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A COMPARATIVE STUDY OF THE LYSOZYME ACTIVITY OF HUMAN GINGIVAL POCKET FLUID, SERUM, AND SALIVA

by

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Fleming's lysozyme (10, 11) is a basic protein of low molecular weight. It functions as a mucolytic enzyme by breaking down aminopolysaccharides in the cell walls of bacteria. This causes the release of cellular contents (29). Lysozyme is active against many species of bacteria; among these are the following common oral microorganisms: Neisseria, Micrococcus, Sarcina, Klebsiella, Streptococcus, Staphylococcus, and Mycobacterium (26). *Epstein & Chain* (9) found that the enzyme may kill bacteria without dissolution; however, the usual bactericidal effect is lytic. In either case, the enzyme affects the cell wall.

Tears, sputum, and nasal, gastric, and intestinal secretions exhibit strong lysozyme activity, whereas saliva and serum have relatively little activity (10, 38). The lysozyme activity of saliva varies considerably. Some authors (6, 12) have reported it to be about the same as that of blood; others (18) have found the activity to be eight times greater in saliva. These variations may be partly attributed to different handling of the samples. *Simmons* (35) reported that uncentrifuged saliva was 50 per cent more active than centrifuged saliva. He explained that the lytic agent

formed a complex with mucin and that the entire complex, not mucin alone, was precipitated and removed on centrifugation. Other variations in lysozyme activity of saliva may be due to the varying mucopolysaccharide content of the salivary fluid (17).

The highest lysozyme activities in mammalian tissues have been described (25, 27) for parts of the gastric and duodenal mucosa and for nonspecific granulation tissue. The values were from 20 to more than 500 times as high as serum values and occasionally reached those measured in tears. Few studies have dealt with lysozyme in gingival tissues. *Burnett, Gougé & Toye* (5) found that normal rat gingiva contained 17,662 μg lysozyme per gram, whereas rat kidney, spleen, and liver contained only 370, 163, and 40 μg per gram, respectively. They reported a very wide range of lysozyme concentrations (43 to 9,036 μg per gram) in 14 specimens of inflamed human gingiva. Apparently, the only values for healthy human gingiva have been reported by *Bernardini & Mingari* (2). According to these authors, the mean lysozyme concentration in normal gingiva was 2.7 μg per gram; in inflamed gingiva, 4.5 μg per gram. These measurements obviously differ from those of *Burnett et al.* (5), but the increase of lysozyme activity in inflamed tissues conforms with accepted opinion (6). Several workers (10, 16, 38) have stated that high concentrations of lysozyme are present in inflammatory areas and exudates. *Bartels & Buchbinder* (1) found that most specimens of exudate collected from root canals exhibited lysozyme activity; they concluded that this was an indication of an inflammatory reaction at the apex of the root.

From inflamed as well as from clinically healthy gingival pockets, there is a continuous outflow of fluid (3). This tissue fluid exerts a mechanical washing effect on bacteria and other small particles introduced into the pocket (3, 15, 41). Through its content of leukocytes (8, 21) and plasma proteins (4, 22), probably including antibodies, the pocket fluid may also attack local bacteria by phagocytosis and other immune mechanisms.

The present investigation was undertaken to furnish data on the occurrence of lysozyme in the pocket fluid from gingivae with greater or lesser inflammation, and to compare the lysozyme concentrations in three body fluids from the same subjects. It was expected that, if lysozyme is detected in gingival fluid, a com-

parison with saliva and serum levels may indicate its source. Correlation with the degree of inflammation may provide some insight into its function.

MATERIALS AND METHODS

Gingival pocket fluid, blood, and saliva were obtained from each of 12 apparently healthy subjects with various periodontal conditions. Ages ranged from 20 to 60 years.

