Antipeptide antisera define neutralizing epitopes on the adenovirus hexon

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The adenovirus (Ad) hexon contains both group- and type-specific antigenic determinants. To identify the latter, peptides were synthesized corresponding to residues 281 to 292 from loop 1 and 441 to 455 from loop 2 of the Ad2 hexon. These sequences display type-specific variation and have been shown by X-ray crystallography to be present on the surface of the virion. Antisera raised against the peptides bound both peptide and the native hexon in ELISA, and blocked virus infectivity, as determined by immunofluorescence or neutralization assays. The loop 1 peptide was shown to inhibit binding of the corresponding antiserum to the native hexon in ELISA and to abolish its neutralizing activity. Neither the loop 1- nor loop 2-specific antiserum neutralized the infectivity of Ad4 or Ad40. Neutralization did not appear to result from aggregation of virus particles and thus their inability to attach to the cell, because virions treated with immune serum were internalized to the same extent as those treated with preimmune serum. Examination of the immune response elicited by Ad2 infection revealed that antibodies directed against the L1 and L2 epitopes were also present in human serum. Thus, the variable regions exposed on the surface of the Ad2 hexon represent type-specific neutralizing antigenic determinants.

Introduction

The human adenoviruses (Ads) belong to the genus Mastadenovirus and, to date, 47 distinct serotypes have been identified (Hierholzer et al., 1988). These are divided into six subgenera (A to F) on the basis of shared immunological and biochemical properties, and are associated with a variety of acute infections, primarily respiratory, ocular and gastrointestinal, although symptoms are rarely serious. Ads share a common architecture consisting of a non-enveloped icosahedral capsid surrounding a linear dsDNA genome of approximately 36 kbp. The viral capsid is composed of three structural proteins: hexon, penton and fibre. Hexon is the major structural component, forming the 20 facets of the icosahedron, whereas pentons, being complexes of penton base with fibre, form the 12 vertices (Ginsberg, 1984).

Hexon, fibre and to a lesser extent penton have been shown to be the major targets for neutralizing antibodies; however, the epitopes involved remain largely undetermined. Fibres contain a large fraction of the antigenic sites of the virion and all serotypes appear to possess a type-specific moiety located in the knob region. This may be accompanied by inter- and intragroup-specific sites in the longer fibres (reviewed by Philipson, 1983). Fibre also contains the domain responsible for interaction with the host cell receptor, attachment being the first step in Ad infection. Antibodies to the type-specific determinant of fibre prevent attachment and this neutralization pathway appears to be due to aggregation. This is supported by the observation that anti-fibre antibody cannot neutralize virus already bound to KB or HeLa cells (Philipson et al., 1968).

Hexon possesses a complex arrangement of antigenic determinants, including those with genus, type, intersubgenus and intrasubgenus specificities (Norrby, 1969a, b; Norrby & Wadell, 1969). The use of monoclonal antibodies (MAbs) has revealed some of the complexities of the hexon molecule. Use of a collection of MAbs against Ad5 (Russell et al., 1981) has indicated the existence of at least five distinct epitopes, one of which is shared by Ad1 and Ad6 but not Ad2, revealing an intrasubgroup specificity. A more extensive series of MAbs raised against the hexon of Ad1 recognize 19 distinct epitopes, one of which is common to all human Ads. The other epitopes recognized are specific within each subgenus and arranged in characteristic combinations on the surface of the different types of hexon (Adam et al., 1986). Type-specific antigenic determinants of hexon have been demonstrated on the surface of the virion and a common group-specific moiety appears to be buried internally (Haase & Pereira, 1972; Kjellén & Pereira, 1968; Mautner & Willcox, 1974; Norrby, 1969a; Willcox & Mautner, 1976a, b). The antigenic properties of Ads are further complicated by naturally
occurring recombinants between closely related serotypes, which may possess hexon specificities of one type and fibre specificities of another (Norrby, 1968).

