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Direct inactivation of herpes simplex virus type-2 by rat epidermal protein

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Summary

Proteins were extracted from corneocytes of skin of 2-day-old rats and fractionated by gel filtration and cation exchange column chromatography. The different protein fractions were tested for direct inactivation of herpes simplex virus infectivity as determined by reduction of plaque formation. The most effective protein fractions against herpes simplex virus were a neutral pH buffer soluble and mol. wts. ranging from 20 K to 30 K. Amino acid composition of the proteins were virtually identical to epidermal histidine-rich proteins. The activity was significantly ($P < 0.001$) stronger against type-2 than type-1. The activity was most stable at pH 7.2 and the rate of inhibition increased in a time-dependent manner up to 4 h. The 50% effective dose was estimated as 1.1 μg protein/ml.

Epidermal protein; Inhibition of HSV infectivity; HSV type-2

Introduction

Herpes simplex virus (HSV) infection most commonly occurs in oral or genital mucous membrane, and intact skin appears to be relatively resistant to the viruses. Lehrer et al. (1985) and Daher et al. (1986) reported that rabbit leukocytes and human neutrophils, respectively, contain natural peptides which

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directly inactivate HSV infectivity. We have investigated the possibility that the epidermis also may contain a non-immune cellular component that inactivates HSV and functions in host resistance against HSV infection.

In order to test whether antiviral protein(s) exist in skin, we evaluated in vitro activity of rat epidermal proteins on HSV viability. Since the major morphological differences between normal skin and mucous membrane or glabrous skin are the cells in the outer surface layers (Fukuyama and Epstein, 1973; Ham, 1976), we speculated that the effective proteins are components of the terminally differentiated epidermal cells (corneocytes) which are not present in mucous membrane epithelium. The proteins which demonstrate direct inactivation of HSV, especially against HSV type-2 (HSV-2) were partially purified and the biochemical properties characterized.

Materials and Methods

Viruses and cells

The viruses used were wild types, isolated in the clinical virology laboratory at Mt. Zion Hospital, of University of California, San Francisco. HSV type 1 (HSV-1) and HSV-2 isolates, typed by standard immunofluorescence microscopy (Peterson et al., 1983), were cultured in human embryo lung fibroblasts (HEL) purchased from Whittaker Bioproducts (Walkersville, MD) using a maintenance medium consisting of minimal essential medium (MEM) supplemented with 3% fetal calf serum (GIBCO, Grand Island, NY), Vancomycin (250 μg/ml), Gentamicin (250 μg/ml) and Fungizone (50 μg/ml). The stock virus preparations were prepared from HSV-2 and HSV-1 isolates from 7 and 6 patients, respectively. They were titered by plaque forming units (PFU) and stored under liquid nitrogen.

Assay of viral inactivation

HSV inactivation was determined by reduction of plaque formation. Unless otherwise specified, the viral stock was thawed immediately before use and diluted to approximately 2000–3000 PFU in 1 ml of maintenance medium. Approximately 240 PFU/100 μl of the viral suspension was mixed with 10 μg epidermal protein dissolved in 1 ml Puck's saline A (PSA), pH 7.2 and incubated at 37°C for 4 h. The protein tested was first dissolved at 100 μg/ml in PSA, passed through a Millex HA 0.45 μm Filter Unit (Millipore, Bedford, MA) and the protein concentration adjusted by addition of PSA. Protein concentration of the test samples was determined by the method of Lowry et al. (1951). The protein–virus mixture was then diluted to 3 ml with maintenance medium and 1-ml aliquots were added to each of 3 wells of a 24-well tissue culture plate (Corning, NY) which contained monolayers of HEL. These plates were incubated at 37°C in 5% CO_2 for 1 h. The wells were then washed with PSA and overlaid with MEM containing 0.5% agarose and maintained at 37°C for 48 h. The culture was terminated by methyl alcohol fixation, stained with

Wright Giemsa stain, and the plaques counted on an inverted microscope. All results were calculated from triplicate cultures, and expressed as a percent reduction of PFU relative to cultures infected with virus not treated with epidermal protein, but incubated in PSA at 37°C for 4 h before HEL infection.

