Inhibition of equine herpesvirus type 1 subtype 1-induced ribonucleotide reductase by the nonapeptide YAGAVVNDL

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The synthetic nonapeptide YAGAVVNDL [identical to the nine carboxy-terminal amino acids of the small subunit of herpes simplex virus (HSV)-encoded ribonucleotide reductase (RR)] was found to inhibit the RR activity induced by equine herpesvirus type 1 subtype 1 (EHV-1). Parallel experiments with HSV type 1 (HSV-1)-encoded RR established that the concentration of peptide required to inhibit 50% of the RR activity was 28 μM for both enzymes. The optimum pH for the EHV-1 enzyme was found to be between 8.0 and 8.1 which is the same as that of HSV-1 RR. By use of antisera made against peptides corresponding to different regions of the large subunit (RR1) of the HSV-1 enzyme and monoclonal antibodies directed against HSV-1 RR1 we have obtained evidence which suggests that the EHV-1 large subunit has an Mr of approximately 90 000 and lacks the N-terminal domain which is so far unique to HSV.

Introduction

The equine herpesviruses comprise a group of antigenically distinct biological agents which cause a variety of infections in the horse ranging from subclinical to fatal disease. The epidemiology of these viruses has been reviewed by O'Callaghan et al. (1983). Equine herpesvirus type 1 (EHV-1) is a major cause of abortion and respiratory disease and is one of the most serious viruses of the horse from an economic standpoint. It is also associated with neurological diseases and, more rarely, with coital exanthema. The availability of an antiviral agent effective against EHV-1 would be of great value in the treatment of these diseases. Recent work on a novel way of inhibiting the herpes simplex virus (HSV)-encoded ribonucleotide reductase (RR) (Dutia et al., 1986; Cohen et al., 1986; Lankinen et al., 1982) has opened up the possibility of developing an antiviral agent against HSV-1 which could be effective also against EHV-1.

RR catalyses the reduction of ribonucleotides to deoxyribonucleotides and is essential for DNA synthesis in all prokaryotic and eukaryotic systems (Erikson & Sjöberg, 1989; Lammers & Follman, 1983; Reichard, 1988; Thelander & Reichard, 1979). Several herpesviruses induce a novel RR in virus-infected cells. For HSV-1 this activity has been shown to be virus-encoded (Dutia, 1983; Preston et al., 1984, 1988) and comparative analysis of DNA sequence data has shown distinct internal amino acid homologies with equivalent proteins from HSV-2 (McLauchlan & Clements, 1983; Swain & Galloway, 1986), Epstein–Barr virus (Gibson et al., 1984) and varicella-zoster virus (VZV) (Davison & Scott, 1986; Nikas et al., 1986). The novel RR activity induced in cells infected with EHV-1 (Cohen et al., 1977) and pseudorabies virus (PRV) (Lankinen et al., 1982) are also presumably virus-encoded but this remains to be established.

The HSV-encoded enzyme consists of two non-identical subunits (Frame et al., 1985; Bacchetti et al., 1986; Ingemarson & Lankinen, 1987; Darling et al., 1988) termed RR1 and RR2 (Frame et al., 1985) or H1 and H2 (Cohen et al., 1985). Sedimentation analysis of the enzyme suggests that both subunits are homodimers (Ingemarson & Lankinen, 1987) with the same basic αβ structure as the Escherichia coli and mammalian reductases.

The HSV-induced RR activity was found to be specifically inhibited by the nonapeptide YAGAVVNDL which corresponds to the nine carboxy-terminal amino acids of RR2 (Dutia et al., 1986; Cohen et al., 1986). The peptide acts by competing for an RR2 binding site on RR1 and as a consequence disrupts the interaction between the two subunits (McClements et al., 1988; Darling et al., 1988). It has been demonstrated that HSV RR enzyme activity is essential for pathogenicity of the virus in mice which suggests that it is a valid antiviral target (Cameron et al., 1988) and that a drug structurally based on all or part of the inhibitory nonapeptide could be a therapeutically useful agent. Furthermore, serological evidence obtained using antisera made against the RR2 carboxy-terminal sequence and tested against cells infected with different viruses, pointed to extensive
conservation of the carboxy-terminal region of the HSV-1 RR2 in other human herpesviruses (HSV-2 and VZV) and also other animal herpesviruses (PRV and EHV-1) and led to the prediction that the nonapeptide might inhibit a broad range of herpesvirus RR enzymes (Dutia et al., 1986). This was subsequently shown to be the case for the PRV enzyme (Cohen et al., 1987). In the present report we demonstrate that the EHV-1 RR enzyme activity is as effectively blocked by the YAGAVVNDL nonapeptide.

