were used after primary monkey kidney (MK) cell passage in this laboratory. Stock virus was propagated in MK cell monolayers in the cysteine medium and harvested after 2 or 3 days of incubation at 37°C. The culture was frozen and thawed to disrupt cells and centrifuged. Supernatant fluid was stored at -20°C.

Tissue culture

MK cell were used for the experiments. The cells were grown in bottles or tubes with Melnick-Hanks solution containing 3% bovine serum at the temperature of 37°C. Puck's salt solution was used for washing the cultures and for diluting the virus or serum. As a maintenance medium, Melnick-Earle's solution with 10% skim milk solution (10%) was used.
Immune sera for typing

Type specific immune sera against 33 types of ECHO virus were obtained from Japan Live Poliovaccine Research Commission. The sera were extracted from monkeys that were immunized with each type of ECHO viruses. All sera, after dilution to 100 units in Puck's solution, were inactivated at 56°C for 30 minutes.

Serum specimens

In October, 1964, 200 blood samples were collected from healthy people in the northern parts of Kyushu. During May and August, 1965, 111 blood samples were collected from aseptic meningitis patients in Moji, Fukuoka prefecture and Omura, Nagasaki prefecture. The serum was separated from the clot and kept frozen at -20°C during storage.

Materials for virus isolation

During May and August, 235 specimens were obtained from aseptic meningitis patients in Moji and Omura. Virus isolation was attempted on these specimens. The specimens were placed in small test tubes which were then tightly sealed with rubber stoppers and sent to the laboratory by messenger. The specimens were kept frozen at -20°C upon their arrival at the laboratory until use.

Method of isolation of cytopathogenic agent.

The stool specimen was weighted and emulsified with Puck's solution which contained 500 units of penicillin per ml and 500 micrograms of streptomycin per ml to make a 10% emulsion. It was then centrifuged at 3,000 rpm for 20 minutes, and 0.2 ml of the supernate was inoculated into tissue culture tubes. Three or more tissue culture tubes were used for each specimen, and cultures were observed daily for a week. If any cytopathogenicity was noted at the height of cellular damage, the fluid and cells were scraped from the tube and harvested for second passage and storage. If no cytopathogenicity was observed, a second passage was made with culture fluid and the cells were harvested at the end of the first week of cultivation. Tubes that were negative after 2 weeks in the second passage were discarded. Cytopathogenic agents that had undergone 2 or 3 passages were preserved in a frozen state until typed.

Method of isolation of cytopathogenic agent from cerebrospinal fluid (CSF) or blood samples.

The MK cell monolayer grown in the bottle washed once with Puck's solution, maintenance medium added, and sample inoculation was done after 24 hours. After removal of the maintenance medium, the monolayer was washed with Puck's solution and inoculated with 0.2 ml of CSF or blood samples. After adsorption for 2 hours, the bottle was washed once with Puck's solution and agar overlay was added. The overlay consisted of 1.5% agar (Difco Bacto) in Hank's BSS containing 2% calf serum, 0.22% sodium bicarbonate and neutral-red at a concentration of 1:60,000. After the agar solidified, the bottles were kept at 37°C for 3 or 5 days. The number and the size of plaques were observed, and pick plaques if present, and subcultures were made into tube cultures for further identification.
Neutralization tests and typing of the isolated virus agents

Sera were inactivated at 56°C for 30 minutes before use. Neutralization tests were carried out as follows: antibody titration of sera sampled were diluted in 2-fold steps, and the neutralizing effect was tested by 100 TCD₅₀ of the viruses. Titration of the viruses used.

Serial 10-fold dilutions of the infected nutrient fluid to be tested were made, and 0.1 ml of each dilution was introduced into 4 tubes which contained 1.0 ml of the medium. Seven days after inoculation, the tubes were examined microscopically for evidence of specific degeneration, and the end point was expressed in term of TCD₅₀ which was calculated by the Behrens-Kärber formula.

typing of ECHO viruses.

The procedure for typing was almost identical with the neutralization test of the recognized serum. Type-specific monkey immune serum pool (Schmidt pool) was combined with an equal amount of 100 TCD₅₀ virus containing tissue culture fluid which showed cytopathogenicity. The type was identified by a positive neutralization test against one of the type specific immune sera.

