Neutrophil leukocyte collagenase, elastase and serum protease inhibitors in human gingival crevices

KJELL OHLSSON, INGE OLSSON &
GUNILLA TYNELIUS-BRATTHALL

Department of Clinical Chemistry and Experimental Surgery, Malmö General Hospital, Malmö and Department of Internal Medicine, Hospital of Lund, Lund; University of Lund, Sweden

Department of Periodontology, Faculty of Odontology, University of Gothenburg, Gothenburg, Sweden

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Extra-cellular neutrophil collagenase and elastase were demonstrated by immunochemical methods in crevicular material from clinically healthy and chronically inflamed human gingivae. Collagenolytic activity was shown by viscosimetry and elastolysis by its digestive action on elastin agarose plates. Also the serum protease inhibitors, α_1-antitrypsin and α_2-macroglobulin, were demonstrated in the crevicular material. The inflamed gingivae contained increased amounts of enzymes and inhibitors. The protease inhibiting capacity of α_1-antitrypsin was saturated in material from inflamed as well as from healthy gingivae, but free neutrophil collagenase and elastase were found mainly in crevices of the inflamed gingivae. It was suggested that free activities of these two proteolytic enzymes might contribute to collagen break-down in gingivitis and periodontitis.

Key-words: neutrophil; periodontal disease; collagenase; elastase

Gunilla Tynelius-Bratthall, Department of Periodontology, Faculty of Odontology, Fack, S-400 33 Göteborg 33, Sweden

The periodontal lesion is histopathologically characterized by a dense infiltrate of inflammatory cells and destruction of connective tissue substances (*Page, Davies & Allison*, 1972). Collagen is the main structural protein of gingival fibres and alveolar bone (*Fullmer et al.*, 1969; *Melcher & Eastoe*, 1969). Morphometric data have shown that infiltrated gingival connective tissue contains a significantly decreased amount of collagen (*Schroeder & Page*, 1972). The loss of collagen in periodontal disease has been ascribed to destruction of collagen (*Fullmer, Baer & Driscoll*, 1969; *Robertson & Grupe Jr.*, 1972) and/or reduced synthesis of collagen (*Stern*, 1967; *Schroeder & Page*, 1972).

In the infiltrated gingiva the distribution of polymorphonuclear (PMN) leukocytes differs from that of mononuclear inflammatory cells. Most of the cells in the gingival crevice and the superficial portions of the junctional epithelium have been characterized as neutrophil PMN-cells (*Attström*, 1970; *Schroeder*, 1970, 1973; *Schroeder et al.*, 1973a; *Schroeder*,

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Münzel-Pedrazzoli & Page, 1973). The basal portions of the junctional epithelium and the connective tissue, however, are infiltrated mainly by mononuclear leukocytes (*Schroeder*, 1970, 1973; *Schroeder et al.*, 1973 a and b). It has been suggested that lysosomal enzymes from neutrophils (for review see *Attström*, 1971; *Tynelius-Bratthall & Attström*, 1972) and mononuclear leukocytes (*Cowley*, 1972; *Page et al.*, 1972) may contribute to tissue break-down in gingivitis and periodontitis. Collagenase has been isolated and purified from lysosomes of human blood neutrophils (*Lazarus et al.*, 1968 a and b; *Lazarus et al.*, 1972; *Ohlsson & Olsson*, 1973a). Elastase has been separated from collagenase in granules of human neutrophils (*Janoff & Scherer*, 1968) and recently three different neutrophil elastases have been isolated (Ohlsson & Olsson, 1973b). Elastase is thought to be a critical mediator in degenerative processes affecting elastic fibres and arterial elastin (Janoff, 1972a). Neutrophil elastase may also contribute to destruction of collagen after its primary fragmentation (Janoff, 1972a).

α_1-antitrypsin and α_2-macroglobulin are known to inhibit granulocyte collagenase (*Ohlsson & Olsson*, 1973a) and elastase (*Ohlsson*, 1971b; *Janoff*, 1972b; *Ohlsson & Olsson*, 1973b). α_1-antitrypsin may also mediate an inhibition of neutrophil proteases, since sera deficient in α_1-antitrypsin also are deficient in the chemotactic factor inactivator (*Ward & Talamo*, 1973). As gingival fluid originates from blood (*Brill*, 1962) it might be possible to find proteolytic serum inhibitors in material from gingival crevices.

