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Inactivation of herpes simplex virus by protein components of bovine neutrophil granules

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Summary

From an acid extract of granules of bovine neutrophils we isolated fractions of cationic proteins, exhibiting significant anti-herpesvirus activity at concentrations which were devoid of cytotoxicity and of activity against a picornavirus (rhinovirus). The mechanism of action seems to involve a direct neutralization of the virions. Two antiviral peptides with an approximate MW 7500 were purified to homogeneity by reversed phase high-performance liquid chromatography. Proline and arginine accounted for about 43% and 26–27% of their amino acid residues. One of these peptides ($IIIa_2\beta$) had an MIC of 2 μM.

Herpes simplex virus; Neutrophil granule; Antiviral peptide

Introduction

Several lines of evidence exist that suggest that neutrophils may play a significant role in antiviral defence. In herpesvirus infection in man and animals, neutrophils are the predominant cells at the site of the lesion [2,29]. Neutrophils phagocytose opsonized viruses [26] and can prevent virus spread from infected to uninfected cells [13]. Furthermore, like other Fc receptor-bearing leukocytes, they also serve as effectors of antibody-dependent lysis of virus-infected cells [2,9,25,31,32].

Bovine neutrophils are particularly active in such cytotoxicity [6]. Unlike the an-

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tibody-dependent cytotoxicity of human neutrophils, which depends on the generation of hydroxyl radicals, lysis of virus-infected cells by bovine neutrophils in the presence of antibody appears to be carried out by granule cationic proteins [31]. Furthermore, upon culture with kidney cells infected with either bovine rhinotracheitis virus or other herpes-viruses, bovine neutrophils have been shown to release a subcellular mediator, which reduces virus-induced cytopathology and has been suggested to act by either protecting the susceptible cells or suppressing virus replication in infected cells [21,22].

To gain some insight into the nature and mechanism of action of the antiviral granular component(s), we have subfractionated an acid extract of the total granule population of bovine neutrophils, testing the various fractions obtained for antiviral activity. In particular, we have studied a protein fraction with a direct neutralizing activity against herpes simplex virus type 1 (HSV_1), and have purified from it two proline- and arginine-rich antiviral peptides with an approximate MW of 7,500.

Materials and Methods

Isolation and extraction of neutrophil granules

Neutrophils were purified from fresh bovine blood, essentially as described by Carlson and Kaneko [3,6,16]. Upon suspension in 0.34 M sucrose – 0.5 mM $MgCl_2$ (brought to pH 7 with $NaHCO_3$) at a density of about 2×10^8 cells per ml, they were disrupted in a Dounce homogenizer, equipped with a tight-fitting pestle (type B). After about 80% cell disruption, unbroken cells and nuclei were sedimented for 10 min at $200\times g$. The total granule population present in the resulting supernatant was collected by centrifugation for 10 min at 4°C and $10000\times g$.

Granule pellets, derived from 2–3 $\times$ 10^{11} neutrophils, were suspended by homogenization in 80–150 ml of 0.2 M Na-acetate-5 mM EDTA, pH 4, subjected to a brief sonication, and the suspension was then stirred for 2 h in an ice-bath. The insoluble residue, sedimented for 30 min at $30000\times g$, was re-extracted over-night with 50–100 ml of buffer as above, and the solubilized proteins were combined with the first extract. This material is referred to as total granule extract.

CM-cellulose chromatography

The acid-extracted proteins were dialyzed against large volumes of distilled water, acidified with a few drops of concentrated HCl (approx. pH 3), in a Spectrapor 6 membrane tubing (nominal cutoff of 3500 MW; Spectrum Medical Industries Inc., Los Angeles, CA). The dialyzed material was vacuum-dried, and suspended in 100–200 ml of 10 mM Na_2HPO_4-0.2 M NaCl, pH 7. The solution obtained was clarified by centrifugation for 40 min at $30000\times g$, filtered through a Millipore filter (pore diameter 0.45 μm) and applied to a column (1.5 $\times$ 20 cm) of CM-cellulose (CM 52; Whatman, Inc., Maidstone, Kent, U.K.), equilibrated in 10 mM

Na_2HPO_4-0.2 M NaCl, pH 7, at a flow rate of 18 ml/h. Fractions including unbound proteins were pooled and constituted pool I [5]. Elution of bound proteins was carried out first with 0.3 M and then 1 M NaCl (in 10 mM Na_2HPO_4, pH 7). Fractions of each peak obtained during the two elution steps were combined into pools IIa, IIb, IIc and pools IIIa, IIIb, IIIc, respectively.

