Collagenase Activity of Gingival Tissue from Patients with Periodontal Diseases.* (30972)

ERNST H. BEUTNER, CLARK TRIFTSHAUSER AND STANLEY P. HAZEN
Department of Bacteriology and Immunology, Schools of Medicine and Dentistry, Department of Periodontolgy, School of Dentistry, State University of New York at Buffalo

The massive destruction of gingival collagen in periodontal disease has long suggested that some type of collagenase plays a role. Efforts to demonstrate such an enzyme in bacteria endogenous to the subgingival calculus have essentially failed. Some slight collagenolytic activity has been demonstrated in strains of *Bacterioides melaninogenicus*(1) and mixed populations of bacteria from calculus have been found to digest ethylene oxide degraded collagen(2). While these reactions cannot account for the observed dissolution of periodontal collagen they have suggested to some authors that an endogenous collagenase associated with the inflammatory responses may play a role in the tissue destruction(3). The existence of a tissue collagenase active against native collagen has been demonstrated by Gross and Lapiere(4,5). This report summarizes some of the salient observations relating to the endogenous or tissue collagenase activity. The object of these studies was to determine whether gingival tissue from patients with periodontal diseases contains endogenous collagenase. This was found to be the case.

Methods. The procedures for preparation of neutral salt soluble collagen and tests for collagenase activity on this substrate were modeled after procedures described by Gross and Lapiere(4,5), with some modifications. Details of the models will be described later (6). In essence, the procedures employed were as follows:

1) Collagen was extracted from minced, fresh guinea pig skin of young, actively growing animals with cold 1 M saline, the presence of bacteria eliminated by the addition of a crystal of thymol and either a) centrifuged and filtered through coarse medium and fine sintered glass filters and stored at 4°C until ready for use or b) precipitated with 5 M saline, redissolved in 0.15 M phosphate buffered saline pH 7.6, reprecipitated by dialysis against 0.01 M disodium phosphate and redissolved. After recycling this material 2 times, it was dissolved in 0.01 M acetic acid, dialyzed against 0.01 M acetic acid and lyophilized. For most of this work method a) was used because it was felt that this material would be less denatured.

2) For enzyme analysis the collagen was reconstituted to about a 0.15% solution (as determined by hydroxyproline assays), 1.5 ml were mixed with an equal volume of triple Eagle's medium or Tyrode's solution containing 100 I.U. penicillin and 100 mg streptomycin/ml and placed in sterile culture plates 35 × 10 mm. (Also, rings cut from plastic tubing 15 × 7 mm mounted on slides and covered with cover glasses were used for some experiments.) The collagen was precipitated to an opalescent gel by incubating at 37°C for 3 hours.

3) Tests were performed either with tissue specimens suspended in Hanks' balanced salt solution containing penicillin and streptomycin or with specimens quick frozen in liquid N_2 or on solid CO_2 and stored until ready for use. Either viable or frozen tissues were placed on the collagen gel, incubated at 37°C and read for lytic activity at 12, 24 and 48 hours. Under these conditions no bacterial growth was observed. Tests of tooth scrapings were performed on collagen gel without antibiotics.

Viable as well as frozen tissue specimens were obtained from tailfins of actively metamorphosing bullfrog tadpoles and from gingiva of Macacus ira monkeys immediately after sacrificing. Human gingival tissue of normal individuals and of patients with periodontal disease were obtained from surgical specimens. For studies of collagenase, tissues were either placed in balanced salt solution or quick frozen as indicated above. Quick frozen specimens were sectioned in a cryo-

* Supported by USPHS Grant DE-01801.

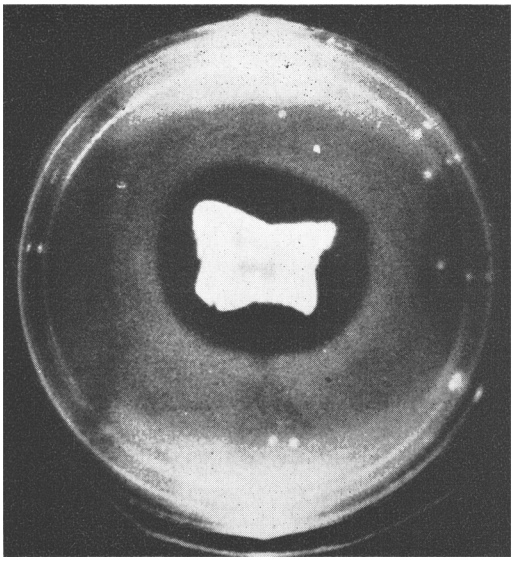

FIG. 1. Collagenolytic activity demonstrated by periodontially involved gingival tissue as demonstrated on reconstituted neutral salt soluble collagen gel.

stat at 4 to 6 μ for immunofluorescent studies.

Samples of tissue for histologic studies were fixed in formalin and processed by standard methods. Immunofluorescent staining was performed with fluorescein labeled rabbit antisera to DEAE fractionated gamma 2 globulin (IgG).

Results. Some but not all specimens of gingival tissue from patients suffering from periodontal disease when applied to collagen gel prepared from neutral salt soluble collagen produced a zone of lysis. A typical positive reaction is depicted in Fig. 1. Collagenolytic activity was manifested as a zone of liquefaction and clearing around the gingival tissue explant. Similar clearing could be demonstrated with non-viable frozen and thawed tissue specimens.

A series of studies was performed on the collagenolytic activity of tadpole tailfin tissue, monkey gingiva, normal human gingiva, gingival specimens of patients with periodontitis and gingivitis and tooth scrapings from patients with periodontal diseases as well as normal subjects. Tissues were placed on collagen substrate containing antibiotics while tooth scrapings were tested on collagen free of antibiotics. The results obtained are summarized in the following Table I.

A total of 15 tadpole tailfin explants tested in a series of 4 experiments yielded uniformly positive collagenase reactions. Conversely, none of 3 monkeys' gingival specimens and none of normal human gingival specimens from 9 surgical patients contained demonstrable enzyme activity as revealed by the standard collagen gel tests. The latter included tissue specimens tested both as viable tissue and as frozen and thawed samples. Thus, it appeared that the test system could detect collagenase but that normal gingival tissue did not contain detectable amounts of such enzyme. Gingival specimens of patients with periodontitis and gingivitis from a series of 41 patients, did, however, yield lytic reactions in 14 (or 34%) of the cases when tested as viable tissue explants. Similarly, 14 of 16 (or 30%) of frozen gingival specimens yielded such reactions. However, none of the scrapings of calculus on materia alba from patients or normal subjects yielded observable lytic reactions. Since the gingival tissue but not the scrapings from the adjacent teeth lysed collagen it would appear that either: 1) the tissue itself or 2) the tissue plus some microorganisms (or microbial products) are necessary to produce the observed lysis. The fact that the tissues were tested in the presence of penicillin and streptomycin and the scrapings were tested in the absence thereof, suggests that the former is more likely.

TABLE I. Collagenolytic Activity of Selected Tissue Specimens on Precipitated Collagen Gel.

Tissue tested	No. of cases	No. of positive	% positive
Tadpole tailfin tissue	4*	4	100
Monkey gingival "	3†	0	0
Normal human gingiva	9‡	0	0
Gingival tissue from periodontitis patients:			
1) Viable tissue	41	14	34
2) Frozen "	46	14	30
Scrapings from teeth of:			
1) