Aims: To investigate the genetic differences among the strains of adenovirus type 8 (Ad8) circulating in Hiroshima city, Japan, and to study their circulation pattern.

Methods: One hundred and twenty nine strains of adenovirus type 8 (Ad8) were isolated in Hiroshima City over a 15 year period (1983–97) from patients with keratoconjunctivitis, and analysed with six restriction enzymes—BamHI, HindIII, PstI, SacI, Sall, and Smal—to investigate possible relations among the isolates and their genetic variability. Seven hypervariable regions of the hexon gene that carry the type specific epitope were also sequenced to investigate the variation among the genome types.

Results: Restriction endonuclease analyses yielded three known genome types (Ad8A, 13 samples; Ad8B, seven samples; and Ad8E, 35 samples) and a novel genome type (Ad8I, 74 samples). Ad8A, Ad8B, and Ad8E were closely related, with 96% homology, whereas Ad8I had only 71% homology. Ad8A, Ad8B, and Ad8E shared 91.8% and 96.4% homology with regard to their amino acid and nucleotide sequences, respectively, with the isolate 1127 (accession no X74663). However, when compared with Ad8A, Ad8B, Ad8E, and isolate 1127, Ad8I shared only 62.7% and 69.9% homology with regard to amino acid and nucleotide sequences, respectively. Ad8A, Ad8B, and Ad8E had a unique 31 amino acid deletion in the hypervariable region 1 of the hexon gene, whereas Ad8I had a 33 residue deletion. The Ad8E strain that circulated from 1984 to 1995 was stable among the study population. Ad8I was isolated from an outbreak of epidemic keratoconjunctivitis in 1995 and was also isolated from sporadic cases until 1997.

Conclusions: These results confirmed that genetic variability occurs in Ad8 in the microenvironment and revealed the emergence of a new genome type (Ad8I).

The 51 serotypes of human adenovirus are classified into six subgenera, A to F, according to their biological, immunological, and biochemical properties. Adenovirus type 8 (Ad8) is the most common agent of epidemic keratoconjunctivitis (EKC). EKC is a highly contagious ocular infection and is often transmitted through ocular facilities, thus causing community epidemics. Type specific neutralisation, which is used for the identification of adenoviruses, cannot distinguish genetic variation among the isolates. Ad8 genomic variants (genome types) have been classified as Ad8A to Ad8H by means of restriction enzyme analysis of prototype and field isolates. This genome typing has provided a powerful and practical tool for the study of the epidemiological and clinical patterns of adenoviruses.

“Epidemic keratoconjunctivitis is a highly contagious ocular infection and is often transmitted through ocular facilities, thus causing community epidemics.”

In our study, Ad8 strains isolated in Hiroshima city over a 15 year period were analysed with six restriction enzymes (BamHI, HindIII, PstI, SacI, Sall, and Smal) to investigate the genetic differences among the isolates, and their circulation pattern. Hypervariable regions (HVRs) of the hexon gene that carry type specific epitope were also sequenced to document the degree of variation among the genome types, which may have some relation to the disease.

Materials and Methods

Samples
In total, 129 strains were isolated from epidemic and sporadic cases of keratoconjunctivitis. The clinical diagnosis was recorded as EKC, acute haemorrhagic conjunctivitis, or acute conjunctivitis. All samples were isolated in Hep2 cells.

DNA extraction
DNA was extracted from the infected cells by a modified Hirt's procedure. Briefly, confluent monolayers of Hep2 cells in 25 cm² flasks were inoculated with virus stock and incubated at 35°C. When a 75–100% cytopathic effect was seen, cells were dislodged with a cell scraper and pelleted by low speed centrifugation. Cells were washed twice with phosphate buffered saline and resuspended in 1 ml of lysis buffer (10mM Tris/HCl (pH 7.4), 10mM EDTA, 1% sodium dodecyl sulfate (SDS)) for 15 minutes at room temperature. The suspension was incubated with 200 μg/ml of proteinase K (Sigma Chemical, St Louis, Missouri, USA) at 37°C for one hour. After incubation, 5M NaCl was added to a final concentration of 1M, and further incubated at 4°C overnight to precipitate cellular DNA.

Abbreviations: Ad8, adenovirus type 8; EKC, epidemic keratoconjunctivitis; HVR, hypervariable region; PCR, polymerase chain reaction; PCRF, pairwise comigrating restriction fragments; SDS, sodium dodecyl sulfate; TE buffer, Tris/EDTA buffer
The suspension was then centrifuged at 15,000 × g for 30 minutes. The supernatant was incubated with 30 µg of RNase A (Sigma Chemical) for one hour and extracted twice in phenol/chloroform. Next, the supernatant was precipitated in two volumes of 100% ethanol. After drying, the DNA was suspended in 50 µl of Tris/EDTA buffer (10mM Tris/HCl (pH 7.4), 10mM EDTA) and measured spectrophotometrically.

