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Genome Analysis with Restriction Endonucleases Recognizing 4- or 5-Base Pair Sequences of Adenovirus Type

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Purpose: To detect the genetic changes in adenovirus type 4 (Ad4) by DNA restriction endonuclease analysis during 1993–1995, when no epidemic outbreak of conjunctivitis occurred.

Methods: We evaluated 16 Ad4 strains from patients with acute viral conjunctivitis at four eye clinics in Sapporo, northern Japan. Seven strains were obtained during the period from December 1993 through March 1994 (first period). Nine strains were obtained during the period from March through May 1995 (second period). These strains were analyzed using DNA restriction endonucleases, *Taq*I and *Hin*fI.

Results: The seven strains obtained during the first period showed identical DNA digestion patterns. The nine strains obtained during the second period showed two DNA digestion patterns using *Taq*I: five strains showed the same digestion pattern as that seen in the strains obtained during the first period, and four showed a different pattern. The genetic changes in Ad4 during 1993–1995 were less frequent than those reported previously during 1985–1989.

Conclusion: It is suggested that the decrease in the incidence of infection in 1993–1995 was related to the decrease in the incidence of mutation in the Ad4 DNA. **Jpn J Ophthalmol 2000;44:463–466**

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

Introduction

Adenovirus type 4 (Ad4) is one of the most common serotypes of adenovirus that cause conjunctivitis in Japan today.[1] Ad4 infection was quite rare before 1979. It is suspected that the Ad4 genome region, which is related to the viral affinity to the human conjunctiva, has changed since that time. Epidemics of conjunctivitis caused by Ad4 have occurred several times since then. Recently, the genome types of Ad4 have been studied using molecular biological techniques. Wadell[2] analyzed the digestion pattern of Ad4 DNA using restriction endonucleases (REs) that recognize 6-base-pair (bp) sequences, and identified two genome types, the Ad4 standard type, Ad4p, and the Ad4 variant, Ad4a. Wang et al[3] determined the genome types of 63 isolates of Ad4 from patients with acute viral conjunctivitis at 10 clinics in Japan, and showed that Ad4a was the more common causative agent of conjunctivitis in Japan. Li and Wadell[4] reported that the Ad4a genomic sequence was highly conserved and that this genome type of Ad4 has been isolated over a 19-year period. Itakura et al.[5] subdivided Ad4a into subtypes using *Taq*I and *Hin*fI, two REs that recognize 4- and 5-bp sequences, respectively, and provisionally called them subgenome types. They showed that the prevalent subgenome types tended to change every few years.

In recent years, the incidence of Ad4 infection has been decreasing and extensive epidemics have not occurred. The reason for this remains unknown. We wondered whether the incidence of genetic change in Ad4 has also been decreasing. We determined the subgenome types of Ad4 strains isolated in the same geographic area during two time periods and investigated the genetic changes. We compared the results

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with the report of Itakura et al,[5] who analyzed the subgenome types of Ad4 in the same area as that considered for the present study when the incidence of Ad4 infection was high.

Materials and Methods

Materials

Sixteen strains of Ad4 isolated from the conjunctival swabs of patients with acute conjunctivitis who attended four eye clinics in Sapporo, northern Japan, were studied. Sixteen strains were isolated during two periods; seven strains during the first period from December 1993 through March 1994, and nine strains during the second period from March through May 1995. These strains were isolated at the Sapporo City Institute of Public Health, and serotyping by the neutralization method led to identification as Ad4.

Methods

Extraction of viral DNA from infected cells. Viral proliferation was allowed to proceed in human epidermoid carcinoma cells in a 75-cm^2 culture flask. The culture medium used was Eagle's minimum essential medium containing 2% fetal bovine serum. The cells were incubated for 2–3 days and when the cytopathic effect was noted to be near completion, the infected cells were collected. Viral DNA was extracted from the infected cells by a method that was modified from that reported by Shinagawa et al,[6] as described previously.

Restriction endonuclease analysis of viral DNA. Four REs recognizing 6-bp sequences, *Bam*HI, *Eco*RI, *Sma*I, and *Xho*I (Takara-Shuzo, Kyoto), were used to identify the genome types of the Ad4 strains. To further classify into subgenome types, two REs, *Taq*I and *Hin*fI (Takara-Shuzo), which recognize 4- and 5-bp sequences, respectively, were used. One unit of RE was added to about 1 μg of viral DNA, which was then digested for 3 hours.

