Induction of neutralizing antibodies by synthetic peptides representing the C terminus of coxsackievirus A9 capsid protein VP1

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The arginine–glycine–aspartic acid motif at the C terminus of coxsackievirus A9 capsid protein VP1 has been shown to play a role in specific attachment of the virus to αvβ3 integrin on the host cell surface. The C-terminal region of the VP1 protein has also been shown to be highly antigenic by using peptide scanning techniques. To find out whether this region contains a neutralizing epitope, three overlapping peptides covering the C-terminal end of VP1 were synthesized and rabbit antisera were raised against these peptides. Neutralization of the virus was observed with all three antipeptide antisera in A549 cells and with two antisera in RD cells.

Coxsackievirus A9 (CAV9) is a member of the genus Enterovirus in the family Picornaviridae. It is a small icosahedral virus with a coat made up of 60 copies of each of four capsid proteins (VP1 to VP4) enclosing an approximately 7450 bases long, single-stranded, positive-sense RNA genome. Although CAV9 exhibits pathogenicity in newborn mice typical of the members of the subgroup A of coxsackieviruses (Hyypia et al., 1993), it is molecularly more closely related to coxsackie B viruses (CBVs) and echoviruses (Chang et al., 1989; Huttunen et al., 1996; Pulli et al., 1995). The disease pattern in humans is also more closely related to CBV infections, including clinical symptoms like meningitis and encephalitis (Grist et al., 1978).

When compared to CBVs, CAV9 has a C-terminal extension in the VP1 capsid protein (Chang et al., 1989, 1992). This extension contains a tripeptide motif of arginine–glycine–aspartic acid (RGD). In general, RGD-containing motifs are known to be involved in multiple biological recognition reactions. In particular, extracellular matrix proteins use their RGD sequence in binding to cell surface receptors known as integrins (Ruoslahti & Pierschbacher, 1987; Hynes, 1992). CAV9 binds with its functional RGD motif to αvβ3 integrin during early virus–cell interactions (Roivainen et al., 1991, 1994). Three other picornaviruses, echovirus 22 (Stanway et al., 1994; Pulli et al., 1997), foot-and-mouth disease virus (FMDV; Fox et al., 1989; Berinstein et al., 1995) and echovirus 9 strain Barty (Zimmermann et al., 1997), are also known to use similar interactions in binding to the host cell.

When antigenic sites of CAV9 were recently investigated using peptide scanning methods, it was found that the C terminus of VP1 is one of the most immunogenic regions of the virus (Pulli et al., 1998). To find out if antibodies that bind to the C terminus of VP1 can neutralize the virus, rabbit antisera were raised against three overlapping peptides covering this region.

Sequences of the peptides used in this study are shown in Table 1. Peptide C represents the C-terminal end of the VP1 polypeptide. Peptide R contains the RGD motif and has three overlapping amino acids with peptide C and two amino acids overlapping with peptide N, which represents the amino acids at the N-terminal side of the RGD motif. In addition, a peptide representing the conserved PALTAVETGHT region in the N-terminal half of CAV9 VP1, which is known to have a high degree of identity among enteroviruses (Hovi & Roivainen, 1998), was included.

Table 1. Amino acid sequences of the synthetic peptides used in this study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence of the peptide</th>
<th>Location of the region in CAV9</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>MSTLNTGCAF</td>
<td>VP1 293–302 C-terminal</td>
</tr>
<tr>
<td>R</td>
<td>QSRRGDMST</td>
<td>VP1 286–295 C-terminal</td>
</tr>
<tr>
<td>N</td>
<td>TDDRKDINTVTVQOS</td>
<td>VP1 272–287 C-terminal</td>
</tr>
<tr>
<td>P</td>
<td>EAIPALTAVETGHTSQVC</td>
<td>VP1 N-terminal</td>
</tr>
</tbody>
</table>

Overlapping amino acids of the peptides are underlined.
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Fig. 1. Reactivity of antipeptide antisera in an ELISA in microtitre wells coated with the peptides used in immunizations or with coxsackievirus A9 or coxsackievirus B4. □, C preimmune serum; ■, C immune serum; △, R preimmune serum; ▲, R immune serum; ○, N preimmune serum; ●, N immune serum; +, P preimmune serum; *, P immune serum.

1993), was included in the study. The linear peptides were synthesized by an Applied Biosystems model 433A peptide synthesizer using Fmoc solid-phase synthesis methodology. Peptides C, N and R were synthesized using multiple antigenic peptide resins (Applied Biosystems). The peptides were purified by reverse-phase high-pressure liquid chromatography and the major peak was recovered for further use.

For production of antisera against the peptides, three sequential 100 µg doses were injected at 2- to 4-week intervals using the popliteal lymph node method (Leinonen, 1985), including the use of Freund’s complete adjuvant only at the first dose. Immunological reactivity of the antisera was tested by an ELISA in which all the steps were done at room temperature. Microtitre plates (96-well MaxiSorp, Nalge Nunc International) were coated overnight with peptides or highly purified (Abraham & Colonno, 1984; Smyth et al., 1993) CAV9 (Griggs strain) or coxsackievirus B4 (JVB strain) (40 ng per well in 100 µl of 50 mM NaHCO$_3$ pH 9.6). The wells were washed three times with PBS containing 0-1% Tween 20 and blocked with 0-25% BSA in PBS for 30 min. Then the wells were washed, serum dilutions were added into the wells in 100 µl dilution buffer (PBS supplemented with 1% BSA, 1% foetal calf serum and 0-1% Tween 20) and the plates were incubated for 1 h. After washings, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:16000 dilution; Bio-Rad) was added into the wells and incubated for 1 h. The wells were washed and H$_2$O$_2$/o-phenylenediamine substrate solution was added. After 30 min, the reaction was stopped by 1M H$_2$SO$_4$ and the absorbance was determined at 492 nm.