Protein purification

Cornified cells from 2-day-old Sprague–Dawley rats were mechanically scraped from epidermis separated from the dermis in 0.24 M NH_4Cl (pH 9.5) as described (Murozuka et al., 1979). The cells kept at −70°C until use were homogenized in 10 times vol. of 0.1% acetic acid by a glass homogenizer. The homogenate was centrifuged at 13,000 × *g* for 30 min and the supernatant once lyophilized (40 mg) was dissolved in 2 ml 1% acetic acid. It was dialyzed against 0.05 M sodium phosphate buffer (pH 7.0), and the precipitate removed by centrifugation at 15,000 × *g*. The supernatant was applied to a Sephacryl S-300 superfine column equilibrated with 0.05 M phosphate buffer (pH 7.0) containing 0.2 M NaCl. The eluent was pooled into 6 fractions (Fr 1 through Fr 6) according to mol. wt. The protein fraction (Fr 1) estimated to be mol. wt. above 80 K, was dialyzed against deionized water and lyophilized. It was dissolved in 0.05 M phosphate buffer (pH 7.0) and applied to a Mono S HR 5/5 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated by the same buffer. The adsorbed proteins were eluted with a NaCl gradient (0–0.5 M) on a fast protein liquid chromatography system (FPLC, Pharmacia). Five fractions (Fr 7 through Fr 11) separately pooled were dialyzed against deionized water and lyophilized. The proteins eluted out from the Mono S HR 5/5 column with 0.125 M NaCl (Fr 8) showed the highest total and specific activity in PFU reduction. Fr 8 was dissolved in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl and applied to a Sephacryl S-200 column equilibrated by the same buffer. Three protein peaks were pooled, dialyzed against water and lyophilized.

Mol. wt. of proteins in the fractions was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed on 10 × 8 cm mini-slabs by the method of Laemmli (1970). For determination of amino acid composition those protein fractions were desalted on a ProRPC HR 10/10 column (Pharmacia) using a linear acetonitrile gradient (0–80%) containing 0.1% trifluoroacetic acid with a FPLC system. All fractions revealed a major protein peak that eluted at 20% acetonitrile. They were separately lyophilized and hydrolyzed for 20 h at 115°C with 6 N HCl in vacuo and analysis was performed.

Effects of protein concentration, incubation time and pH

Modulating factors such as exposure time, protein concentration and pH stability were evaluated. The protein sample (Fr 8) eluted from the Mono S column with 0.125 M NaCl was used. The optimal exposure time for viral inactivation was determined by incubation of HSV-2 in 10 μg/ml of the protein for 1, 2 and 4 h. Control PFU values were obtained for each time point by

incubating the same concentration of HSV-2 in PSA without epidermal proteins. The effects of protein concentrations ranging from 0.3 μg/ml to 10 μg/ml were examined with an incubation time of 4 h. In order to test pH stability of the protein activity, the protein solution was kept at pH 5, 7.2 and 9 at 22°C for 1 h and inactivation of HSV-2 assayed after the pH was adjusted back to 7.2 with HEPES buffer by the standard method.

