Serological and genetic characterisation of a unique strain of adenovirus involved in an outbreak of epidemic keratoconjunctivitis

A K Adhikary, T Inada, U Banik, A Mukouyama, Y Ikeda, M Noda, T Ogino, E Suzuki, T Kaburaki, J Numaga, N Okabe

Aims: To characterise a novel strain of adenovirus (Ad) type Ad8 (genome type Ad8I) involved in an epidemic keratoconjunctivitis (EKC) outbreak in Hiroshima city using serological testing and sequence analysis of the fibre and hexon gene.

Methods: A neutralisation test (NT) was performed in microtitre plates containing a confluent monolayer of A549 cells using 100 tissue culture infectious doses of virus and type specific antisera. The haemagglutination inhibition test was also carried out in microtitre plates with rat erythrocytes using four haemagglutination units of virus and twofold dilutions of serum. The fibre gene was sequenced by generating overlapping polymerase chain reaction products or by direct sequencing of genomic DNA. Primer selection was based on alignment of the fibre genes of human adenovirus serotypes Ad8, Ad19, Ad37, Ad9, and Ad15 available from Gene Bank.

Results: The virus strain was specifically neutralised by anti-Ad8 antibodies, although there was a major crossreaction with anti-Ad9 antibodies. Haemagglutination was equally inhibited by anti-Ad8 and anti-Ad9 antibodies. The predicted amino acid sequences of the hypervariable regions (HVRs) of the Ad8I strain were 83.3% homologous to other Ad8 genome types when REA and sequence analysis of the fibre and hexon gene.

Conclusions: Ad8I is a unique strain of adenovirus because of its lower genomic homology with Ad8, major crossreactivity with Ad9 in NT, and mixed genetic organisation of HVRs of the hexon gene. These factors may have enabled the virus to circumvent acquired immunity, resulting in the outbreak.

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here are 51 serotypes of human adenoviruses (Ads), which are divided into six subgenera (A–F) based on biochemical, immunological, and morphological criteria. Among them, subgenus D consists of 32 serotypes including Ad8, Ad19, and Ad37, the main agents of epidemic keratoconjunctivitis (EKC), and Ad9 and Ad15, which cause acute follicular conjunctivitis. EKC is a clinical disease entity characterised by severe bilateral conjunctivitis, with substantial corneal and extraocular involvement, leading to decreased quality of life and possible economic consequences.

Between 1995 and 1997, in Hiroshima city, a novel strain of Ad8 was isolated from an outbreak of EKC and sporadic cases of EKC; this strain was identified as genome type Ad8I by restriction endonuclease analysis (REA). Before the detection of Ad8I, genome types A8A–H had been identified by REA. The Ad8 genome types usually share 96% and >90% homology when compared by REA or the predicted amino acid sequence of the hypervariable regions (HVRs) of the hexon gene/protein, respectively. In contrast, the novel strain Ad8I was only 71% and 62.0% homologous to other Ad8 genome types when REA and sequence analysis of the HVRs of the hexon gene were carried out. This unusually low degree of homology characterised Ad8I as a unique strain among the Ad8 genome types described so far, prompting us to study this strain in depth at the molecular level.

The hexon protein of adenovirus carries type specific antigenic determinants in its HVRs, which react with neutralising antibody in the serum neutralisation test (NT). Neutralisation of the infectivity of adenoviruses is primarily determined by antibody against the hexon protein. In contrast, the fibre protein is responsible for haemagglutination and also the attachment of the virus to specific cellular receptors through its receptor binding site on the knob region. Together with the hexon protein it is responsible for the serotype specificity of adenoviruses. In our present study, the fibre and hexon genes of the novel strain Ad8I were analysed to compare immunological data (NT and haemagglutination inhibition (HAI) test) with the molecular biology results in an attempt to define any genetic differences that might be relevant to the outbreak of EKC.