The objective of this work was to define the type-specific neutralizing epitopes on the surface of the Ad2 hexon. Based on comparisons between hexon amino acid sequences (Kinloch et al., 1984; Toogood & Hay, 1988; Toogood et al., 1989) and structural data (Roberts et al., 1986), peptides were synthesized corresponding to regions of the hexon considered to be likely candidates for neutralizing epitopes. Antisera raised against these peptides neutralized virus infectivity and fulfilled the prediction that these regions of the Ad2 hexon represent components of the type-specific neutralizing determinants. Examination of human sera indicated that specific antibodies to the synthetic peptides were present in the serum from an individual who had previously been infected with Ad2.

Methods

Cells and viruses. HeLa cells were grown in suspension in Glasgow-modified spinner Eagle's medium (GS-MEM, Gibco) with 7% newborn calf serum. High titre Ad2 was prepared from infected HeLa cells by fluorocarbon extraction of freeze-fractured cells, followed by density gradient centrifugation as described (Hay et al., 1984). Virus was stored at −20 °C in 50% glycerol. The titre of the virus stock was in the region of 1011 to 1012 p.f.u./ml. Monolayer cultures of A549 cells, a human cell line derived from lung carcinoma, were grown in GS-MEM supplemented with 10% foetal calf serum (FCS). For infection, Ad2 was allowed to adsorb to monolayers of A549 cells for 1 h in serum-free medium and thereafter maintained in GS-MEM with 2% FCS. Hexon was obtained from G. Kemp and purified from fluorocarbon-treated cell extracts essentially as described (Pereira et al., 1968), with the exception that chromatography was on Mono-Q rather than DEAE-Sephadex.

Peptide synthesis. Peptides were synthesized by solid-phase fluorenyl methylcarbonyl polyamide chemistry using a CRB Pepsynthesizer II (Atherton et al., 1988). Purification was performed by reverse-phase FPLC, and the composition of peptides confirmed by amino acid analysis. In this way peptides L1 and L2, corresponding to sequences from loop 1 and loop 2, were synthesized.

Immunization. Peptide L1 was coupled via the C-terminal cysteine residue to human albumin using N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP). Peptide L2 was coupled to keyhole limpet haemocyanin using gluteraldehyde. Female Dutch rabbits (two per peptide) were immunized subcutaneously with 100 µg peptide conjugate in Freund's complete adjuvant. Rabbits were boosted after 2 weeks with 100 µg conjugate in Freund's incomplete adjuvant, a test bleed being taken 2 weeks later and tested by ELISA for specific antibodies. Immunizations were repeated a further three times at 4-week intervals, by which time each rabbit was producing a good constant response.

ELISA. The level of specific antipeptide antibody was measured by ELISA using a horseradish peroxidase (HRP) detection system. Ninety-six-well microtitre plates were coated overnight at 4 °C with 0.5 µg/well peptide or purified Ad2 hexon in PBS. After washing with PBS, non-specific binding sites were blocked by incubation with PBS containing 1% low-fat milk (Marvel), 0.1% sodium azide for 3 h at 37 °C. Serial twofold dilutions of each antiserum were allowed to react with peptide or hexon for 1 h at room temperature. After washing, plates were incubated with HRP-conjugated goat anti-rabbit IgG or a similarly conjugated anti-human IgG for 1 h at 37 °C, and the colour was subsequently developed with orthophenylenediamine and hydrogen peroxide for 30 min in the dark. Microtitre plates were read in a Titertek Multiskan. Specificity was assessed by substituting the immune serum with the corresponding preimmune serum, and by testing each serum against an unrelated Ad40 peptide.