Gingival fluid was collected by capillarity into 0.002 ml dis-

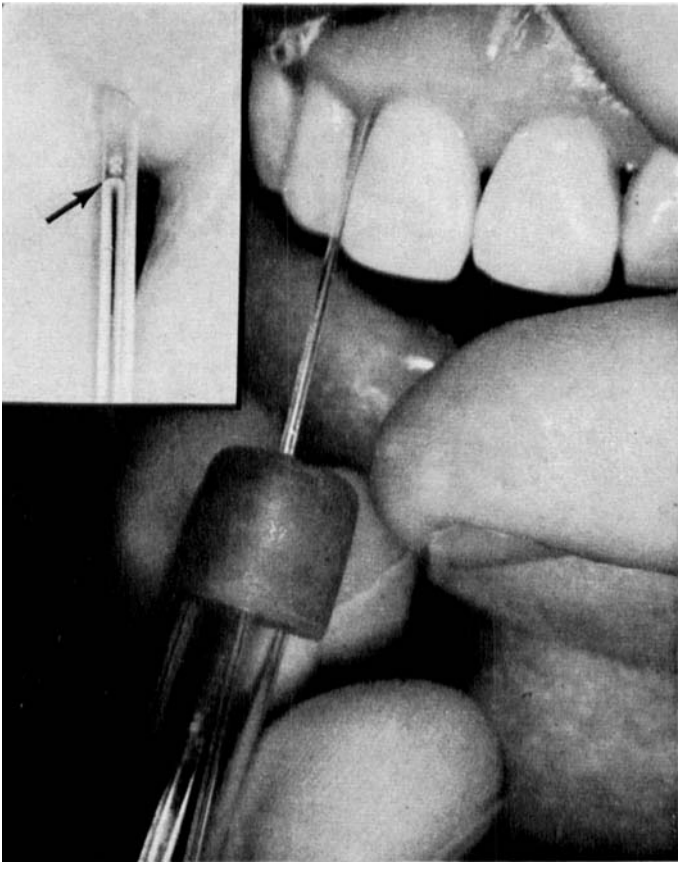

Fig. 1. Collection of gingival pocket fluid.

posable glass micropipettes[1]) (Fig. 1). After the gingiva was isolated and dried, the microdrops of fluid were transferred to non-wettable microcentrifuge tubes[2]) until a total of approximately 0.05 ml was accumulated. This required 45 to 90 minutes. Blood was obtained from the patient's fingertip and placed into a microcentrifuge tube. Whole saliva was expectorated into a test tube without preceding stimulation. All specimens were collected at room temperature.

Saliva was immediately refrigerated (4° C) and centrifuged in the cold for 10 minutes at 3,000 rcf. The lysozyme activity measurements for saliva were made on the day of collection since storage in some cases seemed to decrease the activity. Blood and gingival fluid were left at room temperature for one hour and then centrifuged for 4 minutes at 15,000 rpm in a microfuge.[2]) Occasional storage of these specimens for 24 hours at 4° C did not affect lysozyme measurements.

Lysozyme activity measurements were based on the change in turbidity of a cell suspension of killed and lyophilized *Micrococcus lysodeikticus*.[3]) Turbidity was estimated by a Beckman Spectrocolorimeter.[4]) The color of the gingival fluid and serum specimens varied through several shades of yellow and, in the presence of lysed erythrocytes, assumed a reddish hue. Therefore, a wavelength of 625 mμ was selected at which the specimen colors had the least influence on the light absorbance (Fig. 2). At this wavelength, the substrate turbidity was reduced, but remained large enough for measuring the changes induced by the enzyme. Also, when lysed erythrocytes were added to mixtures of substrate and serum or saliva, the initial optical density was only slightly increased and the net decrease in optical density, produced by lysozyme, was hardly affected.

The substrate concentration of 0.35 mg bacterial cells per ml of phosphate buffer (pH 7.0; ionic strength 0.148)[5]) gave an op-

[1]) Kensington Scientific Corp., 1717 Fifth St., Berkeley 10, Calif.

[2]) Spinco Division, Beckman Instruments, Inc., Stanford Industrial Park, Palo Alto, Calif.

[3]) Worthington Biochemical Corp., Freehold, N. J.

[4]) Model 151, Ultramicro Analytical System; Spinco Division, Beckman Instruments, Inc., Stanford Industrial Park, Palo Alto, Calif.