The present investigation was undertaken to find out

1) whether crevices of inflamed and healthy human gingivae contain
   a) neutrophil collagenase and elastase,
   b) α_1-antitrypsin and α_2-macroglobulin, and
   c) complexes between α_1-antitrypsin and neutrophil collagenase or elastase, and
2) whether the occurrence of these neutrophil enzymes is related to the clinical state of the gingiva.

MATERIAL AND METHODS

Gingival exudate, crevicular leukocytes and gingival crevicular material were sampled from two groups of individuals.

Group I consisted of 10 dental nurses, aged 20—30 years, with clinically healthy gingivae. During a preparatory period of two weeks their teeth were cleaned once every 4 days with rubber cups and pumice. In addition, they were instructed to practice adequate oral hygiene during the period of observation.

Group II consisted of 10 persons of both sexes, aged 30—60, with marked signs of progressive periodontal disease; e.i. severe gingivitis, pathologically deepened pockets and obvious alveolar bone loss.

Gingival exudate was measured in 3 buccal crevices in each jaw (one molar, one premolar and one incisor). The modified intracrevicular technique described by *Löe & Holm-Pedersen* (1965) was used. The exudate was sampled on filter paper strips (1.5×15 mm; Whatman chromatography paper, No. 4, W and B Balston Ltd., England). After a sampling period of 3 minutes the strips were stained with a 0.9% alcoholic solution of ninhydrin. The length of the stained area was measured to the nearest tenth of a millimetre.

Crevicular leukocytes were sampled

according to *Attström & Egelberg* (1971) from the same buccal crevices as the gingival exudate.

Styroflex® films (Norddeutsche Seekabelwerke, AG Nordenhamn, West Germany), 1.5×20 mm, were gently inserted into the gingival crevice as deeply as possible until slight resistance was felt, immediately removed, air-dried, and stained with May Grünwald-Giemsa. The cells were counted according to a method described by *Attström* (1970).

Gingival crevicular material was sampled from all buccal crevices present in each individual according to the technique elaborated by *Tynelius-Bratthall & Attström* (1972). Filter paper strips (Munktell 20 H, Sweden), 1.5×5 mm, were inserted into the gingival crevices and immediately removed. The strips were then placed on a strainer in a test tube and 3 ml 0.15 M sodium-acetate buffer, pH 6, was added. The tube was afterwards shaken in a whirlimixer (Fisons) for 30 seconds and centrifuged at 250 *g* for 10 minutes. Strips and strainer were removed and the supernatant was separated from the pellet. The supernatant was then concentrated to a volume of approximately 300 μl by ultrafiltration (Sartorius-Membranfilter GmbH pore size 8 μ, Sartorius, Göttingen, W. Germany) and used as test material.

This test material was studied in respect of the presence of neutrophil collagenase and elastase as well as collagenolytic, elastolytic and nonspecific proteolytic activity, the presence of α_1-antitrypsin and α_2-macroglobulin and the concentration of total protein.

Neutrophil collagenase and elastase were investigated by immunoelectrophoresis as described by *Johansson* (1972) and the single radial immunodiffusion technique of *Mancini et al.* (1965) using specific rabbit antisera against human neutrophil collagenase and elastase according to *Ohlsson & Olsson* (1973 a, b). The electrophoresis was performed on agarose (A-37 L'Industrie Biologique Francaise, Genevilliers, France) which had been purified on QAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) at 65°C according to *Hjertén & Johansson* (personal communication). Standards in the Mancini technique consisted of human neutrophil collagenase and elastase as purified by *Ohlsson & Olsson* (1973 a, b).

Collagenolytic activity was determined viscometrically with rat tail tendon collagen as a substrate (*Ohlsson & Olsson*, 1973 a). The reaction mixture consisted of 4.9 ml substrate-buffer solution (containing 2 mg collagen per ml) to which 100 μl test material was added. The buffer used was 0.05 M Tris-HCl, pH 8.5, containing 0.015 M $CaCl_2$ and 0.2 M NaCl. Incubation was performed at 24°C in an Ostwald viscosimeter (Jenar Glaswerk, Schott und Gen., Mainz, W. Germany) with a flow time of about 60 seconds for water at 25°C. Controls consisting of substrate-buffer solution and bovine trypsin (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.) in a final concentration of 0.01% were run to evaluate the effect of non-specific proteolytic activity on collagen.