Inhibition of binding by peptide. Each of the two peptides was tested for its ability to inhibit binding of the loop 1 and loop 2 antisera to the native hexon in an ELISA. Serial fivefold dilutions of peptide were placed on a microtitre plate, starting at a concentration of 1 µg/ml, in wells containing a 1:50 dilution of L1/1 or a 1:250 dilution of L1/2 antiserum (the amounts being those giving 75% of the maximum response observed in ELISA using native hexon). Following a 1 h incubation at 37 °C, the peptide/antiserum mixtures were added to microtitre plate wells coated with 0.5 µg purified hexon and blocked as described above. Plates were subsequently treated in the same manner as for ELISA.

Neutralization test. Fifty microlitres of a 10−6 dilution of Ad2 stock was the lowest dilution causing visible c.p.e. in a microtitre well of A549 cells 5 days post-infection. Thus, for neutralization tests 50 µl of a 10−5 dilution of virus stock was mixed with serial twofold dilutions (starting at 1:10) of each antiserum in serum-free medium. Virus and serum were incubated for 1 h at 37 °C and adsorbed onto A549 cells in a microtitre plate for a further 1 h at 37 °C. GS-MEM containing 2% FCS was added and incubation continued for 5 days at 37 °C. Monolayers were fixed with formaldehyde and stained with crystal violet.

Inhibition of neutralization by peptide. Each peptide was serially diluted twofold across a microtitre plate in serum-free medium containing 0.5 µl/well L1/1 or L1/2 antiserum and incubated for 2 h at 37 °C. Ad2 (50 µl, diluted 5 × 10−4) was added, incubated for a further 1 h at 37 °C, and A549 cells were infected with the mixture as described above.

Immunofluorescence. First and second antibodies were pre-absorbed against formaldehyde-fixed, permeabilized uninfected A549 cells overnight at 4 °C, then filter-sterilized and stored at 4 °C. A549 cells were grown overnight to approximately 70% confluence on glass coverslips in 24-well Linbro plates. Ad2 (400 µl, of a 10−4 dilution) was incubated with preimmune or immune serum (1 µl) for 1 h at 37 °C. Cells were infected with virus for 18 h. Monolayers were fixed with 5% formaldehyde, 2% sucrose in PBS for 10 min and permeabilized using 0.5% NP40, 10% sucrose, 1% calf serum (CS) in PBS for 10 min. Following three washes with 1% CS in PBS, coverslips were placed on 10 µl spots of a 1:3000 dilution of a horse anti-Ad5 hexon polyclonal serum (obtained from W. C. Russell) and incubated at room temperature for 1 h. Coverslips were extensively washed with 1% CS and with PBS, and incubated for 1 h with 10 µl of a 1:90 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-horse IgG (Sigma). Coverslips were washed sequentially in permeabilization buffer, 1% CS, PBS and distilled water, and mounted in glycerol/PBS solution (Citifluor). Immunofluorescence was examined using a Nikon Microphot microscope and recorded on a Kodak T-max 400 film.

Results

Choice of peptides

The amino acid sequence of the Ad2 hexon was examined both in the context of its three-dimensional
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Fig. 2. Amino acid sequence of a region of (a) loop 1 and (b) loop 2 of the Ad2 hexon polypeptide and the corresponding regions in Ad5, Ad40 and Ad41. In Ad2 these regions are predicted to be on the surface of the virion. Amino acids identical in all four serotypes are denoted by asterisks, conservative changes are given as dots, and dashes represent the absence of a corresponding residue. The following exchanges are considered to be conservative: exchanges between hydrophobic residues (A, G, L, I, V, C and M), exchanges between aromatic residues (F, Y, W and H) and exchanges between hydrophilic residues of similar bulk (E, Q, N and D; R for K; S for T). The numbering shows the position within the Ad2 polypeptide. The sequences of the peptides used for raising antisera are given. Note the additional cysteine residue at the C terminus of peptide L1.