Ultrogel chromatography

Pool IIIa was dialyzed against distilled water, acidified with HCl as above, lyophilized, dissolved in 5 mM Na-acetate/0.1 M NaCl, pH 4, and applied to a column (2.4 × 113 cm) of Ultrogel AcA 202 (LKB-Producter AB, Bromma, Sweden). Elution of the column, which was carried out at a flow rate of about 8 ml per h, produced five protein peaks absorbing at 280 nm. Fractions belonging to these peaks, labelled $IIIa_1$, $IIIa_2$, $IIIa_3$, $IIIa_4$ and $IIIa_5$ (see legend to Fig. 1), were pooled, dialyzed and lyophilized.

Reversed phase chromatography

Pool $IIIa_2$, dissolved in 0.1% trifluoroacetic acid (TFA), was further resolved into various components by hydrophobic, reversed phase chromatography (RPC) on a PEP-RPC HR 5/5 column of the FPLC system (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Elution of the peptides, which was monitored at 214 nm, was performed with a 0–100% gradient of acetonitrile in 0.1% TFA (see Fig. 3) at a flow rate of 0.7 ml/min. The fractions of the resolved peaks were pooled and, after evaporation of acetonitrile by bubbling oxygen-free nitrogen in the solutions, were lyophilized.

Protein determination and amino acid analyses

Protein was determined by the method of Waddel, as modified by Romeo et al. [20], with bovine serum albumin as standard. This method provided data of protein concentrations, which were very close to the actual wt./vol. concentrations. Furthermore, it permitted assays of concentrations of $IIIa_2\alpha$ and $IIIa_2\beta$, which do not contain amino acid residues (see Results) reactive with the Folin phenol reagent [14].

Dried aliquots (15–20 μg of protein) of antiviral peptides, purified to homogeneity, were hydrolyzed for 22, 48 and 72 h at 110°C in evacuated sealed tubes with 0.5 ml of 6 M HCl, and amino acids were analyzed with a Carlo Erba automatic amino acid analyzer.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) on 16.5 cm-long slabs was carried out in the presence of either sodium dodecyl sulfate (SDS) under alkaline conditions [11] or urea under acid conditions [28].

Antiviral studies

Multiplication of herpes simplex virus type 1 (HSV_1) and type 2 (HSV_2), rhinovirus 1 B (RV1B) and vesicular stomatitis virus (VSV) was examined in MRC-5 cells (supplied by Bio-Mérieux), maintained in basal medium of Eagle with Earle's salts supplemented with 2% fetal calf serum, penicillin and streptomycin sulfate at 40 IU and 40 μg/ml, respectively. A standardized procedure [34] was adopted to test the antiviral activity of protein fractions obtained throughout the purification step. Briefly, confluent cell monolayers (approx. 2×10^6 cells) in 1.7 ml medium, were inoculated with 0.1 ml of the substance under test (concentrated 20-fold) in phosphate buffered saline (PBS) and, 30 min later, with 0.2 ml of virus containing $10 \times TCID_{50}$. Such a preincubation at room temperature was later found unnecessary for expression of antiviral activity, and therefore omitted. Cultures were incubated for 3–4 days (depending upon the virus studies) until a generalized cytopathic effect (CPE) appeared. The minimal inhibitory concentration (MIC) was defined as the minimal concentration which was fully protecting cell monolayers from destruction.

Uninfected cells were used to test the cytotoxicity of the substances and the maximal nontoxic concentration (MNTC) assessed. In some instances, MRC-5 cells were lysed by one cycle of freezing and thawing and the amount of infectious HSV_1 was titrated in other MRC-5 cultures by the $TCID_{50}$ method [34]. Only viral dilutions containing concentrations of substances at least 10 times lower than their MIC were considered.

Virus inactivation was determined by incubating 1 ml of HSV_1 suspension (containing or not containing fraction IIIa) at either 37°C or 21°C. At different times, 9 ml of ice-cold PBS were added and serial dilutions were carried out. Virus was titrated either in MRC-5 cells (as above) or in vivo, by inoculating virus dilutions intraperitoneally (0.2 ml/mouse) in 1-month-old OF_1 mice and determining the LD_{50} by a standard method [19]. The latter procedure was followed specifically to test whether the reduction in virus infectivity by fraction IIIa could be maintained in an animal model.