Elastolysis was determined as the effect of digestive activity on elastin agarose plates (*Ohlsson & Olsson*, 1973 b). The method was modified to demonstrate small quantities (ng) of elastase by using soluble elastin, prepared according to *Keller & Mandl* (1971) as a substrate. The elastin agarose mixture consisted of 1% agarose and 0.01% elastin in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.002 M $CaCl_2$. Samples of 10 μl were analysed and the plates were incubated at 37°C for 15 hours. The standard con-

Table I. *Amount of gingival exudate and number of crevicular leukocytes from healthy and inflamed gingivae. Gingival exudate is expressed as the length (mm) of the stained area of the strip and crevicular leukocytes as the number of squares of the strip containing leukocytes*

gingivae	gingival exudate		crevicular leukocyte score	
	$\bar{x}$	S.E.M.$_k$	$\bar{x}$	S.E.M.$_k$
healthy	0.5	0.2	74	10
inflamed	2.0	0.3	343	58

sisted of human neutrophil elastase, as purified by *Ohlsson & Olsson* (1973b).

Non-specific proteolytic activity was demonstrated as digestion on fibrin agarose plates according to *Astrup & Müllertz* (1952).

α_1-antitrypsin and *α_2-macroglobulin* were studied by the electroimmuno assay of *Laurell* (1972) using specific rabbit antisera against human α_1-antitrypsin and α_2-macroglobulin prepared according to *Ohlsson* (1971a). α_1-antitrypsin was also investigated by crossed immunoelectrophoresis, as described by *Ganrot* (1972).

Protein concentration of the test material was determined according to *Lowry et al.* (1951) with human serum albumin (AB Kabi, Stockholm, Sweden) as a standard.

RESULTS

The amount of gingival exudate was found to be substantially larger in chronically inflamed than in clinically healthy gingivae (Table I). Also the number of crevicular leukocytes was found to be larger in inflamed, than in healthy gingivae (Table I).

Neutrophil collagenase and elastase were demonstrated in crevicular material from chronically inflamed and clinically healthy gingivae. The mean concentration (μg/ml) of each enzyme in samples from the inflamed gingivae was about 7 times that in healthy gingivae according to immunochemical determination (Table II). The immunoelectrophoresis showed the mobility patterns of free collagenase and elastase, mainly in material from inflamed gingivae (Fig. 1). The two enzymes in samples from both inflamed and healthy gingivae were also present in fractions with mobility towards the anode (Fig. 1). This corresponds to the mobility pattern of crevicular α_1-antitrypsin (Fig. 3). The anionic enzyme fractions were also precipitated with antiserum to α_1-antitrypsin (Fig. 1). These data indicate their identity with complexes between α_1-antitrypsin and collagenase and elastase, respectively.

Collagenolytic activity was demonstrated viscometrically in material from

Table II. *Neutrophil collagenase (μg/ml) and elastase (μg/ml) in crevicular material from clinically healthy and chronically inflamed gingivae, determined by the Mancini technique*

gingivae	collagenase		elastase	
	$\bar{x}$	R_k	$\bar{x}$	R_k
healthy	3.0	0.5— 4.8	4.1	0.6— 5.3
inflamed	20.5	10.0—25.0	27.0	15.5—40.0

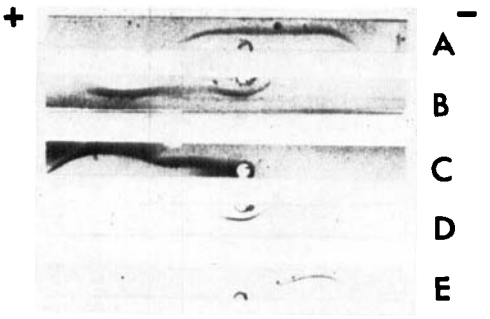

Fig. 1. Immunoelectrophoresis of crevicular material (A-C) from inflamed gingivae with rabbit antiserum against:
A. human neutrophil elastase
B. human neutrophil collagenase
C. human α_1-antitrypsin
The patterns produced by pure neutrophil collagenase (D) and elastase (E) with the corresponding antisera are given as references.

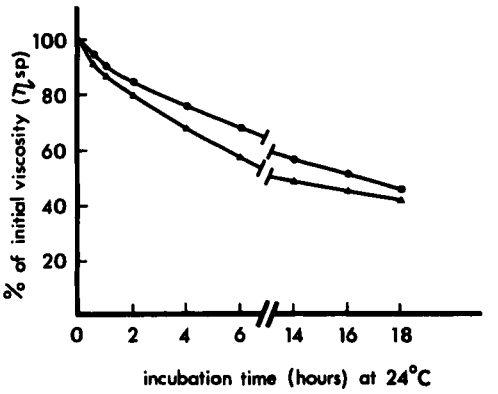

Fig. 2. Collagenolytic activity of crevicular material from two persons with inflamed gingivae. Enzyme activity = reduced viscosity of reaction mixture.

inflamed gingivae (Fig. 2). No activity was found in samples from healthy gingivae. The collagen substrate was not digested by nonspecific proteolytic enzymes as judged from the trypsin control.