Analysis

After digestion of viral DNA, the samples were electrophoresed on 1.2% agarose horizontal plate gels for 2 hours at 125 V (genome typing) or on 5–10% polyacrylamide gels for 2–3 hours at 120–150 V in a cold room at 4°C (subgenome typing). The DNA bands were visualized by staining with 10 mg/mL ethidium bromide and photographed under ultraviolet light irradiation.

Results

The DNA digestion patterns of the representative Ad4 isolates with *Bam*HI, *Sma*I, *Eco*RI and *Xho*I are shown in Figure 1. All 16 isolates showed identical DNA digestion patterns, which were different from that of Ad4p. The digestion patterns corresponded to that of genome type Ad4a reported previously.[2,3,7]

Figure 2 shows the digestion patterns of representative Ad4 isolates with *Hin*fI and *Taq*I. The digestion patterns of all 16 isolates with *Hin*fI were identical (Figure 2, H). On the other hand, the *Taq*I, the isolates were classified into two variants (Figure 2, Ta and Tb). The digestion pattern of Tb showed a missing fragment at about 900 bp and an additional fragment at about 1,100 bp relative to the sequence of Ta. Other fragments were common between the two variants. The digestion patterns of all seven strains obtained during the first period were Ta, while of those obtained during the second period, five were Ta and four were Tb. Thus, the seven strains obtained during the first period were identified as being of the same subgenome type. Of the nine strains obtained during the second period, five were classified into the same subgenome type as that of the isolates of the first period, while the other four strains were classified into a different genomic subtype.

Table 1 shows the distribution according tot he isolation date, clinic, and the subgenome type. Strains showing the DNA digestion pattern Ta were isolated from all four clinics. All four strains showing the DNA digestion pattern Tb were isolated at clinic C.

Discussion

Adenovirus DNA is digested into many fragments with *Taq*I and *Hin*fI and the genome type can be analyzed in greater detail to monitor the alterations of the genome of the prevalent strains. Ad3 and Ad4

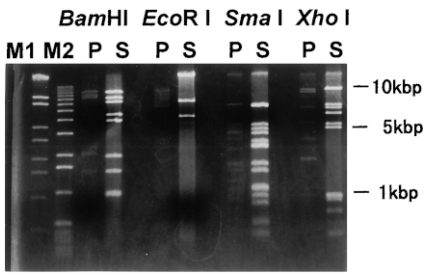

Figure 1. Genome typing of isolated adenovirus type 4 (Ad4) strain. Lane M1: λ *Eco*T14I, lane M2: 1-kb ladder; lane P: Ad4p; lane S: isolated Ad4 strain No. 1. DNA cleavage patterns of isolated Ad4 strain were different from those of Ad4p.

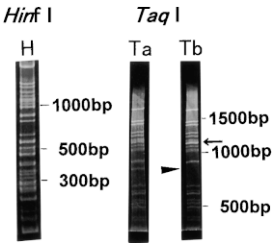

Figure 2. Subgenome typing of isolated adenovirus type 4 (Ad4) strains. Lane H: isolated Ad4 strain No. 4; lane Ta: isolated Ad4 strain No. 10; lane Tb: isolated Ad4 strain No. 9. Cleavage pattern in lane Tb shows missing fragment (arrowhead) and presence of additional fragment (arrow) relative to sequence in lane Ta.

have been analyzed using these REs previously.[5,8,9] In this study, seven Ad4 strains obtained during the first period showed the same subgenome type, and nine strains obtained during the second period were classified into two subgenome types. The differences in the DNA digestion patterns with *Taq*I between the two subgenome types consisted of a missing fragment and the presence of an additional fragment. The DNA digestion pattern with *Hin*fI for all 16 isolates were identical. They may be derivatives of the same subgenome type sharing a common digestion pattern with *Hin*fI.

