THE FIRST EPIDEMIC OF ACUTE HEMORRHAGIC CONJUNCTIVITIS DUE TO A COXSACKIEVIRUS A24 VARIANT IN OKINAWA, JAPAN, IN 1985-1986

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SUMMARY: Epidemics of acute hemorrhagic conjunctivitis due to a coxsackievirus A24 variant occurred in July-November, 1985 and August-October, 1986 in Okinawa Prefecture, Japan. This is the first report of an acute hemorrhagic conjunctivitis epidemic due to a coxsackievirus A24 variant in Japan. The epidemic involved most islands of the prefecture. The prefectural surveillance center was notified of 9,952 cases in 1985 and 6,096 cases in 1986 from three sentinel eye clinics. The neutralizing antibody-positive rate against the coxsackievirus A24 variant of the serum samples collected before and immediately after the 1985 epidemic rose from 1.0% to 8.5%. The coxsackievirus A24 variant was isolated from 48 out of 68 conjunctival swabs collected during the epidemics. The isolates were indistinguishable antigenically in the plaque reduction test from the prototype strain, EH24/70, but had a markedly distinct oligonucleotide pattern.

INTRODUCTION

Since 1970, two human enteroviruses have caused acute hemorrhagic conjunctivitis (AHC); enterovirus 70 (EV70) (1), a new antigenic type isolated...
during the 1971 AHC epidemics in Morocco, Japan and other countries and an antigenic variant of coxsackievirus A24 (CA24v) (2) isolated from the 1970 AHC epidemics in Singapore. These two enteroviruses induce similar symptoms but CA24v tended to cause more explosive and extensive outbreaks than EV70 in Asian countries (3).

While EV70 spread rapidly after its appearance throughout the eastern hemisphere, CA24v was confined to Southeast Asia and Indian Subcontinent where AHC epidemics due to CA24v have been reported approximately every five years.

In March, 1985, the Government Clinic of Singapore noticed many AHC patients and identified CA24v as the causative agent (3,4). A large outbreak of AHC due to CA24v occurred in July-September, 1986, also in Pakistan (5). Epidemics of AHC due to CA24v were reported in some other countries where no case of CA24v-related AHC had been diagnosed; Taiwan (6) during September, 1985-the end of 1986, Caribbean (Trinidad, Jamaica and St. Croix) (7) in October-November, 1986 and Puerto Rico (8) in June-October, 1987. In addition, Brandful et al. (unpublished data) of University of Ghana isolated many strains of CA24v during an extensive AHC epidemic in May, 1987-January, 1988 in Ghana.

In Japan, EV70 was the only causative agent of AHC after introduction of the disease in 1971, although adenoviruses and other enteroviruses were rarely isolated from eye specimens of clinically diagnosed AHC patients. In the summer of 1985, Okinawa islands, the southwest islands in Japan, experienced an unusually extensive AHC epidemic. Many strains of CA24v were isolated from the patients and wide dissemination of the virus on many islands was confirmed sero-epidemiologically. The epidemic started so suddenly and explosively that some schools had to be partially closed for several days. The outbreaks subsided at the end of 1985, but reappeared in July-November, 1986. No outbreak occurred in 1987. Although most islands were involved, the two-year-epidemics due to CA24v were confined to Okinawa Prefecture and the virus did not spread to the rest of Japan.

The epidemiological features and the laboratory findings of the epidemics of AHC due to CA24v on Okinawa Islands will be described herein.

Description of the area

Okinawa Prefecture comprises 48 islands and constitutes southern half of Nansei Islands located between Kyushu, Japan and Taiwan (Fig. 1). It is located at latitude 24-28° north and longitude 122-133° east. Climatologically the area is
subtropical with an average annual rainfall of 2,128 mm; the lowest average temperature is 16.0°C in January and the highest 28.1°C in July. The prefecture covers 2,250 km² in area and has 10 cities, 15 towns and 28 villages. The population numbered 1.179 million in 1985. There are busy international, domestic and inter-island flights and maritime traffic.