[5]) No. R-1784 P; Hellige, Inc., Garden City, N.Y.

tical density of 0.35 at the wavelength of 625 mμ; 0.10 ml of this suspension was transferred to the cuvette of the spectrophotometer. Graded solutions (1.56 to 75.00 μg/ml) of crystalline egg-white lysozyme[3]) were prepared in phosphate buffer[5]) pH 7.0; 0.02 ml of these solutions was added to the cuvette and the con-

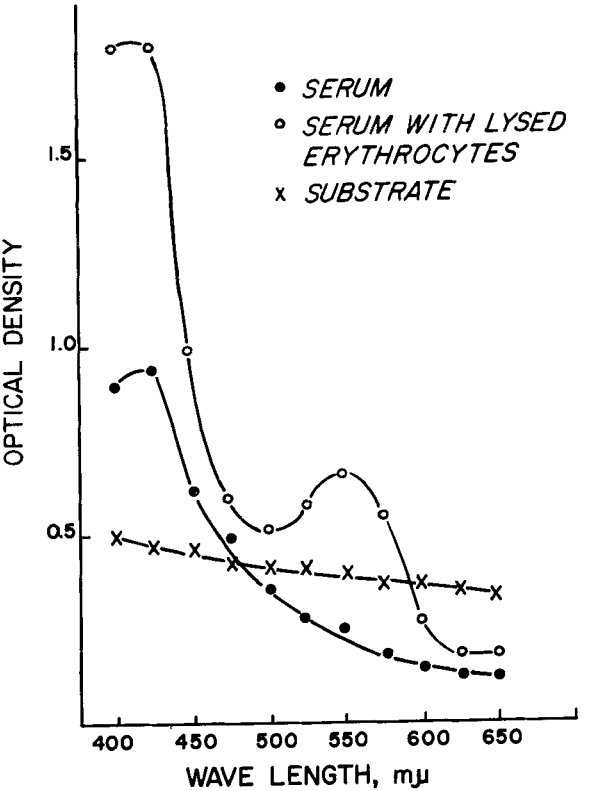

Fig. 2. Spectral absorbance curves for human serum, human serum with lysed erythrocytes, and the substrate (*M. lysodeikticus*, 0.35 mg/ml).

tents were rapidly mixed with a Pasteur pipette. The decrease in optical density during the first two minutes of reaction was recorded and a calibration curve was constructed from the average values of triplicate determinations (Fig. 3). This method was an adaptation of the macromethod of *Shugar* (34).

Gingival fluid, serum, and saliva were diluted 1:2 with the

same buffer and their lysozyme activities were measured in the way described for egg-white lysozyme. Each specimen was measured in triplicate. The average decrease in optical density was used to determine the lysozyme activity by means of the calibration curve. All measurements were performed after the spectrophotometer had been allowed to warm up and the fluids had reached room temperature.

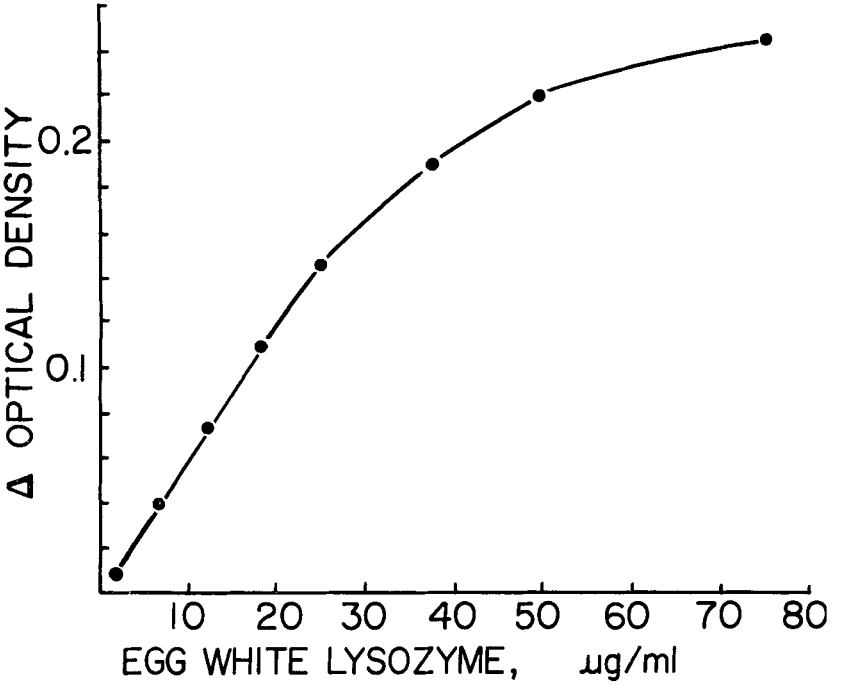

Fig. 3. Calibration curve for estimation of lysozyme concentration by decrease in optical density of substrate.