Presence of bovine interferon (IFN) or IFN-like activity was tested by incubating fraction $IIIa_2$ with MRC-5 cells for 18 h. Cells were then washed twice and challenged with VSV. Bovine βIFN (a preparation titrating 4,000 IU/ml on bovine MDBK cells) was a kind gift of Dr. La Bonnardière. It was used as a reference compound at 10^{-3}, 10^{-2}, 10^{-1} dilution. CPE was determined 72 h after infection.

Results

We first tested the antiviral activity of a total granule extract and found that it was active against HSV_1 and HSV_2, with an MIC of 300 μg/ml (Table 1), but not against RV1B. The MNTC of the extract on stationary MRC-5 cells was ≥600 μg/ml.

Among the protein fractions generated from the extract by CM-cellulose chro-

TABLE 1

Purification and anti-HSV_1 activity of protein fractions extracted from granules of bovine neutrophils.

Sample	mg protein[a]	Anti-HSV_1 activity				
		MIC ± SD (μg/ml)	Log_{10} reduction[b] of infectious titre at (μg/ml) 100	50	15	7
Total granule extract	862–959	300 (n = 1)				
Fraction IIIa (CM cellulose)	8–21	32 ± 19 (n = 7)	>3.14[c]	2.26[c]	n.d.	n.d.
Fraction $IIIa_2$ (Ultrogel)	5–13	19 ± 7 (n = 4)	n.d.	>3.10[d]	2.96[d]	2.34[d]

[a] Data from 2 purifications, normalized to 10^{11} neutrophils.
[b] Determined at 3 days post-infection.
[c,d] Virus titer of untreated cultures = $10^{4.14}$ and $10^{4.10}$, respectively.
n.d. = not determined.

matography, only IIa and IIIa exhibited anti-HSV_1 activity with MICs ≤50 μg/ml and MNTCs ≥100 μg/ml. Fraction IIIa, which emerged from the CM-cellulose column after the change of NaCl concentration in the eluting buffer from 0.3 to 1 M, contained 1–2% of the total protein of the granule extract, and was utilized for further investigations.

As shown in Table 1, which includes data from two different preparations of IIIa, this fraction had an MIC against HSV_1 of 32 ± 19 μg/ml (mean ± SD, from 7 experiments). IIIa was also active against HSV_2 with an MIC of 50 μg/ml (one experiment). At 50 and 100 μg/ml, it caused a log_{10} reduction of HSV_1 infectious titre of 2.26 and >3.14, respectively. At the highest final concentration, at which it was added to the MRC-5 culture medium (100 μg/ml), IIIa did not exhibit any cytotoxicity.

Preliminary experiments on the mechanism of action of fraction IIIa showed that it was capable of directly inactivating HSV_1. This was shown by tests in which a virus suspension was incubated in the absence or in presence of the compound and

TABLE 2

Direct inactivation of HSV_1 by fraction IIIa.

Expt.	Incubation	Sample	Virus titer[a]	log_{10} reduction
1	1h, 21°C	none	$10^{2.84}$	
		IIIa, 150 μg/ml	$10^{2.34}$	0.50
2	20 min, 37°C	none	$10^{2.40}$	
		IIIa, 150 μg/ml	$10^{1.59}$	0.81
3	1 h, 37°C	none	$10^{2.20}$	
		IIIa, 150 μg/ml	$10^{<0.6}$	>1.60
4	2 h, 37°C	none	$10^{3.5}$	
		IIIa, 75 μg/ml	$10^{1.0}$	2.5

[a] Determined by the LD_{50} method (*in vivo*) in expts. 1, 2 and 3, and by the $TCID_{50}$ method (*in vitro*) in expt. 4.

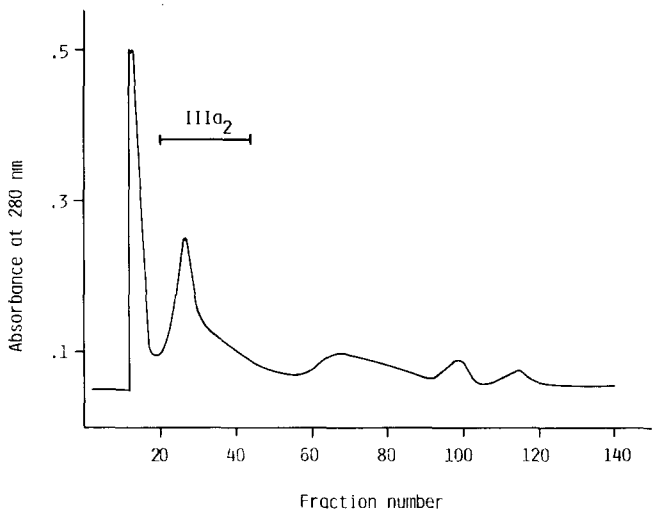

Fig. 1. Gel permeation of fraction IIIa. This was carried out by using a column (24 by 113 cm) of Ultrogel AcA 202, and eluting with 5 mM Na-acetate/0.1 M NaCl, pH 4, at a flow rate of 8 ml h^{-1}. The contents of tubes 13–17, 21–46, 58–90, 93–105 and 108–124 were pooled, and generated fractions $IIIa_1$, $IIIa_2$, $IIIa_3$, $IIIa_4$ and $IIIa_5$.

then titrated either in mice or on MRC-5 cells. As shown in Table 2, when tested by the LD_{50} method, fraction IIIa at 150 μg/ml caused a reduction in the HSV_1 titre of 0.8 and at least 1.6 $\log_{10}$, after an incubation at 37°C for 20 and 60 min, respectively. At a lower temperature (21°C) the neutralizing activity of IIIa was less marked. In $TCID_{50}$ assays, after a 2 h incubation at 37°C fraction IIIa at 75μg/ml reduced the virus titre by 2.5 $\log_{10}$.

To dissociate the antiviral factor(s) from the other components of fraction IIIa, this was subjected to gel permeation through Ultrogel AcA 202. Five peaks were resolved (Fig. 1): $IIIa_1$ (appearing with the void volume), $IIIa_2$, $IIIa_3$, $IIIa_4$ and $IIIa_5$. The antiviral activity was collected in fraction $IIIa_2$, which displayed an MIC of 19 ± 7 μg/ml (mean of four experiments carried out with two different preparations ± SD) (Table 1) and was devoid of cytotoxicity even at 250 μg/ml. Thus the antiviral index of fraction IIIa (MNTC-MIC) was ≥ 13, indicating a significant degree of antiviral selectivity. At 7, 15 and 50 μg/ml, fraction $IIIa_2$ caused a reduction of the HSV_1 titre from $10^{4.10}$ to $10^{1.76}$, $10^{1.14}$ and $10^{<1}$, respectively.

Since fraction $IIIa_2$ included components of MW ≤27000 (see below), an experiment was performed to exclude the participation of bovine IFN in the observed antiviral activity. MRC-5 cells were incubated for 18 h with $IIIa_2$ (30 or 60 μg/ml) or with bovine βIFN (4, 40 or 400 IU/ml), then washed and challenged with VSV at 20×$TCID_{50}$. No protection against the CPE was observed with fraction $IIIa_2$, whereas, as expected, bovine βIFN caused a 50% protection at 4 IU/ml and a 100% protection at 40 and 400 IU/ml.

Fraction $IIIa_2$ accounted for 0.6–1.3% of the proteins of the granule extract and

