Characterisation of hexon and fibre genes of a novel strain of adenovirus involved in epidemic keratoconjunctivitis

A K Adhikary, T Inada, J Numaga, E Suzuki, H Ushijima, U Banik, A Mukouyama, S Matsuno, N Okabe

Aims: To characterise a novel strain (M86) of adenovirus (Ad) involved in epidemic keratoconjunctivitis (EKC).

Methods/Results: The virus strain was neutralised by antisera to both Ad35 and Ad11. Restriction endonuclease analysis of genomic DNA showed 98% and 88% homology with Ad11 and Ad35, respectively. The deduced amino acid sequence of the hypervariable regions of (HVRs) of the hexon gene showed a higher homology with Ad35 (94.4%) than with Ad11 (83.7%). However, it was 100% homologous to Ad35 in HVRs 1, 2, 3, and 6 and to Ad11 in HVRs 4 and 6. In the fibre knob, the isolate was more homologous to Ad11 (99.4%) than to Ad35 (29.1%).

Conclusion: This novel strain of adenovirus showed similarities with both Ad11 and Ad35. The isolation of a novel strain like Ad35+11 is important because of its association with EKC.

A denovirus type 11 (Ad11) and adenovirus type 35 (Ad35) belong to subgenus B2, and cause opportunistic infections mainly among the immunocompromised patients.1 Ad35 was isolated for the first time from a renal transplant recipient with interstitial pneumonia,2 whereas Ad11 was isolated from a faecal specimen of a child with poliomyelitis.3 Here, we report the isolation of a novel strain of adenovirus from a 25 year old otherwise healthy male patient with severe clinical manifestations of epidemic keratoconjunctivitis (EKC) in southern Japan.

METHODS AND RESULTS
Immunochromatography confirmed the causative agent as adenovirus (strain M86).4 Conjunctival scrapings were isolated in A549 cells and the viral titre was also determined in a microtitre plate containing a confluent monolayer of A549 cells. Aliquots (25 μl) of 100 tissue culture infectious doses of virus (100TCID₅₀) were incubated with 25 μl of serially diluted type specific antisera at 37°C for 60 minutes and then inoculated into A549 cells. Viral growth was inhibited by two different type specific antisera, anti-Ad11 and anti-Ad35, at a 256-fold dilution, and the strain was identified as Ad35+11. Although Ad11 infrequently causes keratoconjunctivitis, Ad35 or a novel strain like M86 (Ad35+11) has never been reported as an ocular pathogen.5 Therefore, this strain was subjected to a detailed study at the molecular level.

Viral DNA extraction and restriction endonuclease analysis of M86 with BamHI, BglII, BstEII, EcoRI, SalI, SmaI, XbaI, and XhoI (Boehringer Mannheim, Mannheim, Germany) were carried out to investigate homology with the serologically related prototypes (Ad35 and Ad11), as described previously.6 Genomic homology between M86, Ad35, and Ad11 was calculated from published restriction patterns of Ad11, Ad35, and M86, using the percentage of pair wise co-migrating restriction fragments of a pair divided by the total number of bands in the pair. The isolate (M86) showed 98% and 88% homology with Ad11 and Ad35, respectively (fig 1). Higher homology of the new strain in restriction endonuclease analysis with Ad35 and Ad11 provide supportive evidence that the new strain might have evolved from the recombination of these two parent viruses.7

The fibre knob enables the virus to attach to the cellular receptor and, together with the hexon protein, defines the serological specificity of the adenoviruses. Therefore, the hexon gene and the fibre gene were analysed to compare the immunological data with the molecular biological results, in addition to looking for any possible variation that might be related to ocular pathogenicity. The hypervariable regions

Abbreviations: AA, amino acid; Ad, adenovirus; EKC, epidemic keratoconjunctivitis; HVR, hypervariable region

