HPLC is an effective and fast method for analysis of viral proteins: a study of encephalomyocarditis virus mutants differing in pathogenicity

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We investigated the use of HPLC in analysis of picornavirus variants by comparing structural poly-peptides of three stable mutants of encephalomyocarditis virus (EMCV). The variants are known to differ in their pathogenicity for mice: plaque variant 2 (PV2) is diabetogenic, PV7 is non-diabetogenic and PV21 induces a generalized lethal infection. We first used HPLC to separate the structural proteins at high purity levels. Detailed analysis of these structural proteins by HPLC-peptide mapping revealed differences in all four viral proteins of PV21 as compared with mutants PV2 and PV7. A single amino acid exchange was found in viral protein 1 between PV2 and PV7. Altered peaks were identified by calculating retention times of tryptic peptides using sequence data and a computer program. Since peak alterations could be attributed to the observed amino acid exchanges, the results correlate well with cDNA sequencing data. Thus HPLC proved to be a useful and fast tool for primary or additional characterization of picornavirus variants at the level of whole virus proteins.

Encephalomyocarditis virus (EMCV) variants with different tissue tropisms have been described by several authors (Craighead, 1966; Craighead & McLane, 1968; Yoon et al., 1980). A diabetogenic plaque variant (PV2) and a non-diabetogenic, strongly myocarditogenic variant, PV7, were plaque-purified from the ‘D variant’ (Yoon et al., 1980). We also obtained a virus mutant (PV21) from the ‘M variant’ pool (Craighead, 1966) which causes a generalized infection with 100% lethality in mice within 5 or 6 days after infection.

Although the three variants (PV2, PV7 and PV21) differ markedly in biological properties it has not been possible so far to define structural difference(s) that determine pathogenicity. Viral structural proteins of all three variants were investigated by SDS-PAGE and by Western blotting as described previously (Mertens et al., 1983). Our variants could not be distinguished by analysis using various antisera prepared by hyper-immunization of rabbits with the respective virus mutants. No significant titre differences were found in cross-neutralization tests (Choppin & Eggers, 1962) and ELISAs (data not shown). We report here a method which allows mutations of distinct virus proteins to be recognized, and thereby facilitate discrimination of EMCV variants.

EMCV was propagated, titrated and labelled with L-[35S]methionine (> 1000 Ci/mmol, Amersham-Buchler) by culture in L929 cell monolayers. Cell cultures were maintained in Eagle’s MEM supplemented with 5% fetal calf serum. Virus was purified from cell cultures and then disrupted by freezing and thawing. Cell debris were sedimented and the supernatant was extracted with 1,1,2-trichlorotrifluoroethane (Serva). The resulting virus suspension was concentrated by CsCl gradient centrifugation and subsequently subjected to two isopycnic centrifugations in CsCl (Rosenwirth & Eggers, 1978). Virus-containing fractions were identified by their radioactive label, pooled and then directly subjected to HPLC.

For separation of virus proteins, a Bakerbond WP-C18 column (4.6 x 250 mm) with all eluents containing 60% formic acid was used (Heukeshoven & Dernick, 1983). Eluent A contained 10% acetonitrile and 30% water, eluent B 40% acetonitrile. The flow rate was 1 ml/min and the elution gradient was as follows: 2 min 15% B, 8 min 45% B, 10 min linear gradient to 100% B, 5 min 100% B. The viral structural proteins 1 to 4 (VP1 to VP4) of PV2 and PV7 show an identical elution pattern and coelute when both variants are chromatographed simultaneously (Fig. 1a). However, when PV2 and PV21 are injected as a mixture, VP2 shows two separate peaks (Fig. 1b). The same was found for an injected mixture of PV7 and PV21 (data not shown). SDS-PAGE of purified proteins revealed differences in the molecular weight of the viral structural proteins.
virus particles on the other hand does not reveal any difference in migration of viral proteins.