Methods

Growth of cells and virus. Baby hamster kidney (BHK-21 clone 13) cells were grown in Glasgow modified Eagle's medium containing 10% calf serum. Rabbit kidney (RK13) cells were grown in the same medium supplemented with 1% non-essential amino acids. HSV-1 cells were grown in Glasgow modified Eagle's medium containing 10% foetal calf serum. Rabbit kidney (RK13) cells were grown in the same medium supplemented with 1% non-essential amino acids. HSV-1 strain 17 syn+ (Brown et al., 1973) was propagated in BHK-21 cells (Macpherson & Stoker, 1962). Two strains of EHV-1 subtype 1 were used: strain Kentucky A (O'Callaghan et al., 1986; Henry et al., 1981), which was provided by Professor D. O'Callaghan (Louisiana State University, Shreveport, La., U.S.A.) and strain Vo 1939 (Cullinan et al., 1988). Both strains were propagated in RK13 cells.

Ribonucleotide reductase extraction and assay. RR induced by either HSV-1 or EHV-1 was partially purified from infected cells by ammonium sulphate precipitation as described by Dutia et al. (1986) except that the freezing and thawing cycles for protein extraction were omitted for the EHV-1 enzyme as they appeared to cause rapid loss of RR activity. RR was assayed as described (Darling et al., 1987) with minor modifications. The final assay mixture, in a reaction volume of 90 μl, contained RR, 200 mM-HEPES pH 8.0 (unless specified otherwise), 10 mM-DTT, 110 μM-CDP, 1 μM-[3H]CDP (Amersham; sp. act. 30 Ci/mmol), 1 mM-bacitracin (Sigma; Gaudreau et al., 1987) and where indicated oligopeptides or purified IgG (Dutia et al., 1986).

Neutralization of RR activity by monoclonal antibodies (MAbs). IgG was first purified from mouse ascites fluid by ion-exchange chromatography using a Mono Q column (Pharmacia) with 50 mM-Tris-HCl pH 8.0 buffer and a linear NaCl gradient of 0 to 400 mM. Fractions were assayed for IgG by dot blots probed with horseradish-conjugated goat anti-mouse antibody and substrate using an Immun-Blot kit (Bio-Rad) according to the manufacturer's instructions. Fractions containing IgG were pooled and concentrated using an Amicon ultrafiltration cell fitted with a YM-5 membrane (M, 5000 cutoff) under nitrogen.

The effect of the various IgGs on RR activity was investigated by preincubating the enzyme with the antibody for 10 min at 37°C prior to the addition of the remaining reagents. The concentration of IgG in the final assay mixture was approximately 300 μg/ml.

MAbs. MAbs 7602 (I. Nikas, A. J. Darling, H. Lankinen, A. M. Cross, H. Marsden & J. B. Clemens, unpublished results) and 1100 (Frame et al., 1985) both directed against RR1 were supplied by Dr A. M. Cross.

Radioisotopic labelling of cells. For electrotoblotting, infected and mock-infected cells were labelled between 3 and 24 h after infection with [35S]methionine (50 μCi/ml; Amersham) in Eagle's medium containing 20% of the normal concentration of methionine, and supplemented with 2% foetal calf serum. The cells were washed once with phosphate-buffered saline and suspended in denaturing buffer (0.05 M-Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol; enough bromophenol blue was added to visualize the dye front) at a concentration of 2.5 x 10^8 cell equivalents per ml. This suspension was stored at -70°C and boiled immediately before electrophoresis.

Gel electrophoresis and Western blotting. SDS-PAGE of proteins was conducted with 5 to 12.5% gradient gels cross-linked with 5% (w/w) N,N'-methylene-bisacylamide using the buffer system of Laemmli (1970). Western blots were performed essentially as described (Towbin et al., 1979) with some modifications (Frame et al., 1987). Antisera prepared against synthetic oligopeptides representing different regions of RR1 (Lankinen et al., 1989 and Fig. 4) were diluted as described and bound antibody was visualized with horseradish-conjugated Protein A (Bio-Rad) as specified by the manufacturer. The apparent M, of reactive polypeptides were determined by alignment of the stained strips with an autoradiographic image of the [35S]methionine-labelled proteins bound to the nitrocellulose membranes.

Synthetic oligopeptides. Peptide TSRLSDPNSSAY which corresponds to amino acids 2 to 13 of the 10K protein product of the HSV-1 gene US9 (Frame et al., 1986) was purchased from Cambridge Research Biochemicals. Peptide YAGAVVNDL was a gift from Pharmacia/LKB.

Results

Ribonucleotide reductase was partially purified from RK13 cells infected with either EHV-1 strain Kentucky A or strain Vo 1939 and the virus-induced RR activities were measured and compared with HSV-1 RR activity induced in BHK-13 cells. Fig. 1 shows the enzyme concentration curves for each of the three enzyme extracts. Enzyme prepared from HSV-1-infected cells and EHV-1 (Kentucky A)-infected cells had comparable activities and the relationship appeared linear up to about 500 μg of protein. EHV-1 (Vo 1939)-infected cells had much lower activity (Fig. 1) probably reflecting the poorer growth of this virus (data not shown). Additional experiments with the HSV-1 and EHV-1 (Kentucky A) enzyme were performed with 100 μg of partially purified extract which gave about 10% conversion of the substrate at 110 μM concentration.

The effect of pH on the rate of CDP reduction of HSV-1- and EHV-1 (Kentucky A)-induced RR was determined by varying the pH of the 200 mM-HEPES buffer in the assay mixture between 7.4 and 8.2. Fig. 2 shows that the optimum pH for the HSV-1 enzyme lay between 8.0 and 8.1 in agreement with previous findings (Averett et al., 1983); the EHV-1 enzyme had approximately the same pH optimum (pH 8.1). All subsequent RR assays were performed at pH 8.0 for both enzymes.

To determine the effect of the nonapeptide YAGAVVNDL on the virus-induced RR activity in HSV-1-infected cells, enzyme activity was assayed in the presence of various concentrations of the peptide and the control peptide TSRLSDPNSSAY, previously found not to inhibit HSV-1 RR activity (Dutia et al., 1986). In parallel experiments the effect of these peptides on HSV-1 RR activity was determined and the results (Fig. 3)
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Fig. 1. Virus-induced RR activities. BHK-21 cells were infected with HSV-1 (▲) and RK13 cells were infected with EHV-1 strain Vo 1939 (○) or Kentucky A (●). Partially purified enzyme was assayed for RR activity at various concentrations of protein. The rates of conversion of CDP to dCDP are given in nmol/h at 37°C.

Fig. 2. Effect of pH on EHV-1- and HSV-1-induced RR activities. Partially purified enzyme from HSV-1- or EHV-1 (Kentucky A)-infected cells was assayed in 200 mM-HEPES over the pH range shown. Each assay contained approximately 100 μg protein. (○) HSV-1-infected; (■) EHV-1 (Kentucky A)-infected.

Structural features of the EHV-1 RR polypeptides were investigated using antisera made against oligopeptides from different regions of the HSV-1 RR1. Fig. 4 shows the coding region of RR1, the boundary of the N-terminal 311 amino acid domain which is so far unique to the HSV-1 and HSV-2 RR enzymes, and the position of the three amino acid sequences against which antisera N1, N2 and C2 were raised.