Results

Inactivation of HSV-2 by purified epidermal proteins

A typical elution pattern of epidermal protein from a Sephacryl S-300 column is illustrated in Fig. 1. Although separation of the proteins was not distinct, we have arbitrarily identified Fr 1 through Fr 6 according to mol. wt. estimated from the calibration curve depicted with protein standards. Total protein recovered in each fraction and the activity against HSV-2 at a concentration of 10 μg/ml protein as determined by % reduction in PFU are summarized in Table 1. Fr 1 attained more than 80% reduction of PFU. Other fractions showed variable activity and Fr 3 had no activity, demonstrating that not all epidermal proteins possess the anti-HSV-2 activity. Fr 1, with the highest total activity, was therefore used for further purification by cation exchange column chromatography. The eluent from a Mono S column (Fig. 2) was pooled into 5 fractions (Fr 7–11). As also shown in Table 1, Fr 8 eluted with 0.125 M NaCl showed 100% reduction at a concentration of 10 μg/ml, while the pass through fraction (Fr 7) as well as Fr 9 through 11 contained low

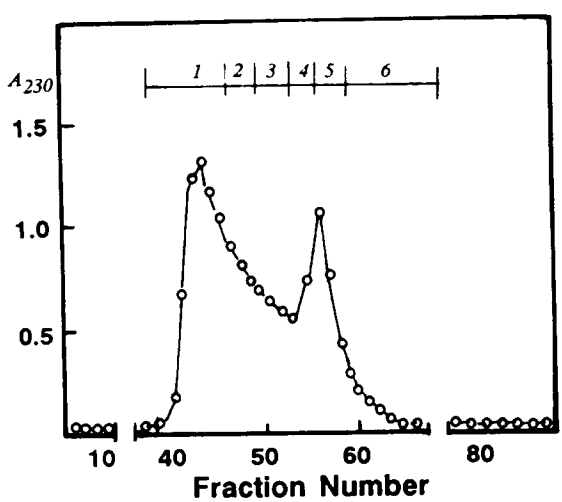

Fig. 1. Elution profile of epidermal proteins from a Sephacryl S-300 superfine column (1.5 × 114 cm) equilibrated in 0.05 M sodium phosphate, 0.2 M NaCl, pH 7.0. The flow rate was 20 ml/h and 3.0 ml/tube collected. Protein concentration (○—○) was monitored by absorbance at 230 nm. The eluents were pooled according to their apparent mol. wt. and named as fraction (Fr) 1 through 6.

TABLE 1

Initial purification of epidermal proteins which inactivate HSV-2

Epidermal proteins[a]	Separation		% Protein[b]	% Reduction of PFU
	By mol. wt.	By NaCl (M)		
Fr 1	170–80 K		36.1	80.9
Fr 2	80–50 K		20.1	63
Fr 3	50–30 K		17.7	0
Fr 4	30–20 K		5.2	12.2
Fr 5	20–13 K		11.4	11
Fr 6	13– 5 K		9.6	56.9
Fr 7		0	5.2	48
Fr 8		0.125	18.7	100
Fr 9		0.175	54.3	18.5
Fr 10		0.225	17.6	27.7
Fr 11		>0.225	4.1	3

[a] Fr 1 to 6 were separated by a Sephacryl S-300 superfine column according to their apparent mol. wt., Fr 7 to 11 were separated from Fr 1 by a Mono S HR 5/5 column with different NaCl concentrations.

[b] % protein in each fraction was calculated taking total recovered protein as 100% for each separation method.

activity. The amount of protein in Fr 8 was about 20% of the total recovered protein. Separation of proteins with different mol. wt. from Fr 8 was possible on a Sephacryl S-200 column, as demonstrated by SDS-PAGE (lane F, G and H) in Fig. 3, but proteins in all fractions exhibited an antiviral effect. At 10 μg/ml concentration, the proteins with mol. wt. 28 K, 23 K and 21 K reduced PFU

