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Genome Analysis of Adenovirus Type 4 Strains Isolated From Acute Conjunctivitis in Japan

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Abstract: Strains of adenovirus type 4 (Ad4) isolated from patients with acute conjunctivitis were studied by DNA restriction analysis. The strains were isolated between July and December 1990 in Japan. All 63 isolates of Ad4 were identified as the genome type Ad4a. This study showed that the same Ad4 genome type, Ad4a, caused acute viral conjunctivitis, even in different areas of Japan. **Jpn J Ophthalmol 1997;41:308–311** © 1997 Japanese Ophthalmological Society

Key Words: Adenovirus type 4, DNA restriction analysis, genome typing, viral conjunctivitis.

Introduction

Certain serotypes of adenovirus (Ad) possess an affinity for the conjunctiva; these are the pathogens most frequently detected in viral conjunctivitis.[1] It is well known that pharyngoconjunctival fever (PCF) is caused by adenovirus type 3 (Ad3), and epidemic keratoconjunctivitis (EKC) by Ad8.

Since 1979, however, Japan has been struck by the prevalence of adenovirus type 4 (Ad4) showing an intermediate clinical profile between PCF and EKC.[2] Because of the recent dramatic progress in molecular biological techniques, by the cleavage of viral DNA using restriction endonucleases and by the comparison of cleavage figures of its base sequence, this virus hitherto judged to be one serotype has been further classified into many subgenome types.[3–6]

Such information by genome typing is more specific and accurate than the data obtained by the conventional serum epidemiology, and a new research field of molecular epidemiology has been established. The Ad4 prototype was mainly reported until 1980; recently a new genome type, Ad4a, was noted. This article reports the results of a study intended to determine which genome type of Ad4 is chiefly responsible for infection in the recent epidemic of Ad4 conjunctivitis in Japan from the viewpoint of molecular epidemiology.

Materials and Methods

Subjects

Between July and December 1990, 63 patients with viral conjunctivitis such as EKC, PCF, and acute conjunctivitis (AC) were clinically diagnosed at 10 clinics in Japan. Isolates of Ad4 from the conjunctiva were obtained from these 63 patients as specimens (Table 1).

Hep-2 cell was used for the isolation and storage of virus. Specimens were stored in Hep-2 primary cells at −80°C until the time of molecular biological analysis. The clinical diagnosis of 63 subjects was PCF in 15 cases (23.8%), EKC in 47 cases (74.6%), and acute conjunctivitis (AC) in one case (1.6%). The regional distribution of patients was, as shown in Table 1, one case in Sapporo, one case in Akita Prefecture, 17 cases in Iwate Prefecture, 19 cases in Tokyo, two cases in Kanagawa Prefecture, 21 cases in Ishikawa Prefecture, and two cases in Kyoto.

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Thirty-four patients were men and twenty-nine were women. Fourteen percent of the patients were younger than 12 years old and eighty-six percent were 12 years or older.

Extraction of Viral DNA From Infected Cells

Viral DNA was extracted from infected cells by a modified method of Hirt[7] (see also Takeuchi et al[8]). That is, the virus was proliferated once in human amnion cell (FL cell) and once in human epidermoid carcinoma cell (He_p-2 cell), and high titer virus was inoculated in He_p-2 cell. It was incubated for 2–5 days in a 75 cm^2 culture bottle and when the cytopathic effect (CPE) was near completion in the He_p-2 cell infected by virus, infected cells were collected. They were washed twice in phosphate-buffered saline (−); 3 mL TE buffer (10 mmol/L Tris Cl [pH 7.4], 1 mmol/L edetic acid [pH 8.0]) was added, and 600 ul of 6% sodium dodecyl sulfate was further added to keep at room temperature for 10 minutes.

Afterwards, 5 mol/L NaCl and proteinase K (5 mg/mL) were added, and the reaction was continued for 1 hour in a water bath at 37°C. After standing overnight at 4°C, the solution was centrifuged for 40 minutes at 18,000 rpm, 4°C, and the supernatant was treated with phenol to remove protein, settled in ethanol, and viral DNA was obtained.