To check the purity of eluted proteins, fractions were collected, desiccated in a vacuum over NaOH pellets, washed with ethanol to remove residual formic acid and separated by SDS–PAGE on 16.5% gels in a modified gel system (Schaegger & van Jagow, 1987). Again, virus proteins of all three variants collected after HPLC are not distinguishable by SDS–PAGE (Fig. 1c).

Purified structural proteins were digested for 2 to 3 days at 37 °C in a trypsin solution (cell culture grade, Boehringer Mannheim) buffered with 0.1 M-borate pH 9.0, and containing 5% protease in relation to virus protein concentration. Tryptic peptides were injected onto a Bakerbond WP-C 8 column and eluted with a linear 0 to 25% gradient of acetonitrile in 0.1% phosphoric acid within 60 min starting 5 min after injection (Fullmer & Wasserman, 1979).

Analysis of the peptide patterns of VP1 consistently reveals 22 major peaks (Fig. 2). PV21 shows a distinct peak (no. 17) which corresponds to the extra peak (no. 17) close to no. 16 of PV2 and PV7. Peak no. 18 of PV21 is shifted towards a higher retention time. An additional peak (no. 21a) is detected in PV21 VP1. The VP1s of PV2 and PV7 were distinguishable in only four of eight experiments as an alteration of peak 6a (Fig. 3).

By analysing tryptic digests of VP2 we reproducibly found 15 major peptides. PV2 and PV7 appear identical (Fig. 4a), whereas the elution profile of PV21 differs in

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Fig. 1. (a) HPLC separation of EMCV structural proteins on a C 18-RP column. A mixture of PV2 and PV7 (100 μl each) was injected; the elution gradient of acetonitrile in 60% formic acid is indicated by the dotted line, the flow rate was 1 ml/min, and detection was performed at 280 nm. (b) PV2 and PV21 (100 μl each) were chromatographed simultaneously using conditions as described for (a). (c) SDS-PAGE of EMCV structural proteins separated by RP-HPLC; proteins were visualized by autoradiography. Lane 1, SDS-dissociated total virus; lanes 2 to 10, protein fractions collected from HPLC separations as marked by numbered bars in (a) and (b).

Fig. 2. EMCV VP1 analysed by HPLC–peptide mapping of tryptic peptides. EMCV PV2 VP1 (upper figure) is compared with VP1 of PV21 (lower figure). The flow rate was 1 ml/min and peptides were detected at 214 nm. Peptide peaks are marked by arrows; additional asterisks indicate L-L[35S]methionine-labelled peptides.
peaks no. 1, 3, 14 and 15 (Fig. 4b). This was confirmed by the changed distribution of radioactively labelled peptides that indicated an altered amino acid composition, and is to be expected from the different behaviour of VP2 regularly detected by HPLC separation of whole virus proteins of PV21 as compared to PV2 and PV7 (Fig. 1b).

VP3 shows two alterations of peaks in PV21 as compared with PV2 and PV7, one resulting in a shift of radioactive labelling. Digestion of VP4 results in only two peaks with significant differences between PV2/7 and PV21, respectively (data not shown).

To verify the results obtained with EMCV protein we prepared cDNA of our variants by use of the Superscript Plasmid System (Gibco-BRL) and by standard cloning techniques (Sambrook et al., 1989). cDNA clones containing the structural genes of EMCV were sequenced (T7Sequencing Kit, Pharmacia). The deduced amino acid sequence data were used for prediction of HPLC retention times for altered tryptic peptides by use of a nucleic acid and protein analysis software system (PC/Gene, IntelliGenetics). Among the exchanges described above, two in VP1 (Table 1), five in VP2 (Table 1) and two in VP3 (not shown) can be easily and unequivocally correlated with peak alterations observed in HPLC. Two additional changes in VP1 have been found in only four out of eight experiments. The two alterations observed in the comparison of VP4 between PV2/7 and PV21 correspond to the predicted peak alterations due to four amino acid exchanges.