MATERIALS AND METHODS

Viruses

Adenovirus prototypes Ad9 and Ad11 and the antisera were obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA); Ad8 was obtained from the collection of the National Institute of Infectious Diseases, Tokyo, Japan. The Ad8I strain was isolated in Hiroshima city, Japan, from the outbreak and sporadic cases of EKC from 1995 to 1997.

Abbreviations: Ad, adenovirus; ATCC, American Type Culture Collection; EKC, epidemic keratoconjunctivitis; HAI, haemagglutination inhibition; HPV, hypervariable region; NT, neutralisation test; PCR, polymerase chain reaction; REA, restriction endonuclease analysis.
Serological tests
For NT analysis the adenovirus stock was grown in A549 cells and titres were determined in microtitre plates containing a confluent monolayer of A549 cells. Aliquots of 25 μl of 100 tissue culture infectious doses of virus (100TCID50) were incubated at 37°C for 60 minutes with 25 μl volumes of twofold serially diluted rabbit antisera, and inoculated into the A549 cells. The adenovirus type was determined by the antisera, which completely inhibited viral growth after 72 hours of incubation. The HAI test was also carried out in microtitre plates with rat erythrocytes. Briefly, four haemagglutination units of virus and twofold dilutions of serum (which had been treated at 65°C for 30 minutes to remove non-specific agglutinin), both in a 25 μl volume, were incubated at room temperature for one hour. Next, 25 μl (final concentration, 1.0%) of erythrocytes was added and the mixture was allowed to stand for one hour at 37°C, after which the results were read. The antiserum to Ad8, Ad9, Ad19, and Ad11 in both the tests were obtained from ATCC. The rat blood cells were obtained from Cosmobio (Tokyo, Japan).

Extraction of viral DNA
Viral DNA was extracted from a confluent monolayer of Hep2 cells inoculated with virus stock. When a cytopathic effect of greater than 75% was observed, cells were dislodged and pelleted by low speed centrifugation. Cells were washed in phosphate buffered saline and resuspended in lysis buffer (10mM Tris/HCl (pH 7.4), 10mM EDTA, 1% sodium dodecyl sulfate buffered saline and resuspended in lysis buffer greater than 75% was observed, cells were dislodged and resuspended in lysis buffer (10mM Tris/HCl (pH 7.4), 10mM EDTA) and measured spectrophotometrically. Next, 5M NaCl was added to a final concentration of 1M, and further incubated at 4°C overnight to precipitate cellular DNA. The suspension was centrifuged at 15 000 × g for 30 minutes. The supernatant was incubated with 30 μl of RNase A (Sigma Chemical) for one hour and then extracted twice in phenol/chloroform, after which the supernatant was precipitated in two volumes of absolute ethanol. After drying, the DNA was suspended in 50 μl of TE buffer (10mM Tris/HCl (pH 7.4), 10mM EDTA) and measured spectrophotometrically.

DNA sequencing
The fibre gene was sequenced by generating overlapping polymerase chain reaction (PCR) products or by direct cycle sequencing of genomic DNA. The selection of the primers was based on available sequences from Gene Bank (Gene Bank accession numbers for the fibre gene: X74660 (Ad8), U69731 (Ad19a), U69132 (Ad37), X74659 (Ad9), and X72934 (Ad15)) of human adenovirus serotypes Ad8, Ad19, Ad37, Ad9, and Ad15. All products were sequenced from both directions with internal and template primers (table 1). Full length adeno viral DNA, extracted by the modified Hirt’s method, was used as a template for PCR. The PCR amplification was carried out in 50 μl reaction mixtures containing 1 μl of DNA, 5 μl of 10× concentrated buffer, 0.5 μl of each primer pair, 200 μM of each dNTP, and 1.25 U of Taq polymerase (Boehringer-Manheim, Mannheim, Germany). The assays were performed in a programmable heat block (Perkin Elmer model 9600-R, 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 extension at 72°C for two minutes, with a final extension at 72°C for seven minutes. The amplification products were analysed on a 1.5% agarose gel. The PCR products were purified using a DNA fragment purification kit (Mag Extractor-PCR and Gel Clean Up; Toyobo Co Ltd, Osaka, Japan), according to the manufacturer’s protocol. 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 Biosystem, Foster City, California, USA). The hexon gene sequence of Ad8I was previously deposited in DDBJ/Genome Bank under the accession number AB090344. The nucleotide sequences of both the hexon and fibre genes were translated into amino acid sequences and compared with the available sequences of Ad8, Ad9, Ad15, Ad19a, and Ad37 by DNASTS (Hitachi Software Ltd, Tokyo, Japan).