Restriction Endonuclease Treatment and Electrophoresis

One unit of restriction endonuclease was added to about 1 μg of viral DNA. Enzymes used in this study were *Bam*HI, *Xho*I, *Eco*RI, and *Sma*I (Takara Shuzo, Kyoto) with *Bam*HI and *Sma*I, the reaction temperature was 30°C and the reaction time was 180 minutes; with *Eco*RI and *Xho*I, 37°C and 180 minutes. After digesting the viral DNA with these enzymes, electrophoresis was conducted for about 18 hours at 90 V, 25 mA, by using agarose horizontal plate gel of 1.2%. The cleavage pattern was observed and photographed by ultraviolet ray irradiation. Ethidium bromide (10 mg/mL) was added at the time of the preparation of agarose gel (final concentration 0.5 μg/mL). To compare with isolates from patients, Ad4 standard strain (RI-67 strain) (Ad4 prototype or Ad4p) was similarly treated with enzymes and studied.

Table 1. Geographic Distribution of Patients With Adenovirus Type 4 Conjunctivitis

	Clinical Diagnosis			
Prefecture	EKC	PCF	AC	Total
Hokkaido			1	1
Akita	1			1
Iwate	12	5		17
Tokyo	18	1		19
Kanagawa	1	1		2
Ishikawa	14	7		21
Kyoto	1	1		2
Total	47	15	1	63

EKC: epidemic keratoconjunctivitis. PCF: pharyngoconjunctival fever. AC: acute conjunctivitis.

Results

The difference in cleavage patterns produced by the four restriction endonucleases of Ad4 isolates and Ad4p is shown in Figures 1–4. With *Bam*HI, all 63 isolates of DNA presented the same cleavage pattern. That is, the isolates were cleaved into nine DNA bands. Ad4p DNA comprised eight DNA bands (Figure 1). With *Xho*I, all 63 isolates of DNA also showed the same cleavage pattern with 10 DNA bands. Ad4p DNA comprised nine DNA bands (Figure 2). With *Sma*I, all 63 isolates of DNA indicated the same cleavage pattern. It can be noted that above the 1107 base-pair (bp), the Ad4p DNA comprised nine bands and the isolates of DNA comprised eight bands (Figure 3). Similarly, with *Eco*RI, all 63 strains of DNA showed the same cleavage pattern. That is, the strain was cleaved into three DNA bands. Ad4p DNA comprised four DNA bands (Figure 4). By comparing the restriction endonuclease cleavage patterns, it is evident that all 63 strains correspond to the genome type Ad4a.[3,6]

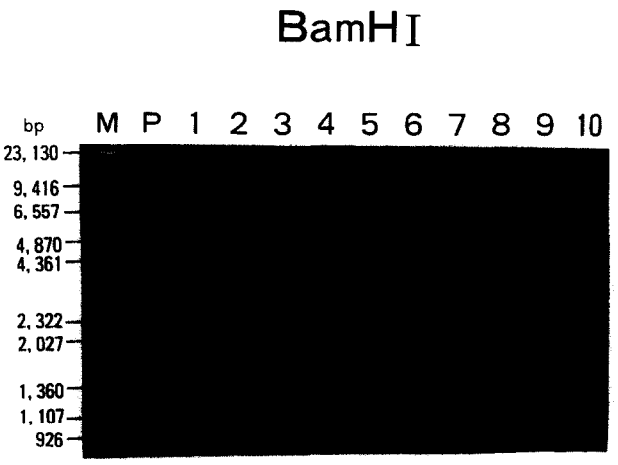

Figure 1. DNA restriction patterns of Ad4 prototype and isolated Ad4 strains digested with *Bam*HI. M: Molecular weight marker (λDNA digested with *Hind*III). P: Ad4 prototype. 1–10: isolated Ad4 strains. DNA fragments were separated by electrophoresis in 1.2% agarose slab gels.

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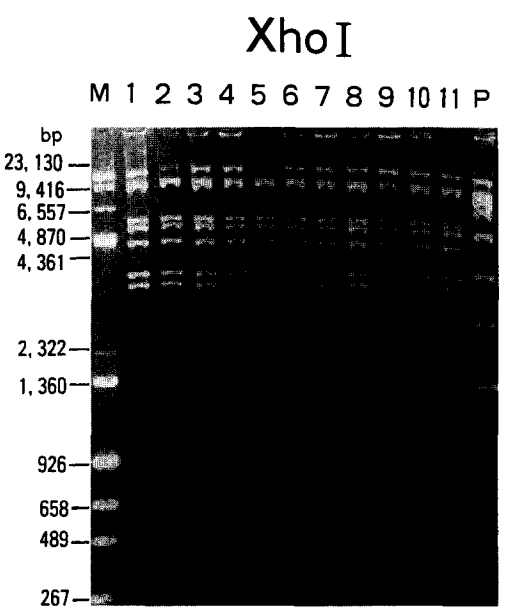

Figure 2. DNA restriction patterns of Ad4 prototype and isolated Ad4 strains digested with *Xho*I. M: Molecular weight marker (λDNA digested with *Hind*III plus PHY marker digested with *Hind*III). P: Ad4 prototype. 1–11: isolated Ad4 strains. DNA fragments were separated by electrophoresis in 1.2% agarose slab gels.

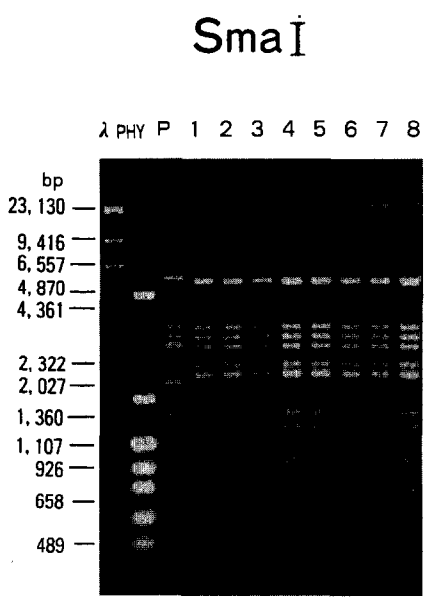

Figure 3. DNA restriction patterns of Ad4 prototype and isolated Ad4 strains digested with *Sma*I. λ: λDNA digested with *Hind*III. PHY: PHY marker digested with *Hind*III. P: Ad4 prototype. 1–8: isolated Ad4 strains. DNA fragments were separated by electrophoresis in 1.2% agarose slab gels.

Discussion

Serum types of human adenovirus are known at present to range from type 1 to type 47. Wadell[6] divided them into six subgroups from A to F. Among these, only group E includes Ad4. Ad4 was reported to cause disease only in very few cases, (i.e., recruit fever), but recently reports of isolates from conjunctivitis are increasing in various countries.[9–12] So far, DNA restriction analysis has revealed four genome types of Ad4 from human material: Ad4p, Ad4a, Ad4a1, and Ad4b.[6]

Wadell[6] reported the genome type of Ad4, isolated between 1965 and 1981 from recruits in California, to be Ad4p. However, Wadell[6] reported the presence of genome type Ad4a, different from the genome type Ad4p of the standard strain (RI-67 strain), in a restriction endonuclease cleavage pattern using *Eco*RI, *Bam*HI, and *Sma*I, and confirmed that all strains isolated from the materials of conjunctivitis in Rome, New York, Chicago, and Atlanta were Ad4a. Since 1979, epidemics of PCF and acute conjunctivitis caused by Ad4 have occurred in Japan. Ren et al[2] analyzed clinical isolates derived from acute respiratory disease (eight isolates) and