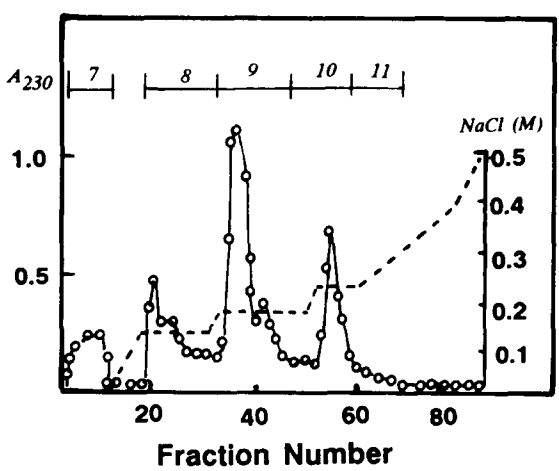

Fig. 2. Ion exchange chromatography of proteins in fraction (Fr) 1. A Mono S HR 5/5 column was equilibrated in 0.05 M sodium phosphate, pH 7.0 and adsorbed proteins were eluted by a step-wise NaCl gradient (- - - -). The flow rate was 1 ml/min and 1.0 ml/tube collected. Protein concentration (○—○) was monitored by absorbance at 230 nm. Five fractions (Fr 7–11) were separately pooled.

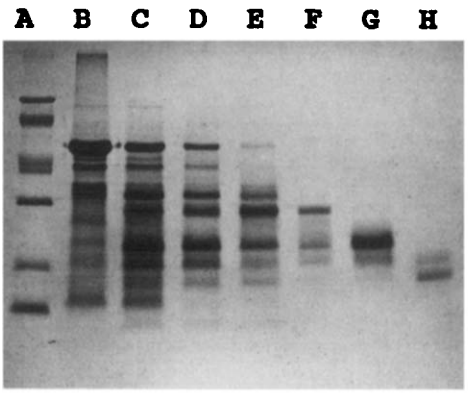

Fig. 3. SDS-PAGE analysis of purification of epidermal proteins. Samples were run on a 12.5% gel and stained with Coomassie brilliant blue R. Molecular weight markers (Lane A): from top, phosphorylase b M_r = 94 K, bovine serum albumin M_r = 67 K, ovalbumin M_r = 43 K, carbonic anhydrase M_r = 30 K, soybean trypsin inhibitor M_r = 20.1 K, α-lactalbumin M_r = 14.4 K. Proteins from cornified cells: crude extract in 0.1% acetic acid (Lane B); pH 7.0 soluble fraction (Lane C); Fr 1 after gel filtration (Lane D); Fr 8 after ion exchange chromatography (Lane E); 28 K protein with minor 23 and 21 K bands (Lane F); 23 K protein with minor 21 K band (Lane G); 18 K protein with minor 21 K band (Lane H).

by 31.3%, 87.5% and 81.3% respectively, indicating that the active protein exists in different mol. wts.

Comparative effects of epidermal protein on HSV-1 and HSV-2

Fr 8 from the Mono S column was used at a concentration of 10 μg/ml to determine the selectivity of the epidermal proteins on herpes simplex viruses. The protein inhibited plaque formation of different isolates of both HSV-1 and HSV-2, but was significantly more effective against HSV-2 than HSV-1 (Table 2). The inhibition of HSV-2 infectivity was dependent on protein concentration at least up to 10 μg/ml (Fig. 4). The 50% effective dose was estimated to be 1.1 μg/ml or 44 nM (calculated from mean mol. wt. = 25 K for the epidermal protein).

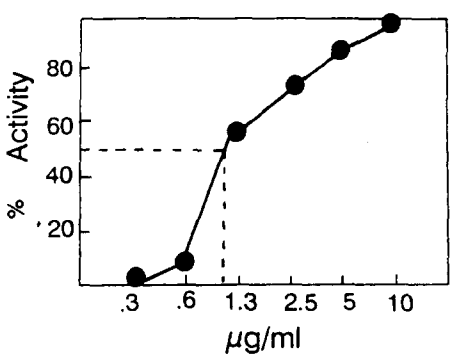

Fig. 4. Dose-dependent activity of epidermal protein against HSV-2. HSV-2 was incubated for 4 h with 0–10 μg/ml of epidermal protein (Fr 8) and % PFU reduction plotted.