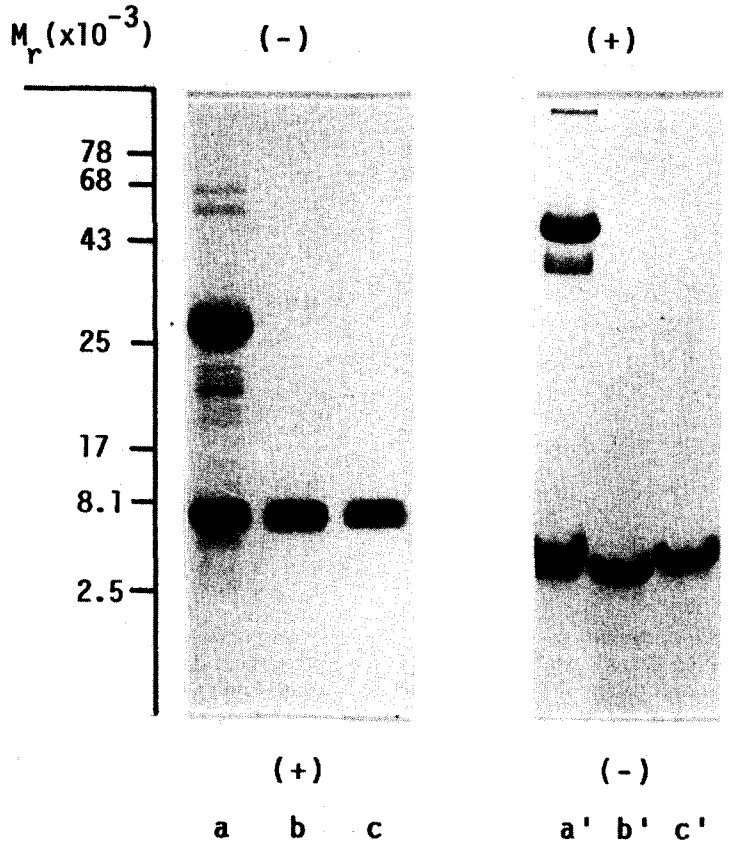

Fig. 2. SDS-PAGE (left) and acid/urea-PAGE (right) of fraction $IIIa_2$ (a,a'=30 μg) and of peptides $IIIa_2\alpha$(b,b'= 10 μg) and $IIIa_2\beta$ (c,c'=10μg). The gel slabs of SDS-PAGE (12–22% acrylamide) and of urea-PAGE (20% acrylamide) were both stained with Coomassie Blue. The gel calibration by MW refers to the SDS-PAGE.

thus for about 0.06–0.13% of the total neutrophil proteins. SDS-PAGE of $IIIa_2$ revealed two major bands, with apparent MW of 27000 and 7500 (Fig. 2a), and other minor components. An attempt was thus perfomed to resolve all these components by hydrophobic reversed phase chromatography. As shown in Fig. 3, this procedure allowed the separation of three peaks: $IIIa_2\alpha$, $IIIa_2\beta$, and $IIIa_2\gamma$.

Since there was a partial overlapping of peaks $IIIa_2\alpha$ and $IIIa_2\beta$, they were further purified by re-chromatography through the PEP-RPC column wherefrom they eluted as sharp peaks. The purified samples were submitted to SDS- and urea-PAGE and behaved as homogeneous peptides with an apparent MW of 7500 (Fig. 2b,c,). Conversely, $IIIa_2\gamma$ contained the other major component of $IIIa_2$ with an approximate MW of 27000, as well as other minor components (not shown).

Fractions $IIIa_2\alpha$, $IIIa_2\beta$, and $IIIa_2\gamma$ were tested for anti-HSV_1 activity at con-

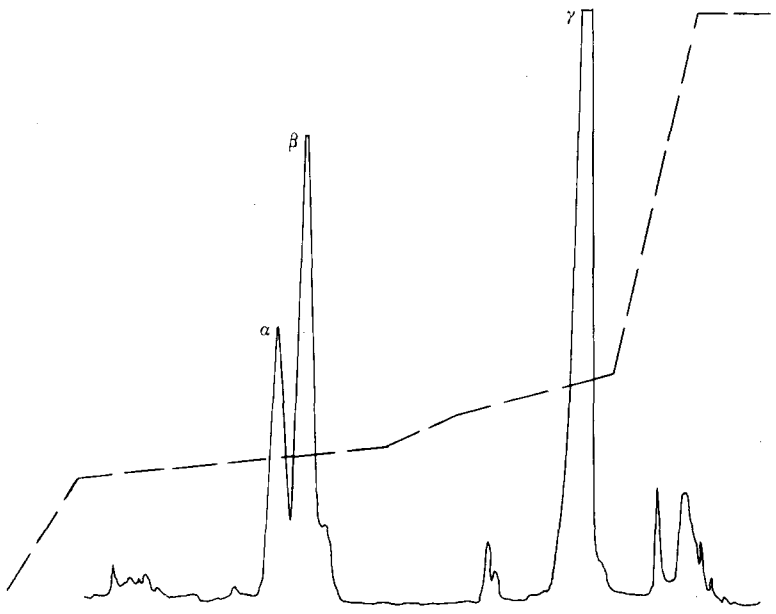

Fig. 3. Reversed phase chromatography of fraction $IIIa_2$. The proteins were dissolved in 0.1% TFA, loaded on a PEP-RPC HR 5/5 column, which was eluted with a gradient (---) of 0–100% acetonitrile in 0.1% TFA at a flow rate of 0.7 ml min^{-1}. Elution was monitored at 214 nm; peaks α and β did not absorb at 280 nm.