Table 1. Isolation Dates, Clinics, and Subgenome Type

Strain No.	Isolation Date	Clinic (Distinct)	Subgenome Type*
First period			
1	27/12/93	A (Shiroishi)	a
2	28/12/93	A	a
3	3/1/94	A	a
4	5/1/95	A	a
5	12/1/94	A	a
6	3/2/94	A	a
7	1/3/94	B (Nishi)	a
Second period			
8	13/3/95	B	a
9	15/3/95	C (Chuo)	b
10	16/3/95	D (Higashi)	a
11	24/3/95	C	a
12	3/4/95	D	a
13	10/4/95	C	b
14	14/4/95	C	b
15	14/4/95	C	b
16	1/5/95	C	a

*a and b indicate subgenome types which show DNA digestion pattern of Ta and Tb with *Taq*I, respectively.

Strains showing the digestion pattern Ta with *Taq*I were isolated from all four clinics. Because each clinic was located in a different district in Sapporo city, this type was considered to be distributed widely in the city and to have survived for more than a year. On the other hand, all four strains showing the digestion pattern Tb with *Taq*I were isolated from one clinic. They were thus considered to be distributed in a small area around the clinic. Subgenome type analysis thus revealed variation in the geographical distribution of the virus.

Itakura et al[5] analyzed 122 Ad4a strains isolated at an eye clinic (clinic A in this report) from 1985 to 1989 and reported that the genomic pattern of prevalent Ad4a strains tended to change and to be replaced by new subgenome types every 2 years. They also reported that almost all the prevalent strains isolated during the epidemic outbreak of Ad4a belonged to a single subgenome type, and that when no epidemic was observed the following year, as many as six subgenome types were found that had been replaced by new subgenome types. This indicates that mutation of the Ad DNA occurred frequently during those years. In the present study, however, during 1993, when no epidemic was observed, only one subgenome type was identified and the same subgenome type was also seen the following year. These findings are different from those during 1985–1989. Ad4 DNA seems to have become more conserved and its mutation less frequent in recent years.

According to data on Japanese surveillance of ocular infection, Ad4 was one of the most frequently isolated serotypes from patients with conjunctivitis during 1984–1988. However, the incidence of Ad4 infection has been decreasing since 1993. We suspect that there are two reasons for this decrease in the incidence of Ad4 infection. First, many Japanese are likely to have developed neutralizing antibodies against Ad4 after consecutive epidemics. Second, although Ad4 has been shown to cause many types of conjunctivitis, such as epidemic keratoconjunctivitis, pharyngoconjunctival fever and acute hemorrhagic conjunctivitis,[10–12] it has shown a tendency to manifest simple conjunctivitis without any extraocular symptoms in recent years. Viral dissemination by tear droplets or eye discharge is less frequent than by nasopharyngeal secretion. In addition, we suspect that the decrease in the incidence of mutations of viral DNA is also related to the decrease in the incidence of Ad4 infection. When the incidence of mutations of viral DNA decreases, then the possibility of appearance of a new Ad4 subgenome type that can cause extensive epidemic outbreaks also decreases.

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Restriction endonucleases that recognize 4- or 5-bp sequences digest viral DNA into several fragments, and furthermore, these enzymes sometimes give complicated or double bands in the gel. It is difficult to compare two DNA digestion patterns in different gels. However, using these enzymes, we can detect slight genetic changes in prevalent strains. It was suggested that the decrease in the incidence of Ad4 infection in 1993–1995 was related to the decrease in the incidence of mutations in the Ad4 DNA. However, the small number of isolates in this study imposes certain limitations on reaching a conclusion. Further studies on larger numbers of cases are necessary to confirm the findings.

Recently, the hexon protein sequences of adenovirus revealed hypervariable regions that differed in length among serotypes, and contained more than 99% of hexon serotype-specific residues among human serotypes.[13] Analysis of the genome sequences of hypervariable regions is done for the detection and serotyping of adenovirus. However, since adenovirus hexons that contain hypervariable regions are conserved in the same serotype of any genome type, it is suggested that genome-type-specific residues exist in a region other than in hexon protein.[14] At this time, virus genome typing is considered more suitable for comparing the differences among genomes than for sequencing of the hexon protein. If all the genome sequences of Ad4 were determined, we could expect to obtain invaluable information concerning the relationship between mutations of the Ad4 DNA and epidemics of Ad4 conjunctivitis. This should be the subject of a future study.

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