Elastolytic activity, reflected by digestion on elastin agarose plates, was demonstrated in crevices of inflamed and healthy gingivae. The activity in inflamed gingivae was ten times that in healthy gingivae (Table III). Similar results were obtained for non-specific proteolytic activity (Table III).

α_1-antitrypsin and α_2-macroglobulin were demonstrated in crevicular material from chronically inflamed as well as clini-

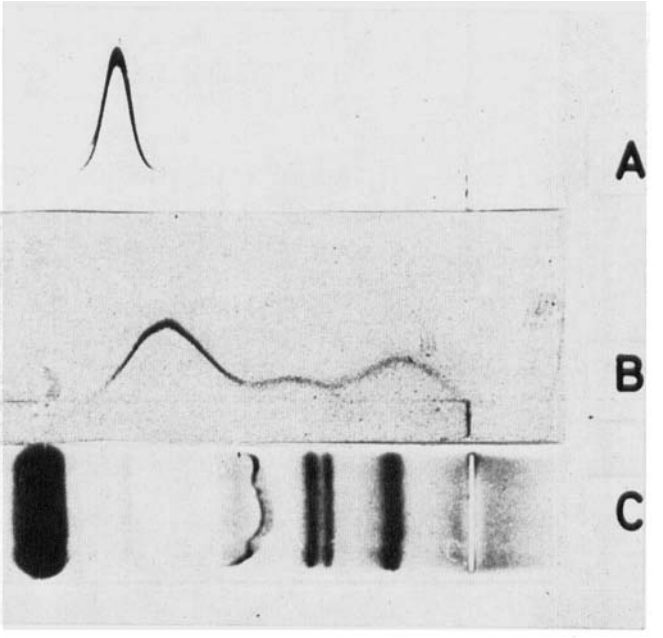

Fig. 3. Crossed immunoelectrophoresis with rabbit antiserum against human α_1-antitrypsin
A. normal human serum
B. crevicular material from inflamed gingivae
The agarose gel electrophoretic pattern of human serum (C) is given as a reference.

Table III. *Elastolytic and non-specific proteolytic (fibrinolytic) activity of crevicular material from clinically healthy and chronically inflamed gingivae. Elastase is expressed as μg/ml and protease as μg bovine trypsin (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.)/ml resulting in the same degree of fibrinolysis*

gingivae	elastase		protease	
	$\bar{x}$	R_k	$\bar{x}$	R_k
healthy	2.5	0— 3.5	1.0	0— 2.0
inflamed	22.0	10—32.5	9.5	7.0—20.0

cally healthy gingivae. The inflamed gingivae contained larger amounts of protease inhibitors (Table IV). Material from inflamed gingivae exhibited pronounced heterogeneity of α_1-antitrypsin, indicating saturation of the inhibitor (Fig. 3). The concentration of α_1-antitrypsin in material from healthy gingivae was too low to permit comparative studies with the method used.

The concentration of the total protein varied considerably within the two experimental groups, but the average protein content of material from inflamed gingivae was fourfold that from healthy gingivae (Table IV).

DISCUSSION

In the present experiments the cellular content of crevicular material from human gingivae was removed to enable analysis for extracellular enzyme activities. The analysis revealed the presence of extracellular collagenase and elastase activities in crevicular material. By using immunochemical techniques it was possible to identify collagenase and elastase originating from neutrophil polymorphonuclear leukocytes. Material from crevices of inflamed gingivae contained higher enzyme activities than that from crevices of healthy gingivae. This is in line with our finding of a larger number of crevicular neutrophils in inflamed compared to healthy gingivae (Table I). The occurrence of neutrophils also in crevices of healthy gingivae is in accord with the findings by *Attström* (1970), *Schroeder* (1970) and *Lindhe, Hamp & Löe* (1973). A relationship between the amount of neutrophils and endogenous protease in the gingival crevice has been reported earlier (*Tynelius-Bratthall & Attström*, 1972) and the neutrophilic origin of this enzyme has also been demonstrated by experimental reduction of crevicular neutrophils using anti-neutrophil serum (*Attström, Tynelius-Bratthall & Egelberg*, 1971).