TABLE 2

Effects of epidermal protein on infectivity of herpes simplex viruses

Virus (no. of strains)	% Reduction of PFU[a] (mean ± SD)
HSV-2 (7)	73.3 ± 14.6[b]
HSV-1 (6)	20.3 ± 16.7

[a]The 50% inhibition dose was estimated to be 1.1 μg/ml.
[b]The difference in % reduction of PFU between HSV-1 and HSV-2 was $P < 0.001$ by the Student's t-test.

TABLE 3

Amino acid composition of epidermal proteins

Amino acid	Mol % of residues						
	Fr 1	Fr 8	28 K	23 K	18 K	HRP I[a]	HRP II[a]
Ala	12.6	10.6	11.0	12.3	12.0	10.8	10.5
Arg	11.6	12.9	14.0	12.7	12.8	14.0	13.1
Asx	4.7	4.8	4.2	4.2	4.1	5.0	5.3
Glx	18.4	21.6	20.7	18.4	19.1	21.9	20.8
Gly	17.0	14.3	12.6	14.4	13.9	14.4	13.6
His	6.9	6.3	6.8	6.5	6.8	6.9	7.3
Ile	2.2	1.9	1.4	1.7	1.1	0.8	1.3
Leu	ND[b]	ND	ND	ND	ND	0.5	0.6
Lys	ND	ND	ND	ND	ND	0.9	1.2
Pro	trace	2.2	6.7	4.8	4.6	0.4	1.9
Ser	19.6	16.9	16.7	19.0	17.2	15.1	16.9
Thr	7.0	7.2	5.8	5.6	6.3	6.7	6.5
Tyr	ND	ND	ND	0.3	ND	1.0	0.4
Val	ND	0.5	ND	ND	ND	0.7	0.5

[a]The values were reported by Ball et al. (1978).
[b]ND, not detected.

Amino acid analysis of epidermal proteins

Amino acid composition of different protein fractions during purification are presented in Table 3. All 5 proteins with activity but different mol. wts. showed essentially the characteristic composition reported for histidine-rich proteins (HRPs) purified from mature epidermal cells (Ball et al., 1978; Londsdale-Eccles et al., 1984; Murozuka et al., 1979; Scott et al., 1982). The amount of histidine residues was 6–7%, and lysine, leucine, tyrosine and valine residues were not detectable.

Effects of exposure time and pH stability

Inactivation of HSV by the protein at pH 7.2 increased with time up to 4 h incubation. Incubation for longer periods under these conditions caused marked reduction of viral infectivity even without epidermal proteins; thus determination of effects of the protein became unreliable. The activity of the protein was most stable in pH 7.2 and loss of the activity was seen at both the acidic and basic pHs after 1 h incubation.

Discussion

Epidermal proteins of mol. wt. 20–30 K directly inactivated HSV-2. The effects were dependent on preincubation time up to 4 h; a longer preincubation was technically difficult. The epidermal proteins which reduced PFU of HSV-2 showed different mol. wts., but they all belong to the HRP family as far as amino acid composition is concerned. HRP has long been regarded as the specific protein marker for epidermal cells (Freedberg, 1983). However, the antiviral proteins have distinct properties different from other members of HRPs already biochemically characterized. Conventional HRP is synthesized in granular cells (Fukuyama and Epstein, 1975) and has been shown to have mol. wt. >300 K (Ball et al., 1978; Londsdale-Eccles et al., 1984). As keratinocytes terminally differentiate, the precursor HRP becomes small mol. wt. with a highly basic charge (Scott and Harding, 1981; Scott et al., 1982). The high mol. wt. and some of the basic HRPs are not soluble at neutral pH. In contrast, the HRP which inhibits HSV-2 infectivity is soluble in a neutral pH buffer and weakly absorbed on a Mono S column under the conditions used. We believe that the basic HRP in corneocytes precipitated during the first dialysis step against pH 7.0 phosphate buffer in the present study and separated from the soluble HRP. The pH range required for solubilization of the basic HRP is not suited for maintaining viral infectivity; thus, it is not possible to test effects of basic HRP on HSV-2 infectivity. Studies to understand the specific biochemical differences between the neutral pH soluble and insoluble HRPs are currently in progress. Histidine residues may account for inactivation of HSV. Docherty and Pollock (1987) reported inactivation of HSV, types 1 and 2 by synthetic histidine-containing peptides.

