Oligonucleotide Fingerprint Analysis of Coxsackievirus A10 Isolated in Japan

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SUMMARY

Eight coxsackievirus A10 strains isolated in 1978 and in 1981 and 1982 from patients with hand, foot-and-mouth disease and with herpangina at a dispensary in Matsue city were compared by RNA fingerprinting techniques. The oligonucleotide maps of the four 1978 isolates were related to each other by 85 to 93% with respect to their large T₁ oligonucleotides. In contrast, the oligonucleotide maps of the four 1981 and 1982 isolates were very different from each other. Co-electrophoresis experiments revealed that the 1981 and 1982 strains shared only 17 to 34% of their large oligonucleotides. In addition, some large oligonucleotides were found in most of the fingerprint maps of isolates from 1978 to 1982, suggesting that there are regions in the genome of coxsackievirus A10 which are not subject to mutational changes.

INTRODUCTION

From June 1981 to January 1982 coxsackievirus A10 (CA10) was isolated from patients with hand, foot-and-mouth disease (HFMD) and with herpangina in San-in district, Japan (mainly in Matsue city) (Itagaki et al., 1983). This was the first report of a clustering outbreak of HFMD caused by CA10, though CA10 had been isolated from patients with HFMD in sporadic cases in New Zealand in 1957 (Seddon & Duff, 1971).

HFMD is primarily caused by CA16 (Robinson et al., 1958) or enterovirus 71 (Schmidt et al., 1974). Other coxsackie group A viruses such as CA4, CA5, CA6 and CA7 have also been reported as the causative agents of sporadic cases of HFMD (Baker & Phillips, 1979; Flewett et al., 1963; Magoffin et al., 1961; Okuhara et al., 1975). However, little is known about what causes the occurrence of HFMD, the differences between viruses which cause HFMD or other diseases, or the ecology of these viruses.

As a first step, we retrospectively reviewed the data on HFMD and CA10 in this area. In 1978, 14 CA10 strains were isolated from patients with herpangina and six from patients with HFMD. In 1981 and 1982, 21 strains were isolated from patients with HFMD and 36 from herpangina cases. In the present study, we have randomly chosen eight strains isolated in a dispensary and report the application of oligonucleotide mapping to the comparison of their genomic RNAs.

METHODS

Viruses. The CA10 isolates studied are listed in Table 1. Clinical specimens were grown in suckling mice and homogenates of these were used as the starting materials. To avoid the arbitrary selection of a single plaque as representative of a heterogeneous virus population, clinical specimens were not plaque-purified. All virus strains were serologically confirmed as CA10 by virus neutralization using both suckling mice and human rhabdomyosarcoma (RD) cells. Each virus strain was grown in about 30 suckling mice to obtain sufficient virus for the viral RNA analysis. After multiplication of the virus, the homogenate was again confirmed as CA10 to exclude possible selective replication of viruses of other serotypes during the second suckling mouse passage.

Isolation of 3H-labelled coxsackievirus B4. Virus was harvested and purified by modifications of the procedures of Chatterjee & Tuchowski (1981). Monolayer cultures of HeLa cells in 60 mm plastic Petri dishes were infected.
Oligonucleotide spots in another strain and C is the number of common large oligonucleotide spots. The number of oligonucleotides was performed according to De Wachter & Fiers (1972) as modified by Lee & Wimmer (1976).

Calculation of similarity ratio (S.R.).

S.R. = 2C/(A + B), where A is the number of large oligonucleotide spots in one strain, B is the number of large oligonucleotide spots in another strain and C is the number of common large oligonucleotide spots. The number of large oligonucleotide spots was determined arbitrarily for each oligonucleotide map.

Table 1. Coxsackievirus A10 strains used in comparative oligonucleotide fingerprint studies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Date of harvest</th>
<th>Age</th>
<th>Sex</th>
<th>Specimen</th>
<th>Clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-1285</td>
<td>18/8/78</td>
<td>9 months</td>
<td>M</td>
<td>Throat swab</td>
<td>Herpangina</td>
</tr>
<tr>
<td>SP-1305</td>
<td>21/8/78</td>
<td>1 year</td>
<td>F</td>
<td>Throat swab</td>
<td>HFMD</td>
</tr>
<tr>
<td>SP-1442</td>
<td>13/10/78</td>
<td>4 years</td>
<td>F</td>
<td>Throat swab</td>
<td>Herpangina</td>
</tr>
<tr>
<td>SP-1533</td>
<td>20/11/78</td>
<td>1 year</td>
<td>F</td>
<td>Throat swab</td>
<td>HFMD</td>
</tr>
<tr>
<td>SP-1789</td>
<td>3/8/81</td>
<td>7 months</td>
<td>M</td>
<td>Throat swab</td>
<td>Herpangina</td>
</tr>
<tr>
<td>SP-2134</td>
<td>31/8/81</td>
<td>1 year</td>
<td>M</td>
<td>Throat swab</td>
<td>Herpangina</td>
</tr>
<tr>
<td>SP-2550</td>
<td>20/11/81</td>
<td>2 years</td>
<td>F</td>
<td>Vesicle</td>
<td>HFMD</td>
</tr>
<tr>
<td>SP-2</td>
<td>4/1/82</td>
<td>2 years</td>
<td>F</td>
<td>Vesicle</td>
<td>HFMD</td>
</tr>
</tbody>
</table>

with 0.5 ml of inoculum at an input multiplicity of 5 to 10 p.f.u./cell. After 1 h incubation at 37°C in a 5% CO₂/air mixture, the virus solution was replaced with 3 ml Eagle's MEM supplemented with 8% foetal bovine serum (FBS) containing 100 μCi [³H]uridine, and incubation was continued. At 24 to 36 h after infection, when 60 to 80% of the cells exhibited cytopathic effect, the cell suspension was frozen and thawed three times and centrifuged at 2000 g for 10 min. The resulting cell pellet was suspended in a small volume (2 to 3 ml) of Dulbecco's phosphate-buffered saline (PBS), sonicated three times for 15 s each, and centrifuged as above. The supernatant was withdrawn and combined with the initial one. Virus from this combined supernatant was pelleted by centrifugation at 150000 g for 2 h at 4°C. The precipitate was suspended in about 4 ml PBS containing 0.5% (v/v) NP40, incubated at 4°C for 16 h, homogenized, and centrifuged at 5000 g to remove the insoluble debris. This clarified virus suspension was layered over an 8 ml linear gradient of CsCl (1.2 to 1.4 g/ml) in PBS and centrifuged at 38000 r.p.m. for 3 h at 8°C in a Hitachi RPS40T rotor.

Virus cultivation in suckling mice and virus purification. One- to 2-day-old ICR/JCL suckling mice were inoculated intraperitoneally with 0.05 ml of diluted starting materials containing 1 × 10³ LD₅₀/ml. Two or 3 days later, moribund mice were frozen. Virus was purified from the mice by a modification of the method of Mattern (1962). After thawing, the heads and legs of the mice were cut off, and the carcasses skinned. All the internal organs were removed and the remains were washed in distilled water, minced and ground in a mortar with 0.8 g of sea sand to each mouse. Two volumes of 8.6% sucrose solution at pH 7.0 were added to the homogenate and mixed thoroughly. The homogenate was centrifuged for 20 min at 1000 g to remove the solid residue. The supernatant fluid was stored frozen. The pellet was resuspended in a small volume of 8.6% sucrose and centrifuged as above, and the supernatants were pooled and stored frozen. After three cycles of freezing and thawing, the thawed supernatant was adjusted to pH 3.0 with 1 M HCl, and 10 g ammonium sulphate was added to each 100 ml of fluid. After mixing for 30 min at 4°C, the precipitate was removed by low-speed centrifugation for 20 min. The supernatant was adjusted to pH 5.0 with 1 M KOH. An additional 30 g ammonium sulphate was added to each 100 ml of the supernatant. After 1 h of mixing and refrigeration, this suspension was centrifuged for 1 h at 1000 g and the result supernatant fluid was discarded. The sediment was resuspended in about 5 ml 5% (w/v) NaCl. This suspension was then adjusted to pH 7.0 with 1 M KOH and dialysed against 5% (w/v) NaCl until free of ammonium sulphate. The non-dialysed fraction was cleared by centrifugation at 1000 g for 20 min, and the virus was collected as the precipitate after centrifugation at 12000 g for 1 h. The precipitate was suspended in about 4 ml PBS containing 0.5% (v/v) NP40 and clarified by the procedures described above in the presence of ³H-labelled coxsackievirus B4 (about 8000 c.p.m.) as an internal marker.