Table 1
Oligonucleotide primers for polymerase chain reaction and sequencing of the adenovirus fibre gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Polarity</th>
<th>Position</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad8F</td>
<td>+</td>
<td>1–17</td>
<td>5’-AAG GGA GGT CAA ATC TCC-3’</td>
</tr>
<tr>
<td>ShoF</td>
<td>+</td>
<td>274–298</td>
<td>5’-CTG GAA AAT TAA CAG TTA ATA CTT GAA A-3’</td>
</tr>
<tr>
<td>Kndb1F</td>
<td>+</td>
<td>513–530</td>
<td>5’-AGA GTC GGA GAA GCC GGC-3’</td>
</tr>
<tr>
<td>Kndb2F</td>
<td>+</td>
<td>813–833</td>
<td>5’-TCAT CTA ACC GGT AAA TCA-3’</td>
</tr>
<tr>
<td>ShoR</td>
<td>+</td>
<td>297–274</td>
<td>5’-CAG TAT TAA CTT GTA ATT TCC GAG-3’</td>
</tr>
<tr>
<td>Kndb1R</td>
<td>+</td>
<td>530–513</td>
<td>5’-GCC GCC TTC TAC AAT CT-3’</td>
</tr>
<tr>
<td>Kndb2R</td>
<td>+</td>
<td>833–813</td>
<td>5’-TGA TCT ACC AAG ATT TGA AAG-3’</td>
</tr>
<tr>
<td>Ad8Rcom</td>
<td>−</td>
<td>1209–1188</td>
<td>5’-TAC YGG YGC TGG TGT AAA AAT C-3’</td>
</tr>
</tbody>
</table>

Nucleotide sequence accession numbers
The nucleotide sequence data reported here will appear in the DDBJ/Genome Bank nucleotide sequence database with the accession number AB098565 (fibre gene).

RESULTS
Serological tests
Ad8I crossreacted with Ad8 and Ad9 antisera in both the NT and the HAI test (table 2).

Sequence analysis
Fibre gene: nucleotide sequence
We determined the nucleotide sequence of the Ad8I fibre gene. Sequence analysis showed that the fibre region of this strain is 1083 nucleotides long, whereas corresponding regions of the fibre genes of Ad8 and Ad9 are 1086 nucleotides long. The difference in length results from the deletion of three nucleotides in the shaft region. Ad8I shows higher homology with Ad8 (overall homology, 95.5%) than with Ad9 (overall homology, 91.5%). However, in the knob region it shows 97.0% and 92.7% homology with Ad8 and Ad9, respectively.

Table 2
NT and HAI titres of the Ad8, Ad8I, and Ad9 strains

<table>
<thead>
<tr>
<th>Adenovirus</th>
<th>Ad8</th>
<th>Ad8I</th>
<th>Ad9</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT titration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad8 antiserum</td>
<td>256</td>
<td>256</td>
<td>16</td>
</tr>
<tr>
<td>Ad9 antiserum</td>
<td>&lt;4</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>HAI titration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad8 antiserum</td>
<td>320</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>Ad9 antiserum</td>
<td>320</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>Ad19 antiserum</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Ad11 antiserum</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

Ad, adeno virus; HAI, haemagglutination inhibition; NT, neutralisation test.
**Fibre polypeptide**
The amino acid sequence of the Ad8I fibre gene was deduced from the nucleotide sequences and aligned for maximum homology (fig 1). The predicted fibre polypeptide comprises 361 amino acid residues, whereas the fibre polypeptides of Ad8 and Ad9 comprise 362 amino acids each. The shaft of Ad8I is 138 residues long, whereas the corresponding regions of Ad8 and Ad9 are made up of 139 amino acids. It also has eight repeats of a 15 amino acid basic structural unit (motif), containing conserved hydrophobic glycine (G) and proline (P) residues. These eight motif repeats in the shaft are consistent with the length of the subgenus D fibre protein. Ad8I showed six mismatches with Ad8 and 16 mismatches with Ad9, resulting in 90.5% and 84.0% homology, respectively.