DNA restriction enzyme analysis
Restriction enzyme analyses were performed with BamHI, HindIII, PstI, SacI, Sall, and Smal (Boehringer Mannheim, Mannheim, Germany). Briefly, a 2 µg aliquot of DNA was incubated with 10 units of restriction endonucleases in 20 µl of reaction mixture at an appropriate temperature (that recommended for each restriction endonuclease) for three hours. After digestion, all products were electrophoresed on a 1.2% horizontal submerged agarose gel at 90 V for three hours in 50 mM Tris acetate EDTA buffer (pH 8.0). The gel was stained with ethidium bromide (1 µg/ml) and photographed under ultraviolet light with a polaroid camera (Funakoshi, Tokyo, Japan). HindIII digests of λ DNA (Boehringer Mannheim) were used as molecular weight markers. Genomic homology between the two strains was calculated using the percentage of pairwise comigrating restriction fragments (PCRF) of a pair divided by the total number of bands in the pair. Genome type identifications were conducted by comparison of the resulting patterns with the published restriction patterns of the prototype and genome types.

PCR, cycle sequencing, and sequence analysis
HVRs were sequenced by generating overlapping polymerase chain reaction (PCR) products and direct cycle sequencing. A set of six primers (forward primers: AdHD1N, 5′-TGG ACC GCG GTC CCA GCT A-3′ (19 to 41); AdHD2F, 5′-ATG AAA CCA TGC TAT GGC TC-3′ (439 to 459); and AdHD3F, 5′-TGG TCG ACT TGC AAG ACA G-3′ (824–842); reverse primers: AdHD2R, 5′-TAG GTT GAC CAT CTT CAG TGG T -3′ (526–505); AdHD3R, 5′-CTG TCC ACC GCA GAG TTC CA-3′ (929–911); and AdH4, 5′-GCC ACG TTC GAG TAC AGA AAA C-3′ (1187–1166)) were selected based on the alignment of hexon gene sequences available from GeneBank (Ad8 (X74663), Ad19 (X98359), Ad37 (X98360), Ad9 (X74664), and Ad15 (X74666)) from human adenovirus serotypes Ad8, Ad19, Ad37, Ad9, and Ad15, respectively. All products were sequenced in both directions with internal and template primers. Full length adenoviral DNA, extracted by Hirt’s method, was used as a template for PCR. The PCR amplification was carried out in 50 µl reaction mixtures containing 1 µl aliquots of DNA, 5 µl of 10x concentrated buffer, 0.5 µM each of the primer pair, 200 µM of each dNTP, and 1.25 U of Taq polymerase (Boehringer Mannheim). The assays were performed in a programmable heat block (model 9600-R; Perkin Elmer, Foster City, California, USA). Thermal cycling consisted of preliminary denaturation for three minutes at 94°C, followed by 35 cycles of denaturation at 94°C for one minute, annealing at 47°C for one minute and at 72°C for two minutes, and a final extension at 72°C for seven minutes. The amplification products were analysed on a 1.5% agarose gel. Next, the

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Figure 1 Identification of adenovirus type 8 (Ad8) genome types in Hiroshima. The open boxes indicate cleavage patterns identical to those of Ad8B. The various shaded boxes indicate cleavage patterns different from those of Ad8B and also distinct from each other.

Figure 2 Restriction fragments of adenovirus type 8 (Ad8) were electrophoresed on a 1.2% agarose gel with a HindIII digest of λ DNA (lane m) as molecular weight standard. Lanes A, B, E, and I contain genome types Ad8A (isolates from 1983), Ad8B (from 1984), Ad8E (from 1994), and Ad8I (from 1995), respectively. Ad8 digested with (A) Sall and PstI; (B) SmaI and BamHI; (C) HindIII and SacI.
PCR products were purified using a DNA fragment purification kit (Mag Extractor-PCR and Gel Cleanup; Toyobo, Osaka, Japan) according to the manufacturer's instructions. The cycle sequence reaction was carried out with an ABI prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Chiba, Japan). The sequences were determined by a genetic analyser 310 (Applied Biosystems). The nucleotide sequences of four genome types (Ad8A, Ad8B, Ad8E, and Ad8I) were compared with the available sequence of the Ad8 isolate, 1127 (accession number, X74663). DNASIS software (Hitachi Software Ltd, Tokyo, Japan) was used for sequence alignment and analysis.

Nucleotide sequence accession numbers

Sequence data from this article have been deposited in GenBank/DDBJ under the accession numbers: hexon gene Ad8A (AB090341), Ad8B (AB090342), Ad8E (AB090343), and Ad8I (AB090344). The amino acid sequences of the residues were deduced.

RESULTS

Prevalence among the age groups

Patients were divided into three age groups: 0–9 years, 10–19 years, and > 20 years. Thirty two (24.8%) of the stains isolated came from the 0–9 year old group, whereas only 9 (6.9%) were in the 10–19 year old group. Most isolates (88 (68.2%)) came from the > 20 years old group.

Cleavage patterns with the restriction endonucleases

Restriction endonuclease cleavage patterns with SacI, PstI, and SmaI divided the isolates into two groups, whereas HindIII and SalI divided them into three groups (fig 1).