MATERIALS AND METHODS

*National epidemiological surveillance for infectious diseases in Japan:* Three ocular diseases, AHC, pharyngo-conjunctival fever and epidemic keratoconjunctivitis, are notifiable infectious diseases under the program. Collaborating
sentinel eye clinics report weekly clinically diagnosed cases to the Surveillance Center. Approximately 250 eye clinics in all 47 prefectures and 10 designated cities serve as sentinel stations of the ocular diseases. Okinawa Prefecture has three sentinel eye clinics.

**Questionnaire:** Filled-in questionnaires were collected in January, 1986, from 3,035 families of students of six schools; two primary and three junior high schools on Okinawa Mainland and one junior high school on Miyako Island.

**Virus isolation:** HeLa, RD-18S (a cloned line of a human rhabdomyosarcoma cell line, RD) (9), human embryo lung (HEL), and primary monkey kidney cells were used in 1985 and HeLa and RD-18S cells in 1986. Conjunctival swabs were inoculated into tube cultures maintained in Eagle’s minimal essential medium supplemented with fetal calf serum (0-2%). The inoculated cells were incubated on a roller drum at 33 C and subcultured at least three times before the results were considered negative.

**Antiserum:** Anti-EH24/70 sera were obtained from a rabbit immunized with crude virus and a guinea pig immunized with purified virus. Anti-J140/85 serum was from a rabbit immunized with crude virus of a plaque-purified clone (#411) of a 1985 isolate. Anti-Joseph horse serum was provided by the American Type Culture Collection (V-027-502-560).

**Serum samples:** Pre- and post-epidemic serum samples for serosurvey were from normal population in Naha, Hirara and Ishigaki cities collected for routine health examinations and stored at -80 C in the serum bank in Okinawa Prefectural Institute of Public Health. One hundred and ninety-eight serum samples collected from four prefectures including Yamagata, Niigata, Nagano and Fukuoka in 1984 were provided by Serum Reference Bank, National Institute of Health.

**Neutralization (NT) test:** Serum samples were titrated by micro-neutralization in HeLa cells. The serum-virus mixtures were incubated for an hour at 37 C and then overnight at 4 C before inoculation. The viruses were EH24/70 strain of CA24v and J670/71 strain of EV70. The plaque reduction test was carried out in HeLa cells.

**Oligonucleotide mapping of isolates:** The procedures for virus purification, virion RNA extraction and oligonucleotide mapping were described previously (10). Briefly, the virus was purified by sucrose density gradient (15-30%) centrifugation. RNA was extracted, purified through 15-30% sucrose density gradient, and precipitated in ethanol. The RNA (1-2.5 μg) was digested with 10 U of T1-RNase and the 5’ end of the resulting oligonucleotides was labeled with 25 μCi of [γ-32P]ATP (3,000 Ci/mmol, New England Nuclear Research Products, USA) by use of 2.5 U of polynucleotide kinase (Toyobo Co., Tokyo) and subjected to two-dimensional polyacrylamide gel electrophoresis. The first-dimensional electrophoresis was conducted at 4 C in 200 × 400 × 1-mm gel (10% containing 6 M urea, pH 3.5, adjusted with citric acid); the second-dimensional electrophoresis
at room temperature in \(350 \times 450 \times 1\text{-mm}\) gel \((22\%, 50 \text{ mM Tris-borate, pH 8.3})\). The gel slabs were autoradiographed to Sakura AO X-ray film at 4°C.

**RESULTS**

*National Epidemiological Surveillance*

Weekly incidences of AHC reported by three sentinel clinics in Okinawa Prefecture in 1985 and 1986 under the national epidemiological surveillance of infectious diseases are presented in Fig. 2.

The AHC epidemic started in the second week of July, 1985, and explosively spread with peak incidence in early October, then subsided rapidly in December.