Table 1. ELISA and neutralization titres of antipeptide antisera

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Specific peptide (t)</th>
<th>Unrelated peptide (t)</th>
<th>Purified hexon (t)</th>
<th>Neutralization (t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1/1</td>
<td>1:8000</td>
<td>&lt;1:32</td>
<td>1:6500</td>
<td>1:4000</td>
</tr>
<tr>
<td>L1/2</td>
<td>1:2000</td>
<td>&lt;1:16</td>
<td>1:1600</td>
<td>1:4000</td>
</tr>
<tr>
<td>L2/1</td>
<td>1:8000</td>
<td>&lt;1:16</td>
<td>&gt;1:2000</td>
<td>&lt;1:32</td>
</tr>
<tr>
<td>L2/2</td>
<td>1:16000</td>
<td>&lt;1:16</td>
<td>1:8000</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

* The ELISA titre is given as the lowest dilution of antiserum that gave a value at least twofold greater than the background reading.
† Neutralization titres are given as the lowest dilution of antiserum to block infection.

Recognition of hexon by antipeptide antisera

The presence of antibodies recognizing the peptides used for immunization and the native Ad2 hexon was determined using ELISA, in which peptides and purified Ad2 hexon were used as the solid phase absorbent. Antiserum directed against each peptide was obtained from each of two rabbits (L1/1 and L1/2, and L2/1 and L2/2) and was tested for binding to immobilized peptide or hexon. The results are summarized in Table 1. The different serotypes, but is bounded by regions of high homology (Fig. 2). Peptide L1 was derived from loop 1 and peptide L2 from loop 2, spanning residues 281 to 292 and 441 to 455 respectively.

structure (Roberts et al., 1986) and of its homology to hexons of other serotypes (Kinloch et al., 1984; Toogood & Hay, 1988; Toogood et al., 1989). Peptides chosen for synthesis were selected from regions of the loop domains predicted to be on the surface of the virion (Roberts et al., 1986) and therefore most likely to be accessible to antibody (Fig. 1). In each case the region corresponding to the selected peptide exhibits little homology between different serotypes, but is bounded by regions of high homology (Fig. 2). Peptide L1 was derived from loop 1 and peptide L2 from loop 2, spanning residues 281 to 292 and 441 to 455 respectively.

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L1/1 and L1/2 antisera bound native hexon more efficiently than peptide, whereas the converse was found for the L2/1 and L2/2 antisera. To establish specificity, each antiserum was tested against an unrelated peptide. In no case was binding detectable at dilutions greater than 1:32. Preimmune bleeds tested against both specific and unrelated peptides did not give detectable binding at dilutions greater than 1:64.

**Neutralization of Ad2 infectivity by antipeptide sera**

Having demonstrated that the antipeptide antisera bound native hexon they were assessed for their ability to neutralize the infectivity of Ad2. Virus was preincubated with a series of dilutions of each antiserum prior to infection of A549 cells and the highest dilution to give protection from infection was determined by staining the monolayer with crystal violet. Both antisera raised against the L1 peptide (L1/1 and L1/2) but only one raised against the L2 peptide (L2/2) neutralized virus infectivity at high dilution (Table 1). Antibodies present in antiserum L2/1 bind to peptide efficiently but bind to hexon relatively poorly and do not neutralize virus infectivity, even at low dilution. Type specificity was established by substituting either Ad4 or Ad40 for Ad2 in the neutralization assay. Neither was neutralized by any of the antisera tested, even at dilutions of 1:2 (data not shown).

As an independent means of demonstrating the neutralizing activity of the peptide antisera, virus was incubated with the various antisera prior to infection of A549 cells and the progress of the infection was monitored by immunofluorescence using a monospecific horse anti-hexon antibody. Cells infected for 18 h at 37°C with virus treated with preimmune serum gave a distribution of fluorescence characteristic of antibody staining hexon (Fig. 3b): intense punctate nuclear foci on a diffuse background and faint cytoplasmic staining...
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Fig. 4. Fate of neutralized and non-neutralized input virus. A549 cells were exposed to 1000 p.f.u./cell purified Ad2 that had been incubated with preimmune sera (a) or antiserum L1/1 (b). After 30 min at 4 °C followed by 30 min at 37 °C, cells were fixed, permeabilized and stained with horse anti-hexon antibody, as described in the legend to Fig. 3.