centrations ranging from 7.5 – 30 μg/ml. Fractions $IIIa_2\beta$ and $IIIa_2\gamma$ inhibited virus multiplication completely at 15 μg/ml (MIC). An incomplete inhibition of the CPE was observed with $IIIa_2\alpha$ at 30 μg/ml. Unfortunately $IIIa_2\alpha$ and $IIIa_2\beta$ appeared to be very unstable and more detailed studies on their mode of action could not be performed. In addition, any attempt to resolve the components of $IIIa_2\gamma$ by changing the conditions of the elution gradient or by employing various ion-exchange columns was unsuccessful.

TABLE 3

Amino acid composition of polypeptides $IIIa_2\alpha$ and $IIIa_2\beta$.

Amino acid	$IIIa_2\alpha$	$IIIa_2\beta$
	(nmoles/100 nmoles)	
Pro	43.6	43.0
Gly	7.0	5.9
Ile	6.1	6.3
Leu	7.0	8.4
Phe	5.6	6.6
Arg	26.0	27.2

Data refer to the highest yields obtained after hydrolyzing samples of the polypeptides for 22, 48 and 72 h. Aspartic acid, serine, glutamic acid, alanine and valine were present each at less than 1% of the total amino acids. Tryptophan was considered absent on the basis of lack of absorbance at 280 nm.

Rechromatographed samples of $IIIa_2\alpha$ and $IIIa_2\beta$ showed a very similar amino acid composition, and were very rich in the amino acid residues Pro and Arg. They also contained Gly, Ile, Leu, and Phe, with traces of other amino acids (Table 3).

$IIIa_2\beta$ had a slightly higher ratio of hydrophobic amino acids (Ile, Leu, Phe) to Arg with respect to $IIIa_2\alpha$. This is consistent with the slightly higher concentration of organic solvent required for the elution of the former peptide from the PEP-RPC column (Fig. 3) as well as with the lower relative mobility of $IIIa_2\beta$ with respect to $IIIa_2\alpha$ in acid/urea-PAGE (Fig. 2, c′ vs. b′).

Discussion

In the present study we describe the purification of antiviral peptides from an acid extract of granules of bovine neutrophils. The antiviral activity of the extract was recovered in two subfractions. IIa and IIIa, of which only the latter was used for further investigations.

The protein fraction IIIa exhibited anti-HSV_1 and anti-HSV_2 activities at concentrations which did not exhibit cytotoxicity or inhibitory effects upon the multiplication of a picornavirus (RV1B).

The overall results indicate that interferon is not involved in the antiviral activity of IIIa. Grounds for this conclusion derive from the observations that 1) fraction IIIa inactivated HSV_1 virions directly (see below) but was not active against an IFN-sensitive virus (RV1B) and 2) that $IIIa_2$, a subfraction of IIIa, did not protect MRC-5 cells against a challenge with VSV. Furthermore (data not shown) we found no loss of activity against HSV_1 when $IIIa_2$ was incubated with a rabbit anti-human αIFN (titer 1:300 000) even at a 1:10 dilution.

The subfraction $IIIa_2$ displayed an MIC of 19 μg/ml and a chemotherapeutic index (MNTC/MIC) of at least 13. All the major components of $IIIa_2$ were found to possess anti-HSV_1 activity. In particular $IIIa_2\beta$, a peptide of approximate MW 7,500, showed an MIC of 15 μg/ml, corresponding to a concentration of 2 μM. While the major, 27 000 MW component of $IIIa_2$ could not be resolved from some contaminating peptides, the two 7,500 MW components of this fraction, $IIIa_2\alpha$ and $IIIa_2\beta$, were purified to homogeneity. The criteria adopted for claiming homogeneity were: (a) elution of the peptides as sharp and distinct peaks after two passages through a reverse phase chromatography column, eluted with a shallow gradient of acetonitrile at a low flow rate, and (b) migration as single bands in high-resolution polyacrylamide gel electrophoresis even after overloading of the gels. Furthermore, association of low MW contaminants with the peptides $IIIa_2\alpha$ and $IIIa_2\beta$ could also be excluded because these peptides (a) were purified from a protein fraction derived from a gel permeation step and exhaustively dialyzed, and (b) were exposed to a solution of high ionic strength in the early steps of purification and to organic solvent during the final reverse phase chromatography.