RESULTS

Lysozyme-like activity was measurable in all 12 gingival fluid specimens. Because of the difficulties in collecting the necessary volume of pocket fluid from subjects with clinically healthy gingivae, normal controls could not be measured. The subjects were, therefore, divided on the basis of severity of disease manifest at

the loci of collection into a periodontitis group and a gingivitis group. The gingivitis group served as a relative control group.

There was an apparent trend toward higher lysozyme activity in gingival fluid with increasing severity of periodontal inflammation and destruction. The average activity was approximately 50 per cent higher in the periodontitis group than in the gingivitis group (Table I).

Table I

Lysozyme Activities (μg/ml) of Gingival Fluid, Serum, and Saliva

Subject	Condition	Degree	Ging. Fluid	Serum	Saliva
1	Periodontitis	Severe	75	15	20
2		Severe	47	11	25
3		Mod-Sev.	47	10	5
4		Mod-Sev.	38	11	40
5		Moderate	34	20	36
Mean ± S.D.			48.2 ± 16.02	13.4 ± 4.15	25.2 ± 13.88
6	Gingivitis	Severe	36	11	13
7		Moderate	5	6	90
8		Mild-Mod.	12	11	67
9		Mild-Mod.	18	8	8
10		Mild-Mod.	16	13	28
11		Mild-Mod.	61	11	23
12		Mild	23	6	36
Mean ± S.D.			24.4 ± 18.79	9.4 ± 2.75	37.9 ± 30.00

The average lysozyme activity of serum was slightly higher in the periodontitis group, whereas the average activity of saliva was about 34 per cent higher in the gingivitis group. However, for both serum and saliva, the values overlapped greatly between the two groups (Table I). On repeated sampling on different days, lysozyme activities varied little in gingival fluid and serum of the same subjects, whereas saliva values fluctuated greatly.

The activity of the pocket fluid was higher than the serum activity in all but one subject. The comparison between gingival fluid and saliva values cannot be summarized as simply. Three of the subjects in the periodontitis group had much higher activities of gingival fluid than of saliva; the remaining two subjects had

similar values for the two fluids. In the gingivitis group, three subjects had gingival fluid activities higher than saliva activities, whereas in four cases the saliva values were higher. No correlation was observed among the activities of the three body fluids in either group.

DISCUSSION

Fluid from inflamed gingivae exhibited a bacteriolytic activity. The activity was measured by lysis of *Micrococcus lysodeikticus*. Suspensions of this test organism are cleared proportionally by egg-white lysozyme. It is customary to express the activities of analogously acting enzymes as if one had ascertained their identity with egg-white lysozyme.

Since the lysozyme activity of gingival fluid showed no quantitative relation to the activities of serum or saliva, the latter body fluids may be excluded as the principal sources of the lytic enzyme in gingival fluid. *Skarnes & Watson* (37) believed that antimicrobial tissue factors do not exist, as such, in normal tissues, but arise in response to physiological changes which accompany stress on the tissues. Gingival tissues are continuously subjected to bacterial influences. As a result, there is an inflammatory infiltration even in clinically healthy gingivae (42). It is assumed (8, 20, 23) that gingival pocket fluid is an exudate caused by an underlying inflammation. This concept may be supported by our finding of relatively high concentrations of lysozyme in the fluid, since inflammatory exudates in general are said to be rich in this enzyme (1, 10, 16, 38). The lysozyme activity of the gingival fluid is shown (Table I) to rise with the severity of inflammation, which, in turn, seems to intensify in response to increased bacterial irritation of the tissues.