The reactivity of these anti-RR1 sera with extracts of cells either mock-infected or infected with HSV-1 or EHV-1 is shown in Fig. 5. As found previously (Lankinen et al., 1989) all three sera reacted with a polypeptide of apparent Mr 136000 in HSV-1-infected cells (lanes 5, 8 and 12) but not in mock-infected cells (lanes 4, 7, 10 and 11). In contrast, sera N1 and N2 were not reactive with any polypeptide in EHV-1-infected cells (lanes 6 and 9) which was not also present in uninfected cells (lanes 4 and 7). Serum C2 reacted with one protein of apparent Mr 90000 (lane 13) which was absent from mock-infected cells (lanes 10 and 11). A faster migrating protein (arrowed) was also detected with the C2 serum but this could be either a proteolytic fragment of the 90K protein or a cellular protein as there

show that the EHV-1 enzyme was specifically inhibited by YAGAVNDL but not by the control peptide. The concentration of peptide required to inhibit 50% of the RR activity was 28 μM for both enzymes.

Fig. 3. Inhibition of (a) HSV-1- and (b) EHV-1 (Kentucky A)-induced RR activity by YAGAVNVL. Increasing concentrations of peptide YAGAVNVL (△) or the control peptide TSRLSDPNSSAY (▲) were added to 100 μg of partially purified enzyme from HSV-1-infected BHK-21 cells or EHV-1-infected RK13 cells. The activity in the absence of oligopeptide is taken as 100%.

Structural features of the EHV-1 RR polypeptides were investigated using antisera made against oligopeptides from different regions of the HSV-1 RR1. Fig. 4 shows the coding region of RR1, the boundary of the N-terminal 311 amino acid domain which is so far unique to the HSV-1 and HSV-2 RR enzymes, and the position of the three amino acid sequences against which antisera N1, N2 and C2 were raised.

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Fig. 4. A schematic presentation of the linearized RR1 protein of HSV-1 strain 17 with the locations of peptides against which the antibodies were raised. The HSV-1 genome is shown with the unique long (UL) and unique short (Us) regions, the terminal repeats (TR_L and TR_S), internal repeats (IR_L and IR_S) and the locus of gene UL39 which encodes the HSV-1 RR1 (McGeoch et al., 1988). The position and sequence of the peptides used to produce the N1, N2 and C2 antisera are shown. The tyrosine (Y) residues in parentheses are not part of the RR1 sequence but were added to facilitate coupling of the peptide to the carrier protein. The amino acid residue numbers of each peptide in this 1137 amino acid protein are also indicated. The inverted triangle (▽) depicts the boundary of the unique N-terminal domain at amino acid 311. The nomenclature used for these antisera and their preparation is documented elsewhere (Lankinen et al., 1989).

<table>
<thead>
<tr>
<th>Antiserum designation</th>
<th>Peptide</th>
<th>Amino acid numbers</th>
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<tbody>
<tr>
<td>N1</td>
<td>MASRPAASSPVE(Y)</td>
<td>1-12</td>
</tr>
<tr>
<td>N2</td>
<td>TQTADVPEAL(Y)</td>
<td>137-147</td>
</tr>
<tr>
<td>C2</td>
<td>RNSQFVALMPTA</td>
<td>959-970</td>
</tr>
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is a weakly reactive band of this mobility in mock-infected cells. The control non-immune rabbit sera (NRS) reacted with some proteins in mock-infected and infected cells (lanes 1, 2 and 3) but none of these bands comigrated with the 90K protein.

To obtain further evidence for the serological relatedness of the HSV-1 and EHV-1 RR1, advantage was taken of the high reactivity of the antisera with the RR1 protein. The results of the neutralization assay are shown in Fig. 6. Neutralization of EHV-1 RR activity by MAb 7602. RR activity in HSV-1- or EHV-1-infected cell extracts was assayed in the presence of IgG purified from control ascites (C) and MAbs 7602 and 1100 as well as in the absence of IgG. The activities shown are the mean of duplicate samples and results of individual samples are shown by the vertical bars in the histogram. The results for HSV-1 RR activity are shown on the left (solid line) and those for EHV-1 are shown on the right (broken line).
taken of the observation (I. Nikas, A. J. Darling, H. Lankinen, A. M. Cross, H. Marsden & J. B. Clements, unpublished results) that MAb 7602, which is reactive with HSV-1 and HSV-2 RR1, inhibits the activity of both the HSV-1 and HSV-2 RR enzyme. We tested whether this antibody would inhibit the activity of the EHV-1 RR enzyme. The data presented in Fig. 6 shows that it did, albeit to a lesser extent than it inhibited the HSV-1 enzyme. The relevant controls, also shown in this figure, are immunoglobulin from control ascites and from MAb 1100 which exhibit only a marginal effect on activity.

Discussion

Cohen et al. (1977) reported the induction of RR activity in cells infected with the Kentucky A strain of EHV-1 subtype 1. Our results confirm this finding which was also demonstrable with another isolate (Vo 1939). The novel virus-induced RR activity was further characterized by us with respect to pH and found to have maximum activity at pH 8.1 (Fig. 2) which is similar to the pH optimum for HSV-1 RR (Fig. 2 and Averett et al., 1983).

The structure of the enzyme was investigated using antisera made against oligopeptides from different regions of the large subunit of the HSV-1-encoded RR1. We were particularly interested in determining whether the EHV-1 RR1 possessed a region equivalent to the N-terminal domain which is so far unique to HSV-1 and HSV-2 (Nikas et al., 1986). Neither of the two sera raised to regions within the unique N-terminal domain of HSV-1 was reactive with the EHV-1 enzyme whereas a serum specifically raised against a region within the conserved carboxy-terminal domain reacted with an EHV-1-induced polypeptide of apparent Mr 90000. A simple interpretation of these data would be that EHV-1 encodes a smaller RR1 polypeptide than either HSV-1 or HSV-2 and that the EHV-1 RR1 does not contain the N-terminal domain found in HSV and so is similar in size to the large subunit of RR enzymes of the type $\alpha_2\beta_2$ from a variety of species. However, until the RR1 gene of EHV-1 is sequenced (work in progress in Dr A. Davison’s laboratory) it seems unjustifiable to draw further conclusions about the N-terminal structure of EHV-1 RR1.

The serological homology between RR1 of HSV-1 and EHV-1 may be limited: two additional sera made against oligopeptides from other regions of the conserved carboxy-terminal domain of HSV-1 RR1 (YPVPLEI-PENAEAVA and KATNSQVFGDDNIV, amino acids 312 to 327 and 1117 to 1131 respectively; Lankinen et al., 1989) and two MAbs, 1100 (Frame et al., 1985) and 1026 (I. Nikas, A. J. Darling, H. Lankinen, A. M. Cross, H. Marsden & J. B. Clements, unpublished results) both specific for HSV RR1, all failed to cross-react with EHV-1 RR1 (unpublished data).

The cross-reactivity of the C2 serum with the EHV 90K polypeptide suggests a structural relatedness between EHV-1- and HSV-1-induced RR1. This view is supported by the partial inhibition of the EHV-1 enzyme by MAb 7602. Earlier it was shown by Dutia et al. (1986) that an antisera raised against the peptide YGAVVNDL precipitated a 33K polypeptide from EHV-1-infected cell extracts. Taken together these data strongly suggest that EHV-1 induces a two subunit RR enzyme and support the assumption that the enzyme is virus-encoded.

The RR activity induced by the Kentucky A strain of EHV-1 subtype 1 was specifically inhibited by the nonapeptide YAGAVVNDL (Fig. 3). Comparable results were obtained with strain Vo 1939 (data not shown). Our data provide further support for the speculation that if a drug based on the nonapeptide could be developed then it might be effective against a broad range of herpesviruses (Dutia et al., 1986) and leads us to further speculate that such a drug might be useful in the treatment of EHV-1 infections in horses. This is of special interest since currently no drug, including acyclovir, is effective therapeutically in such infections (Rollinson, 1989).

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