EXPERIMENTAL RESULTS

Aseptic meningitis in the northern part of Kyushu, during May and August, 1965.

During summer and early autumn 1964, outbreaks of aseptic meningitis due to ECHO virus type 4 were reported from many places in Honshu.

The epidemic of 1965 was first reported in Omura, Nagasaki prefecture, at the end of May and the peak of incidence was reached during the beginning of July. It spread centrifugally to the neighboring districts, including Saga and Fukuoka prefecture where the peak of the epidemic occurred during the begin-
ing of August. The seasonal distribution of aseptic meningitis in Omura and Moji from May to August is shown in Fig. 1.

Fig. 1. Notified cases of aseptic meningitis, by week of onset in the northern parts of Kyushu, 1965.

ASEPTIC MENINGITIS IN SUMMER 1965

ÖMURA

MOJI

May June July August
Fig. 2. Sex difference and age distribution of aseptic meningitis cases in the northern parts of Kyushu, 1965.

Fig. 3. Continuation of clinical symptoms of aseptic meningitis

> Fifty per cent of patients had vomiting for the 2 days.
Outbreaks of aseptic meningitis were reported from many areas throughout Japan in 1965 and virological investigations revealed that those in Hokkaido and Honshu were mainly due to ECHO virus type 6 while those in Shikoku and Kyushu were caused by ECHO virus type 4.

Clinical aspects.

The sex difference and age distribution of aseptic meningitis cases in 1965 are shown in Fig. 2.

In this epidemic, the peak of incidence was in the age group of 2 to 8 years. Male cases were remarkably reported higher than female cases as shown in Fig. 2. Clinical symptoms were mild as tabulated in Fig. 3, but, pleocytosis in CSF persisted for several weeks.

Isolation and typing of ECHO virus

The authors attempted to isolate ECHO virus from feces, CSF and blood specimens of aseptic meningitis patients by inoculation of tissue cultures of MK cells. The results are summarized in Table 1. Eighty-five agents were isolated during May and August in Omura and Moji.

Distribution of infections as measured by neutralizing ECHO type 4 antibody.

Fifty-eight and 53 serum specimens from aseptic meningitis patients living Omura and Moji, respectively, were examined. The results are summarized in Table 1 and indicate that antibody titers against ECHO type 4 was detected among most of the aseptic meningitis patients.

Virological and serological examinations revealed that this epidemic to be due to ECHO virus type 4.

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virological examination from clinical specimens of aseptic meningitis</strong></td>
</tr>
<tr>
<td>number of specimens</td>
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<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Omura civilian hospital</td>
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<tr>
<td>Omura national hospital</td>
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<tr>
<td>Moji civilian hospital</td>
</tr>
<tr>
<td>Total</td>
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<tr>
<td>(%)</td>
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</tbody>
</table>

# positive isolation / number of specimens
## positive identified / number of sera
### positive identified / number of cases examined

Serum neutralization test against 3 strains of ECHO virus type 4.

Sixteen convalescent serum of aseptic meningitis patients were tested against 3 strains of ECHO type 4 viruses. ECHO virus type 4. DuToit strain, was obtained
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from Dr. I. Tagaya of NIH, Tokyo. Two wild ECHO 4 strains (KM-8, KN-6) were isolated in this laboratory from patients ill with aseptic meningitis. The results are summarized in Table 2.

Results represent reciprocals of 50% serum dilution endpoints obtained at the time the final TCD₅₀ endpoint in the control virus titrations was first reached. DuToit strain was neutralized effectively by 16 convalescent serum however 2 strains, KM-8 and KN-6, were not significantly neutralized by any convalescent serum.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serum Neutralization Test Against 3 Strains of ECHO Type 4</th>
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<tbody>
<tr>
<td></td>
<td>Serum Strain</td>
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<tr>
<td>---------</td>
<td>------------------</td>
</tr>
<tr>
<td>C-38</td>
<td>16</td>
</tr>
<tr>
<td>C-49</td>
<td>16</td>
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<tr>
<td>C-27</td>
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<tr>
<td>C-36</td>
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<tr>
<td>C-33</td>
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<tr>
<td>C-35</td>
<td>8</td>
</tr>
<tr>
<td>M-15</td>
<td>&lt;4</td>
</tr>
<tr>
<td>N-15</td>
<td>&lt;4</td>
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<tr>
<td>M-37</td>
<td>32</td>
</tr>
<tr>
<td>M-34</td>
<td>8</td>
</tr>
</tbody>
</table>