The fibre knob of Ad8I comprises 180 amino acids—the same as Ad8 and Ad9—and shows nine and 12 residue mismatches with Ad8 and Ad9, resulting in 94.4% and 91.6% homology, respectively (table 3). In the knob, residues are

| Table 3 Homology (%) between fibre nucleotide and amino acid sequences of Ad8I and those of the members of the adenovirus subgenus D |
|---|---|---|---|---|
| Fibre region | Ad8 | Ad9 | Ad15 | Ad19a/37 |
| Tail DNA | 95.4 | 96.8 | 95.3 | 96.1 |
| Tail Protein | 93.0 | 97.6 | 95.3 | 95.3 |
| Shaft DNA | 94.0 | 88.1 | 59.1 | 53.8 |
| Shaft Protein | 90.5 | 84.0 | 49.2 | 31.8 |
| Knob DNA | 97.0 | 92.7 | 54.2 | 78.9 |
| Knob Protein | 94.4 | 91.6 | 35.5 | 77.6 |
| Overall DNA | 95.5 | 91.5 | 61.0 | 71.1 |
| Overall Protein | 92.7 | 89.4 | 47.9 | 62.0 |

DNA and amino acid homologies were obtained using DNASIS (Hitachi) software.
conserved between Ad8 and Ad8I, including alanine (A) in position 222 and lysine (K) in position 247, which are replaced by aspartic acid (D) and glutamic acid (E), respectively, in Ad9 (fig 1).

**Hexon gene**

The amino acid sequences of the HVRs of the Ad8I hexon protein showed 83.3% homology with Ad9, whereas only 62.0%, 70.9%, and 63.6% homology was seen with Ad8, Ad19, and Ad37, respectively (table 4). Ad8I did not show the unique insertion of 33 residues in HVR1 as is seen in Ad8 (fig 2).

**DISCUSSION**

Adenoviral keratoconjunctivitis is endemic in Japan, with a peak incidence in the summer. According to the National Surveillance System of Infectious Diseases in Japan, adenovirus alone is responsible for 95% of viral conjunctivitis, with a predominance of subgenus D. In contrast to Ad19 and Ad37, Ad8 possesses a higher tropism for the conjunctiva and induces severe clinical manifestations in EKC, whereas Ad9 infrequently causes acute follicular conjunctivitis and mild respiratory infections. Although REA of the genome is infrequently causes acute follicular conjunctivitis and mild respiratory infections. In a previous study, we have shown that the outbreak by the novel strain Ad8I helped the virus to escape the acquired immune response present in the population, enabling the outbreak of epidemic keratoconjunctivitis to occur.

In conclusion, Ad8I is a unique strain because of its lower genomic homology with other Ad8 genome types, major cross-reactivity with Ad9 in the neutralisation test, and mixed genetic organisation of hypervariable regions of the hexon gene.