(Hayashi & Russell, 1968). Cells infected with virus preincubated with immune serum raised against loop 1 (Fig. 3c) or loop 2 (Fig. 3d) showed a pattern of fluorescence indistinguishable from that obtained with the uninfected control (Fig. 3a), confirming that antibodies directed against regions in both loop 1 and loop 2 were capable of neutralizing virus infectivity.

To determine whether the neutralizing effect of the antipeptide antisera was simply due to the inability of the antibody-bound virus to penetrate the cell, the fate of the input virus was followed by immunofluorescence. Cells were exposed to 1000 p.f.u./cell of purified Ad2 virions that had previously been treated with preimmune serum or sufficient L1/I antiserum to neutralize infectivity. After absorption of antiserum-treated virus to cells for 30 min at 4 °C the temperature was shifted to 37 °C for 30 min to allow penetration of virus (Patterson & Russell, 1983). Cells were washed extensively to remove unbound virus, fixed in formaldehyde and treated with NP40 to permeabilize cells and expose internalized virus. Input virus was detected with a horse anti-hexon serum and an FITC-conjugated anti-horse IgG. It is apparent that virus treated with preimmune or neutralizing antipeptide antiserum were internalized to the same extent (Fig. 4), thus indicating that the neutralizing antipeptide antiserum acts at a stage in the infectious cycle subsequent to internalization.

Inhibition of binding and neutralizing activity by peptide

To examine the specificity of the interaction between antipeptide antisera and the Ad2 hexon the ability of free peptide to block this interaction was assessed by ELISA and a neutralization test. In an ELISA using purified hexon as the solid phase absorbent the binding of antibodies raised against loop 1 was inhibited by incubation with the L1 peptide, but not the L2 peptide (Fig. 5a). Preimmune serum displayed negligible binding activity and was unaffected by the presence of either peptide. A similar result was evident for the neutralization assay: the neutralizing activity of the L1/I antiserum was reduced by incubation with the L1 peptide, but was unaffected by the presence of the L2 peptide (Fig. 5b).

Presence of antibodies to L1 and L2 in human sera

Although it has been demonstrated that neutralizing antipeptide antibodies can be generated in rabbits, it is not clear whether similar antibodies are produced in the
Fig. 5. Peptides were used to inhibit the binding of antibody to hexon in ELISA (a) and to abolish neutralizing activity in a neutralization test (b). For the ELISA, the L1/1 antiserum (diluted 1:500) or preimmune serum (diluted 1:500) was preincubated with a range of concentrations of peptide L1 or L2 before application to microtitre plates coated with purified Ad2 hexon. The assay was developed as described in Methods. ○, L1/1 antiserum plus L2 peptide; ■, L1/1 antiserum plus L1 peptide; □, preimmune serum plus L1 peptide. (b) For the neutralization inhibition test, peptide L1 (top line) and L2 (bottom line) were serially diluted twofold and incubated with 0·5 µl L1/1 antiserum for 2 h. Virus was added and incubation continued for a further 1 h, and the total mixture was used to infect approximately 50% confluent microtitre wells containing A549 cells. Cells were fixed and stained with crystal violet after 7 days. Clear wells denote neutralization inhibition.

course of a normal human Ad infection. Therefore, the presence of antibodies to the L1 and L2 peptides in two human sera was determined by ELISA. In parallel, the presence of type-specific neutralizing antibodies was determined in a neutralization assay with Ad2 and Ad5. Although serum EW was capable of neutralizing Ad5 it did not neutralize Ad2, and no antibodies specific to Ad2 peptides L1 and L2 were detected by ELISA (Table 2). In contrast, serum JB neutralized Ad2 but not Ad5, and antibodies that recognized the L1 and L2 peptides were detected by ELISA (Table 2). Thus, a human antibody response is mounted against the L1 and L2 regions of the hexon during Ad infection.