Cleavage pattern with HindIII

Upon digestion with HindIII, 20 isolates showed identical restriction patterns shared by Ad8A and Ad8B. Thirty five isolates, classified as Ad8E, showed a distinct restriction pattern. However, 74 isolates showed a different restriction pattern. These isolates are a novel genome type, designated Ad8I (fig 2C).

Cleavage pattern with Sall

Upon digestion with Sall, 48 isolates showed a similar restriction pattern to that of Ad8A and Ad8E. The pattern of seven isolates was identical to that of Ad8B. However, 74 isolates showed a new restriction pattern (fig 2A).

Cleavage pattern with PstI, Smal, and SacI

Fifty five isolates showed restriction patterns that correspond with Ad8A, Ad8B, and Ad8E. However, 23 isolates showed a new restriction pattern (fig 2A–C).

Cleavage pattern with BamHI

BamHI was not useful for distinguishing between the different isolates because they all showed an identical restriction pattern (fig 2B).

Genome type circulation

The analysis of 129 isolates from Hiroshima using six restriction enzymes (Bam HI, HindIII, PstI, SacI, SalI, and SmaI) yielded three known genome types, namely: Ad8A (13 isolates), Ad8B (seven isolates), and Ad8E (35 isolates) and a new genome type, designated Ad8I (74 isolates) (fig 2). Ad8A and Ad8B circulated between 1983 and 1988, and Ad8E between 1984 and 1995. Ad8I was first isolated from epidemic keratoconjunctivitis in 1995, and then from sporadic cases of EKC until 1997 (fig 3).

Homology among the genome types

DNA homology studies of serotype 8 are often difficult, owing to its growth properties. Some strains grow well but others replicate slowly in the laboratory, as reported previously.12 In our study, there was not enough DNA for the analysis of small fragments with low molecular weight.

Table 1  

<table>
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Ad8, adenovirus type 8.
Pairwise comparison of PCRF showed that Ad8A has a total of 48 fragments for the six restriction enzymes. Ad8B and Ad8E shared 46 (96%) fragments with Ad8A, whereas Ad8I shared 34 (71%) fragments with Ad8A (table 1).

**Nucleotide sequence analysis**
Ad8A, Ad8B, and Ad8E share 91.8% and 96.4% homology in their amino acid and nucleotide sequences, respectively, with the isolate 1127 (accession number, X74663) at the HVRs of the hexon gene. However, when compared with Ad8A, Ad8B, Ad8E, and isolate 1127, Ad8I shared only 62.7% and 69.9% homology in amino acid and nucleotide sequences, respectively. Ad8A, Ad8B, and Ad8E showed a unique deletion of 31 amino acids in HVR 1, whereas Ad8I showed a 33 residue deletion (fig 4).

**DISCUSSION**
Ad8 has a much higher tropism for conjunctival cells and produces more severe clinical manifestations and pathological
Ad8 also has more genomic variants than Ad19 and Ad37 types. Thus, Ad8 has been the target of extensive studies, including those at the molecular level. This serotype persists in the population and causes sporadic epidemics, whereas Ad19 and Ad37 seemed to pass through the population in individual waves. This typing offers a chance to follow the epidemiological distribution of the virus in different geographical regions and time periods. Ad8 is currently endemic in Japan and other East Asian countries. Among four genome types detected in Hiroshima, Ad8A and Ad8B circulated for a long period of time, from 1984 to early 1995. In comparison, Ad8E circulated in Taiwan (Kaoshiung) and Korea (Pushan) in 1981–1987 and in 1983, respectively.

The isolation of a new genome type is medically and epidemiologically important because the appearance of new genome types can result in more severe attacks of conjunctivitis.

The novel genome type Ad8I, which replaced Ad8E, circulated in Hiroshima for a longer period of time, from 1984 to 1995. Ad8I was isolated from an outbreak of epidemic keratoconjunctivitis in Sapporo. Ad8E share 96% PCRF with Ad8A, whereas Ad8I shares only 62.7% with the isolate 1127 (accession no X74663), whereas Ad8I was more distantly related. Ad8I was isolated from an outbreak of epidemic keratoconjunctivitis in 1995 and was also isolated from sporadic cases until 1997. The Ad8E strain that circulated from 1984 to 1995 was distant from Ad8A, Ad8B, or Ad8E. Sequence variation in the hexon gene of Ad8I may have played a series of genetic changes, although the presence of Ad8E in Hiroshima over a 12 year period indicated that this genome type was very stable. The isolation of a new genome type is medically and epidemiologically important because the appearance of new genome types can result in more severe attacks of conjunctivitis. Continued investigations into the genome types of adenoviruses will help to define the unique evolutionary tendencies of these viruses.

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REFERENCES


Table 2 Distribution of Ad8 genome types in Asia/Pacific

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Ad8A-Ad8I, genome types; Ad8P, prototype strain (Trim)


Genetic characterisation of adenovirus type 8 isolated in Hiroshima city over a 15 year period


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