It may be permissible to conclude that at least part of the lytic enzyme in the pocket fluid originates in leukocytes which infiltrate the gingivae and migrate out through the pocket epithelium (8, 21, 33). Lytic enzymes from two types of leukocytes have convincingly been shown to correspond to the definition of lysozyme. First, *Ralston, Baer & Elberg* (28) found that in rabbits oil-induced peritoneal exudates, which are very rich in monocytes, contained large amounts of material lytic for *M. lysodeikti-*

cus. Second, *Zeya & Spitznagel* (43) demonstrated, by means of zone electrophoresis, the presence of lysozyme in lysosomes of neutrophilic granulocytes. Furthermore, *Hiatt et al.* (16) and *Kerby* (19) reported that injured granulocytes released an active enzyme which behaved like lysozyme, and the former authors concluded that the high concentration of lysozyme in stools of patients with ulcerative colitis was derived primarily from invading granulocytes. *Egelberg* (8) recently observed that the number of disintegrated leukocytes was much higher in fluid from chronically inflamed gingivae than in fluid from clinically healthy gingival pockets. Thus, an increased number of leukocytes in the gingival tissues and in the pocket fluid may explain the trend toward higher lysozyme activity of gingival fluid with increasing severity of periodontal inflammation.

It appears (Table I) that there were three exceptions (in subjects no. 7, no. 11, and no. 12) to this trend. In subject no. 7, the lysozyme activity was surprisingly low. This subject's oral hygiene was much poorer than would usually correspond to a moderate degree of gingivitis. It is possible that the large bacterial masses in his gingival pockets or some acidic macromolecules (36) may have lowered the measurable lysozyme activity. The values for this subject were verified by a second sampling. In subjects no. 11 and no. 12, the lysozyme activity of the pocket fluid was higher than expected. Both these subjects had persistent inflammation of their interdental papillae although their oral hygiene was very good. These exceptions suggest that the association of high lysozyme activity of gingival fluid with periodontal disease may be complex. Inflammatory infiltration may explain the presence of lysozyme; the enzyme may, in turn, be harmful to the tissues and accelerate the inflammatory and destructive processes.

A detrimental effect of lysozyme on animal tissues has been investigated by *Meyer et al.* (25). They succeeded in producing ulcerations in the intestines of dogs by oral administration of egg-white lysozyme. The high concentrations of lysozyme in stools of patients with ulcerative colitis indicated to these workers that lysozyme may initiate the local lesions in this disease. However, *Hiatt et al.* (16) held that the concentration of lysozyme in the bowel merely reflected the extent of leukocytic infiltration and that the enzyme did not initiate the lesions. They admitted,

though, that the enzyme might contribute to the continuation of the inflammatory process.

Hoerman, Englander & Shklair (17) speculated that a high parotid lysozyme titer could play an important role in the etiology of ulcerative gingival disease. One may similarly conjecture that lysozyme in gingival fluid may exercise a histolytic activity on the lining of the gingival pocket and thus produce ulcers in the pocket epithelium. Furthermore, lysozyme may, by its mucolytic activity, reduce epithelial stickiness and thereby jeopardize the optimum contact between pocket epithelium and tooth surface. Epithelial detachment is thought to be the initial step in the pathogenesis of periodontal disease (32).

These hypotheses derive their justification from the probable occurrence of the lysozyme substrate in mammalian tissues as well as in bacterial cell walls. According to *Salton* (30), the molecular substrate in the cell wall of *M. lysodeikticus* is a polymer which is split at the bonds between N-acetyl-muramic acid and N-acetyl-glucosamine. Similar polymers are present in gingival tissues (31, 39, 40). However, it remains to be investigated whether lysozyme can affect these substances in mammalian tissues.

Lysozyme of gingival fluid may also exert adverse effects through its bacteriolytic activity. Although primarily lytic for gram-positive organisms, lysozyme can also lyse certain gram-negative bacteria (14, 37) under suitable conditions. Many gram-negative gingival bacteria contain endotoxins (24). These may conceivably be released in locally injurious quantities by the action of lysozyme in the gingival pocket. In a similar way, lysozyme of gingival fluid may accelerate the local release of endocellular bacterial enzymes. Hyaluronidase, chondroitin sulfatase, gelatinase (7), and collagenase (13) of gingival bacteria are mainly endocellular enzymes and are freed during lysis of bacterial cells. All these endocellular toxins and enzymes may play a role in the etiology or perpetuation of periodontal disease.