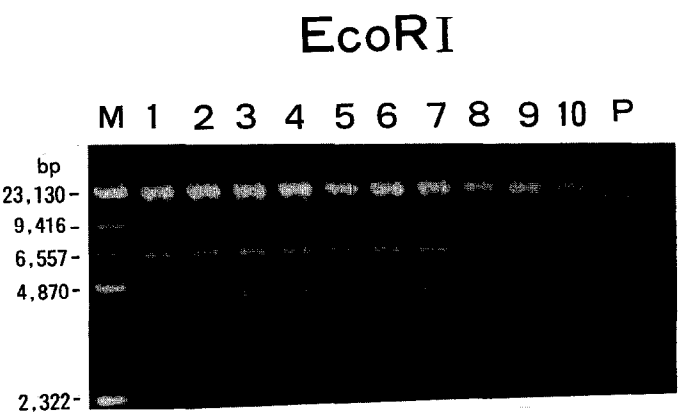

Figure 4. DNA restriction patterns of Ad4 prototype and isolated Ad4 strains digested with EcoRI. M: Molecular weight marker (λDNA digested with *Hind*III). P: Ad4 prototype. 1–10: isolated Ad4 strains. DNA fragments were separated by electrophoresis in 1.2% agarose slab gels.

conjunctivitis (11 isolates) from 1979 to 1983 throughout Japan with *Eco*RI, *Hind*III, and *Bam*HI. As a result, acute respiratory diseases and conjunctivitis were found to share the same genome type, although differing in the clinical disease type. It was the same Ad4a as reported by Wadell.[6]

Guo et al[13] analyzed 10 strains of Ad4 derived from EKC and acute conjunctivitis between 1982 and 1986 using *Sma*I, *Hind*III, and *Bam*HI, and reported that all 10 strains also belonged to Ad4a. It was not possible to find reports about Ad4p, Ad4a1, and Ad4b in Japan at that time. On the other hand, Gomes et al[14] analyzed five isolates of Ad4 obtained from patients with PCF and respiratory disease in Brazil, confirming them to be Ad4a. Li and Wadell[15] classified 50 Ad4 isolates collected around the world and from two strains of chimpanzee into eight genome types using 16 restriction endonucleases. Among the 16 restriction endonucleases, only *Eco*RI could distinguish Ad4a from Ad4a1. Itakura et al,[16] using four or five base recognition restriction endonucleases, cleaved the DNA of Ad4 from PCF and EKC patients, and reported the presence of subgenome types. These are the results of Ad4 isolates in one eye clinic. Ad4 showed mutation in 1 or 2 years, and the same subtype did not appear 2 years later.

In the present study, we analyzed 63 strains of Ad4, using *Bam*HI, *Xho*I, *Eco*RI, and *Sma*I, and found the same DNA cleavage patterns in all of them. Comparing the molecular weight of the cleavage DNA using restriction endonucleases, we found that the clinical isolates in the present study were all Ad4a, the same as in the report by Adrian et al[3] and Wadell.[6] In the present study, even with *Eco*RI, the pattern of Ad4a1 was not recognized. Therefore, it seems necessary to continue further investigations in more clinical isolates, increasing the types of DNA restriction endonucleases. Adenovirus type 4 undergoes minor variation, and carriers of serum antibody against Ad4 are still at least in the order of 10%.[9] Therefore, the outbreak of Ad4 infection may occur in the future. Variations of Ad4, as other adenoviruses, may be discovered at genome level by analysis with four base recognition enzymes.

In this study, the clinical diagnosis of 63 patients with conjunctivitis was PCF in 23.8%, EKC in 74.6%, and acute conjunctivitis in 1.6%, and the clinical symptoms were varied. But the results of molecular epidemiological analysis indicated the same genome type, Ad4a. Although specimens were collected throughout Japan, from Hokkaido to Kyoto, the Ad4 conjunctivitis epidemic in the Summer, Autumn, and Winter of 1990 was due to the same Ad genome type. It was concluded, therefore, that the genome type of Ad4 isolates in Japan was Ad4a.

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