Figure 1 Restriction patterns obtained after cleavage of Ad35p (P) and M86 (C). (A) Restriction endonucleases XhoI, BstEII, EcoRI, SalI, and SmaI; (B) XbaI, PstI, BamHI, HindIII, and BglII. The samples were electrophoresed on a 1.2% agarose gel. A HindIII digest of λ DNA (lane M) was run as a molecular weight standard.
(HVRs) of the hexon gene and all regions of the fibre gene were sequenced by overlapping primers from genomic DNA by direct cycle sequencing. Multiple sets of primers for the hexon and fibre genes were selected based on alignment of the hexon gene (x76549 (Ad3), x76551 (Ad7), AB018424 (Ad11), AB018425 (Ad14), x74662 (Ad16), AY008279 (Ad21), AB018246 (Ad34), and AB018427 (Ad35)) and fibre gene sequences (m12411 (Ad3), m23696 (Ad7), L08231 (Ad11), AB065116 (Ad14), u06106 (Ad16), u06107 (Ad21), u10271 (Ad34), and u10272 (Ad35)) available from GeneBank. The sequences were determined by a Genetic Analyser 310 (Applied Biosystem, Foster City, California, USA). DNASIS software (Hitachi Software Ltd, Tokyo, Japan) was used for sequence alignment and analysis. The amino acid (AA) sequences of these residues were deduced.

The AA sequences of M86 were compared with the available sequences of Ad3, Ad7, Ad11, Ad34, Ad35, Ad8, Ad19a, and Ad37 involved in keratoconjunctivitis. The nucleotide sequence data reported in our paper will appear in the DDBJ/GeneBank nucleotide sequence database with the accession numbers AB098564 (hexon gene) and AB098563 (fibre gene).

In HVRs of the hexon, M86 showed an overall 94.4% AA homology with Ad35 and 86.7% with Ad11 (table 1). However, it was 100% homologous to Ad35 in HVRs 1, 2, 3, and 6 and to Ad11 in HVRs 4 and 6 (fig 2). The fibre knob showed high AA homology (99.4%) with Ad11, but only 29.1% homology with Ad35 (table 2).

**DISCUSSION**

Members of subgenus D adenoviruses (Ad8, Ad19, and Ad37) are the common agent of EKC. Occasionally, the members of subgenus B (Ad3 and Ad7) and subgenus E (Ad4) are also related to EKC. The tropism of adenoviruses for conjunctival or corneal cells depends on the presence of certain amino acids in the knob, which attaches the virus to the specific cellular receptor. The fact that the fibre knob of M86 has 99.4% homology with Ad11, with only a single AA difference, but only 29.1% homology with Ad35, means that it is able to attach to conjunctival and corneal cells (fig 3).

### Table 1  Hexon gene amino acid homologies (%) of M86 with other members of subgenus B and subgenus D

<table>
<thead>
<tr>
<th></th>
<th>HVR1</th>
<th>HVR2</th>
<th>HVR3</th>
<th>HVR4</th>
<th>HVR5</th>
<th>HVR6</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ad</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad3</td>
<td>11.7</td>
<td>36.8</td>
<td>33.3</td>
<td>28.5</td>
<td>12.5</td>
<td>36.3</td>
<td>20.0</td>
</tr>
<tr>
<td>Ad7</td>
<td>11.7</td>
<td>41.1</td>
<td>33.3</td>
<td>38.0</td>
<td>31.2</td>
<td>50.0</td>
<td>16.2</td>
</tr>
<tr>
<td>Ad11</td>
<td>45.4</td>
<td>50.0</td>
<td>86.6</td>
<td>100</td>
<td>87.5</td>
<td>100</td>
<td>85.0</td>
</tr>
<tr>
<td>Ad14</td>
<td>48.9</td>
<td>50.0</td>
<td>100</td>
<td>82.5</td>
<td>66.2</td>
<td>50.0</td>
<td>15.3</td>
</tr>
<tr>
<td>Ad21</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>42.8</td>
<td>87.5</td>
<td>100</td>
<td>95.0</td>
</tr>
<tr>
<td>Ad16</td>
<td>12.7</td>
<td>9.5</td>
<td>12.1</td>
<td>42.2</td>
<td>12.5</td>
<td>41.6</td>
<td>10.0</td>
</tr>
<tr>
<td>Ad19</td>
<td>10.8</td>
<td>22.2</td>
<td>18.1</td>
<td>28.5</td>
<td>25.0</td>
<td>66.6</td>
<td>10.0</td>
</tr>
<tr>
<td>Ad27</td>
<td>10.8</td>
<td>41.1</td>
<td>18.1</td>
<td>25.0</td>
<td>10.6</td>
<td>66.6</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Per cent of homology between the indicated hypervariable regions (HVRs) of M86 and the HVRs of adenovirus (Ad) subgenus B and subgenus D.