5' end-labelling of RNase T₁-resistant oligonucleotides. Virion RNA was extracted from purified virus with SDS in the presence of 100 μg/ml proteinase K and phenol–chloroform as previously described (Kamahora et al., 1979). RNase T₁-resistant oligonucleotides were end-labelled by a modification of the method of Pedersen & Haseltine (1980). Viral RNA (0.4 μg) was dissolved in 2 μl digestion buffer (20 mm-Tris–HCl pH 7.5, 2 mm-EDTA), heated in boiling water for 1 min, immediately cooled in ice-cold water, and digested with 2 units RNase T₁ at 37°C for 30 min. Thirty-four μl kinase buffer [10 mm-Tris–HCl pH 8.0, 10 mm-Mg(CH₃COO)₂, 1 mm-dithiothreitol, 2 μl polynucleotide kinase (5 units), and 10 μl containing 50 to 100 μCi [γ-³²P]ATP (Amersham, sp. act. 5000 Ci/mmol)] was added to the digested RNA, and incubation was continued for 30 min at 37°C. The reaction was terminated by the addition of 100 μl phenol and 50 μl of stop mixture (0.6 M CH₃COONH₄ containing 100 μg yeast carrier RNA). After mixing and centrifuging, the aqueous phase was precipitated with 2.5 vol. ethanol at −20°C for over 2 h.

Oligonucleotide fingerprinting. Two-dimensional (2D) PAGE of the ³²P-labelled RNase T₁-resistant oligonucleotides was performed according to De Wachter & Fiers (1972) as modified by Lee & Wimmer (1976). This procedure was described previously by Kamahora et al. (1979).

Calculation of similarity ratio (S.R.). The similarity ratio between two strains was calculated as S.R. = 2C/(A + B), where A is the number of large oligonucleotide spots in one strain, B is the number of large oligonucleotide spots in another strain and C is the number of common large oligonucleotide spots. The number of large oligonucleotide spots was determined arbitrarily for each oligonucleotide map.
Fig. 1. Oligonucleotide maps of the 1978 isolates of coxsackievirus A10. For each pattern, electrophoresis in the first dimension (7-6% polyacrylamide, 6 M-urea pH 3.3) was from left to right, and in the second dimension (22% polyacrylamide, 50 mM-Tris-borate pH 8.2) from the bottom to the top. Large oligonucleotides have been assigned arbitrary numbers for reference purposes.

RESULTS

We could not obtain highly radioactive virions in a CA10-infected cell system and furthermore the presence of some virions of different densities has been reported in this system (Hagiwara et al., unpublished results). However, all virions obtained from suckling mice inoculated with CA10 equilibrated at 1.34 g/ml (Mattern, 1962; Kamahora, unpublished results). Unlabelled RNA was prepared from CA10 virions purified from suckling mice in parallel with 3H-labelled coxsackievirus B4 (CB4) as an internal marker as described in Methods. The RNA was then digested with RNase T1, labelled at the 5' end with [γ-32P]ATP and the resulting radioactive oligonucleotides were analysed by 2D PAGE. The CB4 used as internal marker was not present in sufficient amount to influence the fingerprint, since a map of CB4 RNA at the concentration used for the marker had no visible spots (data not shown).

A comparison of RNAs of eight CA10 isolates (listed in Table 1) was provided by 2D PAGE of RNase T1-resistant oligonucleotides generated from the viral genomes. As seen in the fingerprint maps, the apparent molarity of the larger oligonucleotide was not uniform. This is assumed to be due to the secondary structure of the oligonucleotides affecting the relative efficiency of the kinase reaction. Similar observations have been noted when the genomes of several RNA viruses were analysed by this method (Frisby, 1977; Stephenson & ter Meulen, 1982). However, these variations in intensity were judged not to affect the validity of the
conclusions since the fingerprint patterns were reproducible in repeated analyses of the same RNA sample and for different RNA preparations from the same virus stock.

Oligonucleotide maps of the genomes of four strains isolated in 1978 were compared (Fig. 1). The patterns of the large oligonucleotides of the four strains were quite similar to each other. The SR between the large unique oligonucleotides ranged from 0.81 (SP-1285 compared to SP-1305) to 0.90 (SP-1305 compared to SP-1533) (Table 2).

On the other hand, the 1981 and 1982 isolates differed in their oligonucleotide maps (Fig. 2). When the oligonucleotide maps were similar to each other, as with the 1978 strains, it was possible to see differences by direct visual comparison. But when the maps were clearly different from each other, it was not possible to estimate the extent of spot homology. This is the case for the 1981 and 1982 strains. In order to compare directly two genomic RNAs of these isolates, we co-electrophoresed the digests of their respective RNAs. The results are shown in Fig. 3. The SR between the large unique oligonucleotides ranged from 0.17 (SP-1789 and SP-2143) to 0.34 (SP-2 and SP-2550) (Table 3). Of the shared spots, two (indicated by arrows in Fig. 3) were also found in most of the maps of the 1978 isolates.