![Weekly reported number of AHC patients in Okinawa Prefecture during 1985-1986 (data based on National Epidemiological Surveillance of Infectious Diseases).](image)

Fig. 2. Weekly reported number of AHC patients in Okinawa Prefecture during 1985-1986 (data based on National Epidemiological Surveillance of Infectious Diseases).
The outbreak recurred in 1986, with the highest incidence in August and September, and ceased in November. The prefectural surveillance center was informed from the three sentinel clinics of 9,952 cases in 1985 (11) and 6,096 cases in 1986 (12). The figures accounted for 70% and 61%, respectively, of the total AHC cases in Japan and for 0.84% and 0.52%, respectively, of the total population of the prefecture. The past prefectural surveillance data revealed a preceding small increase of cases in the autumn of 1984.

The age distribution of cases in 1985 and that in 1986 were very similar (Table I). The percentage of schoolchildren, i.e., the 5-9 and 10-14 year groups, among AHC cases were two to three times higher than those in other areas (15.2%-16.1% vs 7.8% and 30.5-31.6% vs 9.1%, respectively). AHC in the areas outside of Okinawa Prefecture was considered to be due to other agents than CA24v, because no CA24v isolation was accompanied with them.

School and Household Surveys

According to the inquiry conducted by the Prefectural Education Bureau, most of the 260 primary schools and 154 junior high schools on 38 islands in the prefecture were involved in the 1985 epidemic with the attack rates ranging from 17 to 54%. The survey by questionnaires of the families of schoolchildren conducted immediately after the 1985 epidemic confirmed the surveillance through the sentinel eye clinics. The age-specific attack rate was highest in the 10 to 14-year group (almost 20%) and lower in adults (6-8%). The most probable place of infection was schools (50%), followed by homes (46%). The duration from the onset of the index case to the peak incidence in schools was 21 days and to the cessation of the outbreak 50 days on average. In most cases, both eyes were affected either concomitantly or in succession but about 10% of patients had unilateral conjunctivitis. Major clinical symptoms were conjunctival redness, subconjunctival hemorrhage and ocular pain as in AHC caused by EV70.
### Table I. Age distribution of AHC patients reported by sentinel eye clinics (National epidemiological surveillance of infectious diseases)

<table>
<thead>
<tr>
<th>Age group</th>
<th>Cases in Okinawa Prefecture (%)</th>
<th>Cases in Japan excluding Okinawa Prefecture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>340 (100.)</td>
<td>9,952 (100.)</td>
</tr>
<tr>
<td>&lt; 1</td>
<td>5 (1.5)</td>
<td>106 (1.1)</td>
</tr>
<tr>
<td>1—4</td>
<td>23 (6.8)</td>
<td>639 (6.4)</td>
</tr>
<tr>
<td>5—9</td>
<td>47 (13.8)</td>
<td>1,512 (15.2)</td>
</tr>
<tr>
<td>10—14</td>
<td>138 (40.6)</td>
<td>3,039 (30.5)</td>
</tr>
<tr>
<td>15—</td>
<td>127 (37.4)</td>
<td>4,656 (46.8)</td>
</tr>
<tr>
<td>Unknown</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table II. Isolation of CA24v

<table>
<thead>
<tr>
<th>Year</th>
<th>Place of residence</th>
<th>Patient</th>
<th>Specimen (conjunctival swab)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Age (median)</td>
<td>Date of collection</td>
</tr>
<tr>
<td>1985</td>
<td>Miyako Island</td>
<td>7-72 (14)</td>
<td>Oct. 25</td>
</tr>
<tr>
<td>1986</td>
<td>Okinawa City</td>
<td>6-40 (17)</td>
<td>Sept. 9</td>
</tr>
<tr>
<td></td>
<td>Ishigaki City</td>
<td>3-79 (33)</td>
<td>Aug. 29-30</td>
</tr>
</tbody>
</table>
Virus Isolation

In 1985, 17 of 25 conjunctival swabs collected on Miyako Island were virus positive. In 1986, the virus was isolated from 15 of 23 conjunctival swabs in Okinawa City and 16 of 20 in Ishigaki City (Table II). Virus was isolated most frequently in RD-18S cells, followed by HEL and HeLa cells. No CPE was observed in primary monkey kidney cells.