Sera from rabbits immunized with peptides C, N and P reacted well with the corresponding peptide (Fig. 1). However, the binding of serum R to the homologous peptide was inefficient, suggesting weak immunogenicity in rabbits. On the other hand, serum C bound efficiently to peptide R, but serum R did not recognize peptide C. This could be explained by high immunogenicity of the three overlapping amino acids MST in peptide C shared with peptide R. This cross-reactivity was not, however, seen in the reversed configuration. In our peptide scanning studies (Pulli et al., 1998), an antiserum raised against native CAV9 also recognized peptides representing the extreme C-terminal end of VP1, indicating that this region is also immunogenic in the native virus. Serum P also reacted weakly with peptide R, suggesting that there may be some common structures present in these two peptides but, again, serum R did not recognize peptide P.

Reactivities of the peptide antisera with purified coxsackievirus A9 and B4 are shown in Fig. 1. The serum against peptide P bound efficiently to both of the viruses, as could be expected
Neutralizing antibodies to CAV9 VP1

Fig. 2. Neutralizing activity of antipeptide antisera. Plaque reduction tests were performed with CAV9 in A549 (a) and RD (b) cells and with CBV4 in A549 cells (c). The data represent means of three experiments and bars indicate SD. □, Preimmune serum, 1:10 dilution; ■, immune serum, 1:10 dilution; □, preimmune serum, 1:30 dilution; ■, immune serum, 1:30 dilution.

Neutralizing activity of antipeptide antisera. Plaque reduction tests were performed to determine whether the sera raised against the peptides could neutralize the infectivity of CAV9. Preimmune or immune serum dilutions in 50 µl Hank’s balanced salt solution, supplemented with 2 mM HEPES (pH 7.4) and 0.6% foetal calf serum, were incubated with approximately 100 p.f.u. CAV9 or CBV4 (control) for 1 h at 36 °C. Human lung carcinoma cells (A549; obtained from the ATCC) or human rhabdomyosarcoma cells (RD; from the WHO), grown as monolayers in 3.5 cm diameter wells, were washed with Hank’s balanced salt solution containing 2 mM HEPES (pH 7.4). The serum dilutions containing the virus were added onto the cells and incubated for 15 min at room temperature. The cells were then overlaid with 0.5% carboxymethylcellulose in the culture medium and the incubation was continued in a CO₂-humidified incubator at 36 °C. After 24 h (RD cells) or 48 h (A549 cells), the medium was removed and the cells were stained with crystal violet solution prior to counting the number of the virus plaques. The assays with CBV4 were performed only in A549 cells because RD cells are not susceptible to this virus.

When 1:10 dilutions were used, the antiserum against peptide C caused over 70% and antiserum against peptides R or N over 30% neutralization of the virus in A549 cells (Fig. 2). When 1:30 dilutions of the antiserum were used, the inhibition of infectivity was reduced to approximately 30% with antiserum C and < 20% with antiserum R and N. Although the antiserum against peptide P reacted efficiently with the virus in an ELISA (Fig. 1), it did not neutralize the virus. None of the sera had any effect on the infectivity of CBV4. The antiserum against the VP1 C-terminal peptide C was the most effective inhibitor of the CAV9 infection. Although the reactivity of peptide-specific antibodies against peptide R (containing the RGD motif) in an ELISA was remarkably lower when compared to the antiserum against other peptides (Fig. 1), it exhibited detectable neutralizing activity. The ability of the antiserum raised against peptides C and N to neutralize CAV9 may be a result of steric hindrance of the RGD-mediated interactions between the virus and cell surface integrin. Especially, the high cross-reactivity of serum C with peptide P suggests that it binds efficiently to amino acids in the shared MST sequence, which is adjacent to the RGD motif. It has also been shown by mutagenesis that the C-terminal amino acid immediately following the RGD motif has an effect on growth efficiency of the virus, indicating that amino acids surrounding the motif could influence the receptor interactions (Hughes et al., 1995).

Because the interactions between the RGD motif and the integrin are not involved in CAV9 binding and entry into RD cells (Hughes et al., 1995; Roivainen et al., 1996), the virus has to use some alternative regions of the capsid for internalization into these cells. Interestingly, neutralization efficiencies of the antiserum C and R were only slightly reduced in RD cells when compared to the results obtained in A549 cells, implying that
these two antisera could neutralize the virus by some other route than direct blocking of virus–receptor interactions. This may be accomplished by preventing uncoating of the virus by antibodies binding to neighbouring capsid pentamers or by forming aggregates of the viruses as has been observed in other picornaviruses (Thomas et al., 1985; Smith et al., 1993; Hewat & Blaas, 1996). Another possibility is that the binding site of another receptor is so close to the RGD-containing region that antibodies binding to the region can prevent receptor interactions by steric hindrance.

Although the RGD motif of FMDV participates directly in the interactions with a neutralizing monoclonal antibody (Verdaguer et al., 1995), amino acids surrounding the motif are also crucial for antibody binding (Mateu et al., 1990; Martínez et al., 1997; Verdaguer et al., 1998). In our study, neutralization of CAV9 was achieved with the antisera raised against peptides surrounding the RGD motif, implying that direct binding of antibodies to this motif may not be essential for neutralization in this antigenic region. Furthermore, two antisera inhibited CAV9 infection in RD cells, in which the RGD motif of the virus appears not to be involved in the entry process, indicating that antibodies in these sera may neutralize the virus by a different manner than by directly blocking receptor interactions.

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References


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