Table 2. ELISA and neutralization titres of human sera

<table>
<thead>
<tr>
<th>Human serum</th>
<th>Neutralization*</th>
<th>ELISA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>EW</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>JB</td>
<td>160</td>
<td>320</td>
</tr>
</tbody>
</table>

* The neutralization titres are given as the reciprocal of the lowest dilution to block infection.
† The ELISA titre is given as the reciprocal of the lowest dilution of antiserum giving a value at least twofold greater than the background reading.

Discussion

Although determinants for type- and group-specific neutralizing antibodies have been mapped to the Ad hexon (Haase & Pereira, 1972; Kjellén & Pereira, 1968; Willcox & Mautner, 1976b), the precise location of these epitopes on the hexon molecule has not been determined. To date four human Ad hexons have been sequenced, those of types 2, 5, 40 and 41. The homology between these proteins has allowed the sequences to be aligned (Kinloch et al., 1984; Toogood & Hay, 1988; Toogood et al., 1989) and the location of common structural domains to be predicted. However, the three loop domains identified in the Ad2 hexon, which form the virion surface, show great variability when compared to those of other serotypes, suggesting their susceptibility to immunological pressure. The study of these variable regions, in particular loops 1 and 2, was of prime importance for the choice of peptides for this study. The two most promising regions showed little conservation between types but were flanked by homologous sequences, suggesting that the loops might be similarly constrained. The variable regions of loops 1 and 2 of Ad2, Ad5, Ad40 and Ad41 vary not only in sequence but also in length (Kinloch et al., 1984; Toogood & Hay, 1988; Toogood et al., 1989), suggesting that they are outside the rest of the loops, which are involved in a complex interweaving. In intact virus these sequences are predicted to be located on the extreme surface of the virion, but their faint electron density indicates that these regions are likely to be highly mobile (Roberts et al., 1986). Together these observations suggested that these regions of loops 1 and 2 are structurally not very complex, are at the extreme outer surface of the virion and may contain linear stretches ideal for the design of peptides capable of inducing antibodies which would recognize and potentially neutralize virus.

On this basis, peptides were synthesized which were predicted to be components of the neutralizing epitopes on the surface of hexon. We have demonstrated that
antibodies raised in rabbits against two synthetic peptides derived from the major capsid protein of Ad2 neutralize virus infectivity in a type-specific fashion and that antibodies with similar specificity are present in the sera of Ad2-infected humans. It is worth noting that although anti-L1 antibodies efficiently bind both peptide and native hexon and neutralize virus infectivity, this is not the case for anti-L2 antibodies. Although the anti-L2 antibodies bind well to peptide they bind to hexon and neutralize virus infectivity less well than the corresponding anti-L1 antibodies (Table 1). An explanation for this behaviour is that the L2 peptide region of native hexon is held in a conformation that is poorly accessible to antibody. Alternatively the conformation of the L2 peptide region in native hexon may not have been adopted when the peptide was coupled to carrier, thus resulting in a lack of antibodies that recognize the native protein.

It has been shown previously that Ad2 virions neutralized by anti-hexon antibody attach and penetrate cells to the same extent as virions treated with preimmune serum, but are unable to escape from intracellular vesicles (Wohlfart et al., 1985). Neutralization by anti-hexon antisera contains single-hit kinetics with, on average, 1-4 antibody molecules bound per virion, and it is thought that bound antibody inhibits a low pH-induced conformational change that takes place in the acidic endosomes (Wohlfart, 1988). During this conformational change the N-terminal region of the protein is exposed and antibodies directed against an N-terminal 15K proteolytic fragment can neutralize virus infectivity (Varga et al., 1990). The antipeptide antibodies described here also allow internalization of the virus, but are not directed against the N-terminal region of the protein, although they may also block the acid-induced conformational change.

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References


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