**Plaque Characteristics of Isolated ECHO Virus Type 4 Strains.**

The plaque morphology of isolated ECHO virus type 4 strains was not distinguishable from that obtained by the prototype Pesascek strain. The plaques were almost round with an irregular margin and the lysed center was rather well defined.

In bottles stained after incubation for 4 to 5 days, the plaque varied in size from 4 to 8 mm. A different plaque morphology was obtained with DuToit strain. Irregularly sharply defined degenerated areas approximately 0.2 to 0.5 mm were seen when viewed by direct illumination.

**ECHO Type 4 Antibody Status Before Epidemic of Aseptic Meningitis.**

In October, 1964, 200 blood samples were collected from healthy people in the northern part of Kyushu, and a comparison of children having antibody to ECHO type 4 Pesascek strain and DuToit strain was determined. The results are shown in Fig. 4.
Antibody titers of 4 or higher are referred to as "positive" and lower as "negative". Twenty-eight per cent of total children were "positive" and the remaining 72% "negative".

Among children from 0 to 1 years 10% were "positive" while none of those from 2 to 6 years had antibodies, suggesting the transfer and the following decline of maternal antibodies. One fifth of the children from 7 to 19 years of age were "positive" and with increasing age over 20 years the "positive" becomes extremely high.

**Fig. 4.** The antibody possessing ratio in each age group against ECHO 4 in northern part of Kyushu, 1964. (200 healthy people)

**DISCUSSION**

During summer and early autumn, 1965, outbreaks of aseptic meningitis were reported from many places in Japan. Virological investigation revealed that those in Hokkaido and Honshu were mainly due to ECHO virus type 6 and those in Shikoku and Kyushu were caused by ECHO virus type 4.

An aseptic meningitis epidemic in Kyushu occurred during the months of May to August. A detailed account of this epidemic and the important clinical findings has been reported.

The present paper reports the results of our study on those epidemics of Omura, Nagasaki prefecture and Moji, Fukuoka prefecture. Twenty four strains
were isolated from 34 feces samples by inoculation of a MK cell tube culture, and 52 strains were isolated from 119 CSF samples by inoculation of a MK cell bottle culture. Nine strains were isolated from 82 blood samples by inoculation of a MK cell bottle culture. The virus in CSF and blood were isolated by plaque method in MK cell bottle culture, but not as frequently as by inoculation into the MK cell tube culture. Our data on the isolation of ECHO virus using MK plaque method confirm the results published by Ishii et al\textsuperscript{4}). This plaque method appears to be more useful for isolation of virus from CSF and blood samples than the use of normal tube method.

Discrete outbreaks of aseptic meningitis due to ECHO virus type 4 have occurred in many parts of the world\textsuperscript{7) 8) 9).} ECHO virus type 4 have been reported to be weakly antigenic\textsuperscript{6) 10),} and neutralizing antibody responses in man and experimental animals have been difficult to demonstrate by the tube neutralization test.

The virus, ECHO type 4, isolated in the present work, was indistinguishable from that obtained with the prototype Pesasek strain with regard to weakly antigenicity and plaque characteristics.

\textbf{Fig. 5. Geographical distribution of outbreaks of aseptic meningitis in Japan in 1964 and 1965.}
SUMMARY

Aseptic meningitis became epidemic in Omura, Nagasaki prefecture and Moji, Fukuoka prefecture in the summer of 1965.

Eighty five agents were isolated during the epidemic of May to August in the northern part of Kyushu. All of those agents were ECHO virus type 4.

The 111 serum specimens from aseptic meningitis patients were examined. Antibody titer against ECHO virus type 4 was detected at the highest percentages of this epidemic.

The virus, ECHO type 4, isolated in this study had characteristic weakly antigenic and plaque character on MK monolayer as prototype Pesascek strain.

REFERENCES