The virus was recovered from those ranging from three to 79 years in age. The rate of virus isolation from males and that from females were similar.

The isolates did not react with any set of pooled antisera against enteroviruses or type-specific antisera against EV70, or adenovirus type 1, 3, 4, 7, 8, 11, 19 or 37. The isolates obtained in the two years were identified as CA24v with strain-specific antisera against EH24/70 prepared in the rabbit and the guinea pig. All 17 isolates of 1985 and 31 of 1986 were equally neutralized with anti-EH24/70 rabbit serum.

Three adenovirus type 3 strains, one in 1985 and the other two in 1986, were isolated from CA24v-negative patients.

Comparison between Isolates and the Prototype Strain

(1) Plaque reduction test. To identify antigenically the isolates, neutralization tests by plaque reduction were carried out (Table III). A plaque-purified clone of the representative of 1985 isolates, J140/85-411, and the prototype strain, EH24/70, showed similar plaque reduction with either anti-J140/85-411 or anti-EH24/70 rabbit immune serum, but not with the antiserum against the standard strain of coxsackievirus A24 (Joseph strain). Hu-39 strain, another antigenic variant of coxsackievirus A24, was neutralized by both antisera against J140/85-411 and EH24/70 at a titer of 1:200. Thus, antigenically no difference was discernible between EH24/70, isolated 15 years ago in Singapore, and J140/85, prevalent in Okinawa in 1985.

(2) Oligonucleotide mapping analysis. To analyze the nucleotide sequence variation among strains, RNase T1-resistant oligonucleotide mapping of viral RNA was attempted. The 5'-ends of T1-RNase-digested virion RNA were labeled and subjected to two-dimensional gel electrophoresis. Large T1-resistant oligonucleotides of EH24/70, J140/85 and J60/86 (a representative of 1986 isolates) strains were compared (Fig. 3). The common spots between strains were identified by co-electrophoresis.
Table III. Cross neutralization tests of the isolates with other coxsackie A24 viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>J140/85-411 (rabbit)</th>
<th>EH24/70 (rabbit)</th>
<th>Joseph strain (horse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J140/85-411</td>
<td>800</td>
<td>1,600</td>
<td>&lt;25</td>
</tr>
<tr>
<td>EH24/70</td>
<td>400</td>
<td>3,200</td>
<td>&lt;25</td>
</tr>
<tr>
<td>Joseph strain</td>
<td>20</td>
<td>20</td>
<td>2,560</td>
</tr>
<tr>
<td>Hu-39 strain</td>
<td>200</td>
<td>200</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

*Reciprocals of the highest serum dilution with 80% plaque reduction.

The two Okinawa isolates gave closely related oligonucleotide patterns; J60/86 differed from J140/85 in only three spots (two missing and one additional). On the other hand, the maps of these two isolates markedly differed from that of EH24/70. Among 48 spots on the map of EH24/70, 35 were missing and 25 newly appeared on the map of J140/85. Thus, J140/85 and EH24/70 shared only 13 spots.

**Seroepidemiology**

The serum samples collected before and after the first epidemic were examined for the neutralizing antibody against CA24v (Table IV). Before the summer, 1985, all samples but two were negative for CA24v NT-antibody. The antibody-positive (≥1:4) rate of serum samples collected during October, 1985-February, 1986 increased to 8.5%. However, of the three cities from which the serum samples were obtained, Naha differed from the other two cities in the antibody-positive rate; the positive rates against CA24v increased significantly in post-epidemic serum samples collected in both Ishigaki and Hirara cities, but that of Naha was as low as 3.0%, indicating low distribution of the virus. On the other hand, the positive rate against EV70, another causative virus of AHC, in pre- and
Fig. 3. Left: Oligonucleotide maps of the prototype strain of CA24v, EH24/70, and two Okinawa isolates obtained in 1985 (J140/85) and in 1986 (J60/86). The origin is the bottom left. X’s are the position of bromophenol blue and xylene cyanol FF dye markers. Right: Diagrams of oligonucleotide maps. The large, intense spots under the dotted line were compared. Filled spots represent oligonucleotide spots shared with EH24/70. Arrows indicate the spots which are different between J140/85 and J60/86.
post-epidemic serum samples from Naha increased from 4.0 to 12.1% (Table IV). The result indicates that AHC due to EV70 was also prevalent in Naha in 1985.

Age-specific incidences of NT antibodies against CA24v in post-epidemic serum samples are shown in Fig. 4. The antibody-positive rates to CA24v were highest in both the 15 to 19- and >60-year-old groups, although those in children under 15 years were not known. The highest antibody titer was 1:64 and the geometric mean titer was 1:8.8. The antibody-positive rate in all ages against CA24v after the first outbreak was approximately half of that of EV70 (8.5% vs 17.3%).

For comparison, 198 serum samples collected from other prefectures than Okinawa in 1985 were assayed but no serum had CA24v antibody (data not shown).

Fig. 4. Age-specific antibody-positive rates against CA24v and EV70 in Okinawa sera collected during October, 1985 - February, 1986, immediately after the 1985 epidemic and prior to the 1986 epidemic of CA24v. *No serum tested.
<table>
<thead>
<tr>
<th>Area</th>
<th>Time of serum sampling</th>
<th>Number tested</th>
<th>NT antibody positives (%) against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>EH24/70* (CA24v)</td>
</tr>
<tr>
<td>Total</td>
<td>pre (Jan.'85-July '85)</td>
<td>192</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>post (Oct.'85-Feb.'86)</td>
<td>284</td>
<td>8.5</td>
</tr>
<tr>
<td>Naha City</td>
<td>pre (June '85-July '85)</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>post (Nov.'85-Jan.'86)</td>
<td>99</td>
<td>3.0</td>
</tr>
<tr>
<td>Ishigaki City</td>
<td>pre (Jan.'85-Feb.'85)</td>
<td>87</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>post (Jan.'86)</td>
<td>94</td>
<td>12.8</td>
</tr>
<tr>
<td>Hirara City</td>
<td>pre (May '85-June '85)</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>post (Oct.'85-Feb.'86)</td>
<td>91</td>
<td>9.9</td>
</tr>
</tbody>
</table>

*: $\geq 1:4$, **: $\geq 1:8$. 
DISCUSSION

The epidemic of ARC due to CA24v in Japan started in July, 1985, soon after a large outbreak in Singapore in March, 1985 in Okinawa Prefecture, and the epidemic peak coincided with that in Taiwan (5). Thereafter, CA24v was isolated in various areas including Central America and Africa, where CA24v had never been prevalent before. Thus, the epidemics of CA24v in Okinawa in 1985 and 1986 seemed to have been a part of a pandemic involving for the first time the areas outside of Asia.

Our preliminary genetic analysis of the virus demonstrated that the isolates from Okinawa and Taiwan, located very near each other, had very similar oligonucleotide maps of genome RNA, indicating that the viruses that caused Okinawa and Taiwan outbreaks were recently branched from a common progenitor. This, together with the fact that the first ARC outbreaks due to CA24v occurred in both areas concurrently (6), suggests that the virus was introduced to both areas at nearly the same time.