Table 2. Similarity ratios among the 1978 isolates as determined by RNA oligonucleotide fingerprint analysis

<table>
<thead>
<tr>
<th></th>
<th>SP-1285</th>
<th>SP-1305</th>
<th>SP-1442</th>
<th>SP-1533</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-1285</td>
<td>-</td>
<td>0.81</td>
<td>0.88</td>
<td>0.85</td>
</tr>
<tr>
<td>SP-1305</td>
<td>0.81</td>
<td>-</td>
<td>0.86</td>
<td>0.90</td>
</tr>
<tr>
<td>SP-1442</td>
<td>0.88</td>
<td>0.86</td>
<td>-</td>
<td>0.88</td>
</tr>
<tr>
<td>SP-1533</td>
<td>0.85</td>
<td>0.90</td>
<td>0.88</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 3. Co-electrophoresis of the RNA digests of the 1981 and 1982 isolates. Oligonucleotide maps of the RNase T₁ digests of the two RNA species are given in the left panels with schematic representations of the resolved oligonucleotides shown in the right panels. Those large oligonucleotides which are shared by both viruses are indicated by closed circles; those which are unique to either of the viruses are indicated by open circles or half-filled circles. Arrows indicate the large oligonucleotides which are also found in most of the 1978 isolates.

Among the 1981 and 1982 isolates, only SP-1789 seemed to have a map similar to those of the 1978 isolates.

In order to determine the SR between the isolates from two epidemic periods, we directly compared SP-1305 (one of the 1978 isolates) with the 1981 and 1982 isolates by
Fingerprinting of coxsackievirus A10 isolates

Table 3. Similarity ratios among the 1981 and 1982 isolates, and between SP-1305 and these isolates as determined by co-electrophoresis experiments

<table>
<thead>
<tr>
<th></th>
<th>SP-1789</th>
<th>SP-2134</th>
<th>SP-2550</th>
<th>SP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-1789</td>
<td>0.17</td>
<td>0.17</td>
<td>0.27</td>
<td>0.22</td>
</tr>
<tr>
<td>SP-2134</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SP-2550</td>
<td>0.21</td>
<td>ND</td>
<td>0.34</td>
<td>0.34</td>
</tr>
<tr>
<td>SP-2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>*</td>
</tr>
<tr>
<td>SP-1305</td>
<td>0.68</td>
<td>0.05</td>
<td>0.18</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* ND, Not done.

co-electrophoresis experiments. The results are shown in Fig. 4. Only spots from SP-1305 are numbered according to Fig. 1. Their SR are summarized in Table 3. Among the 1981 and 1982 isolates, only SP-1789 had a similar map to SP-1305 (SR 0.68) but others showed lower similarity ratios ranging from 0.05 to 0.22 (Table 3).

DISCUSSION

In this report, we have compared several CA10 strains isolated in a limited area from 1978 to 1982 by 2D PAGE of the RNase T1 digests of their genomic RNAs. Firstly, it was observed that CA10 underwent genome variation, probably upon replication in human beings as has been shown for type 1, 2 and 3 polioviruses (Nottay et al., 1981; Kew et al., 1981). The second finding was that two different types of CA10 were prevalent in 1978 and in 1981 to 1982 respectively. The oligonucleotide maps of the 1978 isolates resembled each other (SR between 0.81 and 0.90); on the other hand, those of the 1981 and 1982 isolates were different from each other (SR from 0.17 to 0.34). Because the oligonucleotide maps of the 1978 isolates resembled each other, the differences between the maps of the 1981 and 1982 isolates seemed more evident. To explain these phenomena, we suggest that (i) two or more types of CA10 entered this area in 1981 and 1982, and (ii) many more people were infected by CA10 in 1981 to 1982 than in 1978, so that the genome of CA10 was subject to stronger selective pressure and hence more frequent mutations than in 1978. In this case, however, most of the cases of infection with CA10 might have been inapparent because no more cases of HFMD or herpangina were reported in 1981 and 1982 than in 1978.

Among the 1981 and 1982 strains, SP-1789 had a similar fingerprint map to SP-1305, a representative of the 1978 strains, but the rest of the 1981 and 1982 strains showed rather low similarity ratios (from 0.05 to 0.22). As SP-1789 was the first of these 1981 and 1982 strains to be isolated (Table 1), it might be speculated that the 1978 strains had been latent in this area or accumulated mutations slowly in their genome over 3 years and changed rapidly in a few months.

We also found two spots shared by seven out of eight fingerprint maps. These two spots may indicate a conserved sequence in the genomic RNA, but we have not yet been able to define whether these spots are actually the same or co-migrate by chance.

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REFERENCES


Fig. 4. Co-electrophoresis of the RNA digests of the 1981 and 1982 isolates with SP-1305. Those large oligonucleotides which are shared by both viruses are indicated by closed circles; those which are unique to either of the viruses are indicated by half-filled circles.


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