**Table 4** Hexon gene amino acid homologies (%) of Ad8I with the members of adenovirus subgenus D

<table>
<thead>
<tr>
<th>Ad</th>
<th>HVR1</th>
<th>HVR2</th>
<th>HVR3</th>
<th>HVR4</th>
<th>HVR5</th>
<th>HVR6</th>
<th>HVR6</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad8</td>
<td>8.8</td>
<td>11.7</td>
<td>18.1</td>
<td>52.3</td>
<td>10.5</td>
<td>41.6</td>
<td>42.8</td>
<td>62.0</td>
</tr>
<tr>
<td>Ad9</td>
<td>61.7</td>
<td>94.1</td>
<td>81.8</td>
<td>23.8</td>
<td>71.4</td>
<td>41.6</td>
<td>77.2</td>
<td>83.3</td>
</tr>
<tr>
<td>Ad15</td>
<td>45.4</td>
<td>29.4</td>
<td>27.2</td>
<td>26.3</td>
<td>33.3</td>
<td>58.3</td>
<td>75.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Ad19</td>
<td>41.1</td>
<td>11.7</td>
<td>45.4</td>
<td>38.0</td>
<td>33.3</td>
<td>41.6</td>
<td>65.9</td>
<td>70.9</td>
</tr>
<tr>
<td>Ad37</td>
<td>14.7</td>
<td>41.1</td>
<td>63.6</td>
<td>20.0</td>
<td>14.2</td>
<td>58.3</td>
<td>29.5</td>
<td>63.6</td>
</tr>
</tbody>
</table>

Amino acid homologies were obtained using DNASIS (Hitachi) software.

Ad, adenovirus; HVR, hypervariable region.

**Take home messages**

- Adenovirus 8I (Ad8I) is a unique strain of adenovirus because of its lower genomic homology with Ad8, major crossreactivity with Ad9 in the neutralisation test, and mixed genetic organisation of hypervariable regions of the hexon gene.
- The high homology between Ad8I and Ad8 in the fibre knob may explain the higher tropism of this strain for conjunctival cells.
- Illegitimate recombination in the hexon and fibre genes may have helped the virus to escape the acquired immune response present in the population, enabling this outbreak of epidemic keratoconjunctivitis to occur.

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We have shown that the hypervariable regions of the Ad8I hexon are more homologous to Ad9 than to Ad8.

In the HA1 test, haemagglutination was equally inhibited by antisera against Ad8 and Ad9, a typical characteristic of Ad8 and Ad9 in this test. Comparison of the predicted amino acid homology of the isolate with the prototypes, in addition to the presence of specific residues in the fibre knob, apparently provided a guideline to distinguish EKC (Ad8) from non-EKC (Ad9) strain. The fibre knob amino acid sequence of the new strain shares 94.4% and 91.6% homology with Ad8 and Ad9, respectively. Seven conserved sequences (fig 1) found in the subgenus D fibre knob are also present in Ad8I. The new strain carries residues A (alanine) and K (lysine) in positions 222 and 247, which are conserved among EKC strains Ad8, Ad19, and Ad37, but are replaced by other residues in Ad9; these residues are thought to play an important role in the higher tropism of these strains for corneal and conjunctival cells. Hence, it seems likely that the Ad8I strain has the same Ad8 fibre ancestor, but has been modified slightly.

We have shown that the outbreak by the novel strain Ad8I can be explained by the immunological data and the molecular biology of the hexon and the fibre genes. The high homology between Ad8I and Ad8 in the fibre knob possibly played a key role in the higher tropism of this strain for conjunctival cells. In contrast, illegitimate recombination helped the virus to escape the acquired immune response present in the population and caused the outbreak of EKC. In conclusion, Ad8I is a unique strain because of its lower genomic homology with other Ad8 genome types, major crossreactivity with Ad9 in the NT, and unique genetic
organisation at the HVRs of the hexon and the fibre genes, which possibly enhanced the pathogenic potential of the virus. It is of utmost importance to accumulate gene sequence data on the HVRs of the hexon and the fibre genes of EKC causing adenoviruses to help predict future outbreaks of adenovirus infections.

Figure 2 Comparison of predicted amino acid sequences of seven hypervariable regions (HVRs) of adenovirus 8 (Ad8), Ad9, Ad8I, Ad15, Ad19, and Ad37 hexon polypeptide. The sequences of loop 1 and loop 2 have been aligned to obtain maximal homology. The HVRs are marked above.
ACKNOWLEDGEMENTS
Supported by grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), Japan.

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doi: 10.1136/jcp.2003.012500

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