Investigations under way in our laboratory have indicated that several cationic proteins of bovine neutrophil granules share an antigenic determinant, thereby being very likely generated by processing of a common precursor protein. It is thus

possible that the peptides $IIIa_2\alpha$ and $IIIa_2\beta$ are also derived from the major component of fraction $IIIa_2\gamma$ by proteolytic splitting.

The activity of $IIIa_2\alpha$ and $IIIa_2\beta$ decreased substantially even when they were kept at −20°C. Such a loss in activity may depend on either an unspecific absorption of the peptides to the tube walls or an instability of their conformation due to their unusual amino acid composition. We are presently studying storage conditions which would allow prevention of activity loss, particularly of $IIIa_2\beta$, and to pursue further antiviral studies.

The mode of action of the antiviral peptides contained in IIIa seems to involve a direct inactivation of the virus, which is greater at 37°C than at 21°C, and is time-dependent. Although the precise mechanism of the inactivation remains to be elucidated, the different sensitivity of HSV_1 and HSV_2 towards RV1B may suggest an interaction of the IIIa peptides with the envelope constituents of the virions. In this respect, HSV inhibition might result from a strong binding of granule peptides to some glycoprotein(s) at the viral surface, which would prevent virus attachment and/or penetration through specific receptors at the host cell surface [4,18]. In HSV_1 virions, the five major, antigenically different, glycoproteins gA, gB, gC, gD and gE may represent candidate target molecules for the virus-inactivating effect of the granule proteins [8,23,27]. Alternatively, the cationic antiviral peptides of the bovine neutrophil granules may bind to the lipid phase of the viral envelope. This type of interaction would be dependent on the fluidity of the envelope, thereby justifying the decrease in antiviral activity observed when the temperature is lowered from 37 to 21°C. Interaction with the lipid moiety of the envelope would also explain the greater activity of the more hydrophobic peptide, $IIIa_2\beta$, with respect to $IIIa_2\alpha$.

The antiviral peptides $IIIa_2\alpha$ and $IIIa_2\beta$ have an unusual high content in Pro, thereby resembling the proline-rich proteins (PRPs) coded by a multigene family in salivary glands and secreted into the saliva [1,10,17]. PRPs are characterized by a predominance of Pro residues (25–42%), present in up to heptaproline sequences [10], followed by Gly (16–22%) and Glu/Gln (15–28%) [1]. Basic PRPs also contain varying amounts of Arg plus Lys (7–9%) [17]. Among the potential biological roles of PRPs, it was suggested that they might protect oral tissues from bacterial infection [10]. It remains to be established whether neutrophil and salivary gland PRPs have some sequences and/or biological functions in common.

Natural defences against virus infections in the host are partly provided by antiviral proteins, which include well known molecules such as antibodies, interferons, and complement components. Some of the latter appear to directly neutralize enveloped viruses [15,33]. Furthermore, a protein with properties distinct from interferon and acting in the late stages of virus replication has been purified from serum (serum protective factor) [30].

Finally, peptides with an approximate MW of 3,800, capable of exerting both antibacterial and antiviral activity [12], have been purified from rabbit neutrophils [24]. These peptides, although rich in Arg residues, bear no resemblance in amino acid composition with $IIIa_2\alpha$ and $IIIa_2\beta$.

Both our results and those reported by Lehrer and co-workers [12] point to the

presence of peptides in the neutrophil granules that upon discharge in the extracellular fluid or into the phagocytic vacuoles could inactivate viruses. This process might result in either intravacuolar inactivation of endocytosed viruses or inhibition of viral spreading from infected to uninfected cells.

In this connection, it is interesting that from our data of protein recovery, 10^{11} bovine neutrophils should contain 5–13 mg of protein pertaining to fraction $IIIa_2$. Considering that 10^{11} neutrophils have a cellular volume of about 40 ml, of which less than one tenth is likely to be occupied by the granule population containing the antiviral polypeptides, their concentration in the granule would exceed 100- to 200-fold the MIC for anti-HSV_1 activity. Effective concentrations of the antiviral peptides could thus be reached after partial discharge of the granule content in either the phagocytic vacuole or the space surrounding the cell.

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