| Ad35 | 1  | GGTYNSLAP | KQAPAWQWQ | AKGVTAAAG | QNGDEEHHTE | EFTATTTCT | 50  |
| M86  | 1  | GGTYNSLAP | KQAPAWQWQ | AKGVTAAAG | QNGDEEHHTE | EFTATTTCT | 50  |
| Ad11 | S1 | AYKPAEAQAKT | KEGPILGEI | SAEENKSPIY | ADKQVPQKPO | VODGETWLDLD | 100 |
|      |   |             |           |           |            |            |     |
| Ad14 | S1 | AYKPAEAQAKT | KEGPILGEI | SAEENKSPIY | ADKQVPQKPO | VODGETWLDLD | 100 |
|      |   |             |           |           |            |            |     |
| Ad35 | S1 | AKTKEYQAGRA | LDPYTMNKP | YSGYFAPKYN | KOSQAKPKNS | EPSSKIVEDY | 150 |
| M86  | S1 | AKTKEYQAGRA | LDPYTMNKP | YSGYFAPKYN | KOSQAKPKNS | EPSSKIVEDY | 150 |
| Ad11 | S1 | AKTKEYQAGRA | LDPYTMNKP | YSGYFAPKYN | KOSQAKPKNS | EPSSKIVEDY | 150 |

**DISCUSSION**

Per cent of homology between different fibre regions of M86 and those of adenovirus (Ad) subgenus B and subgenus D.

<table>
<thead>
<tr>
<th>Ad11</th>
<th>Ad34</th>
<th>Ad35</th>
<th>Ad3</th>
<th>Ad7</th>
<th>Ad8</th>
<th>Ad19/37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail</td>
<td>DNA</td>
<td>100</td>
<td>99</td>
<td>99</td>
<td>80.6</td>
<td>97.6</td>
</tr>
<tr>
<td>Shift</td>
<td>DNA</td>
<td>98.1</td>
<td>71.7</td>
<td>71.7</td>
<td>55.2</td>
<td>90.1</td>
</tr>
<tr>
<td>Knob</td>
<td>DNA</td>
<td>99.8</td>
<td>45.0</td>
<td>45.4</td>
<td>50.6</td>
<td>93.7</td>
</tr>
<tr>
<td>Protein</td>
<td>DNA</td>
<td>99.4</td>
<td>28.9</td>
<td>28.9</td>
<td>28.8</td>
<td>91.6</td>
</tr>
<tr>
<td>Overall</td>
<td>DNA</td>
<td>99.3</td>
<td>59.7</td>
<td>59.9</td>
<td>56.3</td>
<td>93.2</td>
</tr>
<tr>
<td>Protein</td>
<td>DNA</td>
<td>99.3</td>
<td>47.8</td>
<td>47.8</td>
<td>41.8</td>
<td>92.3</td>
</tr>
</tbody>
</table>

In HVRs of the hexon, M86 showed an overall 94.4% AA homology with Ad35 and 86.7% with Ad11 (table 1). However, it was 100% homologous to Ad35 in HVRs 1, 2, 3, and 6 and to Ad11 in HVRs 4 and 6 (fig 2). The fibre knob showed high AA homology (99.4%) with Ad11, but only 29.1% homology with Ad35 (table 2).

**DISCUSSION**

Members of subgenus D adenoviruses (Ad8, Ad19, and Ad37) are the common agent of EKC. Occasionally, the members of subgenus B (Ad3 and Ad7) and subgenus E (Ad4) are also related to EKC. The tropism of adenoviruses for conjunctival or corneal cells depends on the presence of certain amino acids in the knob, which attaches the virus to the specific cellular receptor. The fact that the fibre knob of M86 has 99.4% homology with Ad11, with only a single AA difference, but only 29.1% homology with Ad35, means that it is able to attach to conjunctival and corneal cells (fig 3).
Neutralisation of the infectivity of adenoviruses is primarily carried out by antibodies against the hexon protein. Antigenic determinants (epitopes) located in two or more of the seven HVRs in loop 1 and loop 2 of the hexon react with antibodies against the hexon protein, enabling it to circumvent existing immunity and might be responsible for outbreaks of EKC in the future; this may be especially important in developing countries, where the detection of adenoviruses in the clinical setting is not available. It is also important to accumulate data on the HVRs of the hexon gene and fibre gene sequences of the EKC strains to predict their possible role in keratoconjunctivitis.