Regarding VP1 and VP2 only five amino acid exchanges are not regularly correlated with peak alterations in HPLC analysis. Three amino acid exchanges are Arg/Lys exchanges which are not detectable by reversed phase (RP) HPLC owing to the high hydrophilicity of these amino acids (Table 1). Two consecutive amino acid exchanges were not detected (Asp_{12} → Asn_{12} and Thr_{13} → Ala_{13}; PV2/7 to PV21) for unknown reasons. One amino acid exchange (Ser_{260} → Thr_{260}; PV2/7 to PV21) was found in four out of eight HPLC experiments (Table 1). The only definite difference between PV2 and PV7 (Gln_{63} → Lys_{63}; PV2 to PV7) was detected by HPLC, but also only in four out of eight experiments (see above). The correlation of peak 6a with this amino acid exchange is confirmed by the observation that peak 6a of PV7 is found to have the same retention

<table>
<thead>
<tr>
<th>Amino acid exchange</th>
<th>Retention time (min)</th>
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<tbody>
<tr>
<td>VP1</td>
<td>Predicted</td>
</tr>
<tr>
<td>Arg_{5} → Lys_{5}</td>
<td>49.2 ± 46.9</td>
</tr>
<tr>
<td>Asp_{12} → Asn_{12}</td>
<td>49.2 ± 46.9</td>
</tr>
<tr>
<td>Thr_{13} → Ala_{13}</td>
<td>29.0 ± 28.0</td>
</tr>
<tr>
<td>Gln_{63} → Lys_{63}</td>
<td>55.2 ± 55.6</td>
</tr>
<tr>
<td>Ala_{152} → Thr_{152}</td>
<td>58.5 ± 59.2</td>
</tr>
<tr>
<td>VP2</td>
<td>21.1 ± 25.2</td>
</tr>
<tr>
<td>Ala_{38} → Thr_{38}</td>
<td>64.1 ± 64.6</td>
</tr>
<tr>
<td>Ala_{139} → Thr_{139}</td>
<td>59.6 ± 60.2</td>
</tr>
<tr>
<td>Thr_{13} → Ala_{13}</td>
<td>59.6 ± 60.2</td>
</tr>
<tr>
<td>Lys_{114} → Arg_{114}</td>
<td>7.7 ± 7.5</td>
</tr>
<tr>
<td>Arg_{161} → Lys_{161}</td>
<td>7.7 ± 7.5</td>
</tr>
</tbody>
</table>

* NP, Retention time not predictable.
† ND, Not detectable by HPLC.
‡ NF, Not found by HPLC.
§ Found in only four out of eight experiments (see text).
time as peak 6a of PV21 (Fig. 3b). This behaviour must be expected since both PV7 and PV21 have a lysine at position 63 of VP1. Thus, we believe that this inconsistency in the analysis of VP1 is due to minor differences in digestion conditions.

HPLC analysis is more sensitive than conventional protein analysis methods such as SDS–PAGE and isoelectric focusing (IEF). Additionally, IEF is not useful with these proteins since the viral proteins are insoluble even in 8 M-urea. Two-dimensional fingerprint analysis could be similarly sensitive but, unlike HPLC, requires radioactive detection methods. Our data demonstrate that EMCV VP21, in contrast to the immunological and protein analytical methods mentioned above, can definitely be distinguished from the other two variants by HPLC. Furthermore, the peptides isolated by HPLC are directly accessible to amino acid analysis and protein sequencing. Preliminary results suggest that the peptides prepared by HPLC may be suitable for immunological analysis of EMCV, even if detailed epitope analysis may require the use of synthetic peptides. The HPLC method is also applicable to other picornaviruses. Coxsackievirus B4 proteins have been separated and peptide-mapped successfully in our laboratory (data not shown).

Approximately 230 different serotypes of picornaviruses have been described. Sequencing data have been published for only a few of these. Additionally, for many of them subvariants differing in pathogenicity or drug resistance are known. Thus, we are convinced that HPLC is a very fast and highly sensitive method which allows the detection of minor differences in the structural proteins of picornaviruses.
The significance of these protein differences for the pathogenic properties of the variants remains undetermined, since four virus proteins are shown to be different in PV21, but this, of course, is also true for sequencing data. The significance of the single amino acid exchange in VP1 between PV2 and PV7 remains to be clarified.

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References


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