Table IV. *α_1-antitrypsin, α_2-macroglobulin and total protein in crevicular material from clinically healthy and chronically inflamed gingivae. α_1-antitrypsin and α_2-macroglobulin are expressed in μg/ml and the total protein in μg human serum albumin/ml*

gingivae	α_1-antitrypsin		α_2-macroglobulin		total protein	
	$\bar{x}$	R_k	$\bar{x}$	R_k	$\bar{x}$	R_k
healthy	1.3	1.0—2.1	0.7	0.5—0.8	200	110—275
inflamed	6.8	2.5—9.5	5.4	2.8—6.1	750	350—950

The present investigation also demonstrated that gingival crevices contain the serum protease inhibitors α_1-antitrypsin and α_2-macroglobulin. In addition, it showed that, in the crevicular material, α_1-antitrypsin forms complexes with neutrophil collagenase and elastase. The protease-inhibiting capacity of α_1-antitrypsin was found to be saturated in material from both inflamed and healthy gingivae, but free neutrophil enzymes were present mainly in crevices of inflamed gingivae.

Earlier investigations have shown that α_1-antitrypsin and α_2-macroglobulin are capable of inhibiting most collagenases (*Eisen, Bloch & Sakai*, 1970) with the exception of neutrophil collagenase (*Lazarus et al.*, 1972) and synovial fluid enzyme (*Harris, Dibona & Krane*, 1969). *Ohlsson & Olsson* (1973a) have found, however, that these two serum proteins can also block neutrophil collagenase activity. *Paunio & Mäkinen* (1969) investigated collagenase in normal and diseased periodontal membranes apart from areas of inflammation and found a higher enzyme activity in the normal material. They suggested that inhibitory mechanisms connected with the inflammatory process might be present in the diseased material. *Robertson & Grupe Jr.* (1972) reported on the presence of tissue collagenase of healthy and slightly inflamed gingivae. No increase of collagenolytic activity was demonstrated in association with the development of *initial* gingivitis. It was therefore suggested that changes in the enzyme activity might be regulated by specific inhibitors in serum and tissue fluids.

As dental plaque bacteria and their products are the main etiologic factors in periodontal disease (for review see *Socransky*, 1970) the occurence of bacterial collagenases in gingival inflammation has been studied in a number of investigations. Collagenolytic activity has been demonstrated in microbial dental plaque and also in isolated crevicular bacteria in inflamed, but not in healthy, gingivae (*Schultz-Haudt, Bibby & Bruce*, 1954; *Schultz-Haudt & Scherp*, 1955). *Roth & Myers* (1956) reported that crevicular microorganisms were not able to degrade collagen, but *Mäkinen & Paunio* (1966) showed that insoluble and soluble collagen from human periodontal membrane was hydrolysed by plaque enzyme extract. *Mergenhagen, Scott & Scherp* (1960) suggested that bacterial collagenase might be responsible for a secondary attack on collagen in periodontitis, whereas the primary cleavage of collagen might be caused by endogenous substances originating from the inflammatory response.

The periodontal tissues also exhibit collagenolytic activity, demonstrated in inflamed (*Fullmer & Gibson*, 1966), but not in healthy gingivae (*Bennick & Hunt*, 1967). Collagenases have been identified in both epithelial cells and connective tissue of human gingivae (*Fullmer et al.*, 1969b) and in alveolar bones (*Fullmer, Lazarus & Lightbody*, 1968). *Beutner, Triftshauser & Hazen* (1966) found, however, that collagenase of inflamed gingivae was associated with inflammatory foci of the tissue and suggested that collagenase from inflammatory cells might be responsible for collagen break-down in periodontal disease.

The present study has clearly demonstrated the presence of free neutrophil collagenase and elastase in human gingival crevices. Though the *in vivo* action of these enzymes in gingival inflammation is still obscure there is evidence of digestive activity of neutrophil proteases *in vivo* (for review see Henson, 1972). As col-

lagen loss and inflammatory cell infiltration are the main features in gingival inflammation (*Schroeder et al.*, 1973a) the finding of neutrophil collagenase and elastase in the gingival crevice indicates that these enzymes may be involved in collagen destruction in periodontal disease. The occurrence and capacity of protease inhibitors in gingival crevices may also be of significance in the pathogenesis of gingivitis and periodontitis.

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