In spite of intensive surveillance for ARC throughout the country, no epidemic due to CA24v was reported from any other area than Okinawa during or after these two epidemics, except a few possibly imported cases. In Wakayama Prefecture (13), a 19-year boy developed AHC at the end of July, 1986, immediately after returning from Okinawa. Four other school children who had attended a summer school held in Tokushima Prefecture from July 29 to August 2, 1986, developed AHC and their three family members acquired secondary infection after the children returned home. Foreign participants of the summer school were suspected to be the possible source of infection. In Tokushima Prefecture (14), CA24v was isolated in August, 1986, from a 7-year boy infected through an unknown route. In Kanagawa Prefecture (15), three families acquired CA24v infection in August, 1986. One involved four members with an index case of 18-year boy who developed AHC immediately after his visit to Taiwan. From three other AHC patients of two families, two strains of CA24v were isolated but the route of transmission was unknown. No endemic dissemination followed anywhere in Japan except Okinawa, in spite of the seroepidemiological evidence showing that nobody was immune against the virus.

The serosurvey of pre- and post-epidemic serum samples in Okinawa Prefecture allowed estimation of the seroconversion rate during the first outbreak. The result showed that the antibody-positive rate increased from 1.0 to 8.5% immediately after the 1985 epidemic. The antibody-positive rate, however, should have been higher if the post-epidemic serum samples from children under 15 years,
constituting as high as 55% of CA24v patients, had been tested (Table I). According to the national census in 1985, children under 15 years numbered 322,523 accounting for 27.4% of the total population, 1,178,514 of Okinawa Prefecture. Therefore, the reported AHC cases in 1985 among those under 15 years represented 2.79% of this age group, being 3.2 times higher than that in older age groups (0.85%). From this ratio, antibody acquisition among children under 15 years of age was estimated at 23.7% (7.4% × 3.2 times) from the seroepidemiological finding that the increase in seropositive rate was 7.4% (from 1.0% to 8.5%) in those older than 15 years and that in the whole population in 1985 should have been 12.0%. The percentage was very similar to the attack rate obtained by the questionnaires in 1985. Thus, together with the second-year epidemic in which the reported cases accounted for 0.52% of the total population, approximately 227,500 cases (i.e. 19.3% of the population) were estimated to have contracted the disease during the two-years’ virgin soil epidemic of CA24v in Okinawa Prefecture. The rate thus estimated was similar to that of Singapore (Miyamura et al., unpublished data), where outbreaks of AHC due to CA24v recurred approximately every five years (3,4).

In the serum samples collected from Naha City, the antibody-positive rates against EV70 and CA24v were lower than those in the other two cities. Moreover, in contrast to only a slight increase against CA24v, the EV70 antibody-positive rate in Naha City increased significantly after the 1985 epidemic. This may suggest that in the autumn of 1985, the activity of CA24v was very low, while that of EV70 was high, prevailing in Naha City. Concomitant spread of EV70 and CA24v in Okinawa Prefecture in 1985 was evidenced by serological diagnosis (16). As expected, Naha City and the neighboring areas had extensive ARC outbreaks in 1986 and distribution of CA24v was confirmed by virus isolation in Okinawa City, only 22 km apart from Naha City.

In general, those possessing antibodies to EV70 or CA24v are fewer in Japan than in other Asian countries (17). Within Japan, however, those possessing EV70 antibody were significantly more in Okinawa Islands than in other areas (usually lower than 9% at a titer of 1:8). It has been suggested that high temperature and humidity should have been associated with the large outbreaks of AHC due to both viruses. In fact, summer and autumn seem to have favored AHC epidemics. It was not explained, however, why CA24v spread so suddenly and widely only on Okinawa Islands. When EV70 was introduced into Japan, rapid dissemination of the virus proceeded throughout the country (18). It is conceivable that the climate
of Japan other than Okinawa may be unfavorable for dissemination of CA24v as compared with EV70.

After these two epidemics, AHC cases in Okinawa Prefecture have remained on the base level and no outbreak was reported in 1987. Therefore, it is interesting to find whether CA24v persists there endemically to cause periodical outbreaks.

ACKNOWLEDGEMENT

We are grateful to Dr. Katsuro Natori for his contribution of Table III.

REFERENCES