ACKNOWLEDGEMENTS
Supported by grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), Japan.

Authors’ affiliations
A K Adhikary, T Inada, U Banik, A Mukuyama, S Matsuno, N Okabe, Infectious Disease Surveillance Centre, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
J Numaga, Department of Ophthalmology, Graduate School of Medicine, The University of Tokyo, Tokyo 107-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
E Suzuki, H Ushijima, Department of Developmental Medical Sciences, Graduate School of Medicine, The University of Tokyo

Correspondence to: Dr A K Adhikary, Mailing, Infectious Disease Surveillance Centre, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan; arun.nih.go.jp

Accepted for publication 10 July 2003

REFERENCES

Take home messages
- We have characterised a novel strain (M86) of adenovirus (Ad) involved in epidemic keratoconjunctivitis.
- The virus strain showed homology with both Ad35 and Ad11 using both immunological and molecular biological techniques.
- It high homology with the fibre knob sequences of Ad11 may provide it with the ability to invade ocular cells.
- The mixed antigenic characteristics of virus strains such as this may enable them to circumvent existing immunity.

Figure 3 Comparison of predicted fibre knob sequences of M86, adenovirus type 11 (Ad11p), and Ad35p. The sequences were aligned to obtain maximal homology. The deletions are represented by dashes. The construction of a chimaera in the hypervariable regions of the hexon could change the antigenic specificity of the virus, enabling it to escape type specific neutralisation.

M86 1 WTCVNPTEAN QIQMNSSEND DCKLILTVK TGOAVTFAYV VIGVSNFNM
Ad11 1 WTCVNPTEAN QIQMNSSEND DCKLILTVK TGOAVTFAYV VIGVSNFNM
Ad35 1 WTCVNPTEAN QIQMNSSEND DCKLILTVK TGOAVTFAYV VIGVSNFNM

Figure 3 Comparison of predicted fibre knob sequences of M86, adenovirus type 11 (Ad11p), and Ad35p. The sequences were aligned to obtain maximal homology. The deletions are represented by dashes. The construction of a chimaera in the hypervariable regions of the hexon could change the antigenic specificity of the virus, enabling it to escape type specific neutralisation.

M86 1 WTCVNPTEAN QIQMNSSEND DCKLILTVK TGOAVTFAYV VIGVSNFNM
Ad11 1 WTCVNPTEAN QIQMNSSEND DCKLILTVK TGOAVTFAYV VIGVSNFNM
Ad35 1 WTCVNPTEAN QIQMNSSEND DCKLILTVK TGOAVTFAYV VIGVSNFNM

M86 51 LTTHRXNIP6 AELFFDSGCN LLTRLSKLT PLKHIKOSQNML YGCAITNAC
Ad11 51 LTTHRXNIP6 AELFFDSGCN LLTRLSKLT PLKHIKOSQNML YGCAITNAC
Ad35 51 LTTHRXNIP6 AELFFDSGCN LLTRLSKLT PLKHIKOSQNML YGCAITNAC

M86 101 MPSETAYYPF NDNSREKENY YICTCYTTAS --DTDAFPDI SYMLNRRAIN
Ad11 101 MPSETAYYPF NDNSREKENY YICTCYTTAS --DTDAFPDI SYMLNRRAIN
Ad35 101 MPSETAYYPF NDNSREKENY YICTCYTTAS --DTDAFPDI SYMLNRRAIN

M86 151 DETSYCIRT WSNWYCDAPE VQTSATLTV SYFPPFYIRED DD........ 200
Ad11 151 DETSYCIR WSNWYCDAPE VQTSATLTV SYFPPFYIRED DD........ 200
Ad35 151 DETSYCIRT WSNWYCDAPE VQTSATLTV SYFPPFYIRED DD........ 200
Characterisation of hexon and fibre genes of a novel strain of adenovirus involved in epidemic keratoconjunctivitis

A K Adhikary, T Inada, J Numaga, E Suzuki, H Ushijima, U Banik, A Mukouyama, S Matsuno and N Okabe

doi: 10.1136/jcp.57.1.95