Proteins from leukocytes and macrophages have been investigated extensively as natural peptide antibiotics. A group of proteins called defensins has been purified from different species (Eisenhauer et al., 1989; Ganz et al., 1985; Selsted and Harwig, 1987; Selsted et al., 1984). The defensins consist of 29–34 amino acid residues (Daher et al., 1986; Eisenhauer et al., 1989; Selsted, 1985; Selsted et al., 1983; Selsted and Harwig, 1987), and 8 amino acids, 6 of which are cysteine residues, are conserved among different species (Eisenhauer et al., 1989). The effect of defensins on infectivity of HSV-1 has been tested (Daher et al., 1986; Eisenhauer et al., 1989; Lehrer et al., 1985; Selsted, 1985) and some of the antimicrobial peptides also show in vitro antiviral activity for HSV-2 (Daher et al., 1986; Lehrer et al., 1985), and cytomegalovirus (CMV) (Daher et al., 1986). For defensin action against the virus, the molecular topology with intramolecular disulfide bonds appears to be required as reduction and alkylation of the peptide abolished the activity (Daher et al., 1986; Lehrer et al., 1985). In this respect the action of epidermal protein, HRP, differs considerably; no cysteine residues are found in the HRP molecule. Among antiviral defensins, MCP-1, MCP-2 and HNP-1 were tested for activity against HSV-1, HSV-2 and CMV. HSV-1 and HSV-2 were susceptible to these 3 defensins but CMV was resistant (Lehrer et al., 1985) or susceptible only at

high concentrations (100 and 200 μg/ml) (Daher et al., 1986). The effect of Fr 8 of epidermal protein on HSV was different; it inhibited plaque formation of HSV-2, while HSV-1 was relatively resistant at the concentration of 10 μg/ml.

The mechanism by which epidermal protein inactivates HSV remains to be elucidated. It may be speculated that the neutral pH soluble HRP alters the surface molecules of the virus to prevent infection of HEL, and interferes with attachment on (Fuller et al., 1989; Highlander et al., 1988) and/or penetration into the cells (Highlander et al., 1987; Ligas and Johnson 1988). The inhibitory effect of epidermal protein on HSV-2 was much stronger than on HSV-1, suggesting that there is a difference in the degree of interaction between the protein and these two viruses. In this regard, the differential binding capacity of HSV-1 and HSV-2 for cell receptors is known (Addison et al., 1984; Vahlne et al., 1980; Vahlne et al., 1979). Recently, WuDunn and Spear (1989) reported that cell surface heparan sulfate serves as the initial receptor for HSV. Heparin, a related glycosaminoglycan, binds to HSV and blocks the virion-cell interaction, and heparin binding to HSV-2 is greater than that to HSV-1. It seems possible that a similar selective binding occurs between epidermal protein and HSV-2.

The epidermis covers the surface of the body and has been considered to function as a physical barrier to the external environment. HRP is a component of keratohyalin granules and represents a major non-fibrous protein in terminally differentiated epidermal cells and is not detectable in tissues without keratohyalin granules such as mucous membrane. Presence of this anti-HSV protein in skin may help to explain the relative resistance of epidermis to HSV while mucous membranes appear more sensitive to infection by this virus. Elucidation of biological functions of HRP has been the aim of many investigations and the present findings strengthen an earlier suggestion (Kashima et al., 1989) that portions of this protein may participate in host defense mechanisms against external microbial infection.

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