It is more common to attribute to lysozyme functions which are protective. The lytic effect on oral bacteria has been mentioned in the introduction. In addition, lysozyme also acts on bacteria by agglutination (29), thus preparing them for phagocytosis. Lysozyme in the pocket fluid may help to reduce the numbers of

gingival bacteria or their virulence. This may, to some extent, account for the commonly insidious and chronic, rather than acute, character of periodontal disease.

SUMMARY

The lysozyme activities of gingival pocket fluid, serum, and saliva of 12 systemically healthy individuals with various periodontal conditions were determined by a turbidimetric micromethod using *M. lysodeikticus* as a substrate. The average activity of the gingival fluids from seven subjects with gingivitis was 24.4 μg/ml; the average for five subjects with periodontitis was 48.2 μg/ml. The serum activities were 9.4 and 13.4 μg/ml for the two groups respectively; and the average values for saliva were 37.9 and 25.2 μg/ml. There was an apparent trend toward higher activity of gingival fluid with increased severity of periodontal inflammation and destruction. No such trend was noticeable for the two other body fluids. Lysozyme values in the three fluids were not correlated. Therefore, the enzyme in gingival fluid is assumed to be primarily of local origin, possibly derived from leukocytes.

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RÉSUMÉ

ÉTUDE COMPARATIVE DE L'ACTIVITÉ EN LYSOZYME DU LIQUIDE DU CUL-DE-SAC GINGIVO-DENTAIRE, DU SÉRUM ET DE LA SALIVE CHEZ L'HOMME

Les activités en lysozyme du liquide du cul-de-sac gingivo-dentaire, du sérum et de la salive de 12 sujets en bonne santé du point de vue de l'état général mais présentant diverses affections du parodonte ont été déterminées par une micro-méthode turbidimétrique utilisant M. lysodeikticus.

L'activité moyenne des liquides gingivaux de sept sujets présentant une gingivite était de 24,4 μg/ml; la moyenne pour cinq sujets présentant une parodontite était de 48,2 μg/ml. Les acti-

vités du sérum dans les deux groupes étaient respectivement de 9,4 et de 13,4 μg/ml; les valeurs moyennes pour la salive étaient de 37,9 et de 25,2 μg/ml. L'activité du liquide gingival tendait manifestement à augmenter avec l'aggravation de l'inflammation et de la destruction du parodonte. Les deux autres liquides ne présentaient pas de signes indiquant cette tendance. Les valeurs du lysozyme dans les trois liquides étaient sans corrélation entre elles. Il est donc à supposer que l'enzyme du liquide gingival est principalement d'origine locale, dérivant peut-être des leucocytes.

ZUSAMMENFASSUNG

VERGLEICHENDE UNTERSUCHUNGEN DER LYSOZYMAKTIVITÄTEN IN GINGIVALTASCHENFLÜSSIGKEIT, SERUM UND SALIVA BEIM MENSCHEN

Die Lysozymaktivitäten in Gingivaltaschenflüssigkeit, Serum und Saliva von 12 Individuen ohne Allgemeinkrankheiten wurden bestimmt durch eine turbidimetrische Mikromethode, wobei *M. lysodeikticus* als Substrat benutzt wurde. Die mittlere Aktivität der Gingivaltaschenflüssigkeit von sieben Individuen mit Gingivitiden betrug 24,4 μg/ml; der Mittelwert für fünf Personen mit Periodontitiden betrug 48,2 μg/ml. Die Serumwerte für dieselben zwei Gruppen waren bzw. 9,4 und 13,4 μg/ml, und die Mittelwerte für die Aktivitäten in Saliva betrugen 37,9 und 25,2 μg/ml. Eine scheinbare Tendenz gegen höhere Aktivität der Gingivaltaschenflüssigkeit mit zunehmender periodontalen Inflammation und Destruktion wurde festgestellt. Eine solche Tendenz war bei den anderen zwei Körperflüssigkeiten nicht vorhanden. Die Lysozymwerte in den drei untersuchten Flüssigkeiten waren nich korreliert. Deshalb muss man annehmen, dass das Enzym in Gingivaltaschenflüssigkeit vorwiegend lokaler Herkunft ist, möglicherweise von Leukozyten abgegeben.

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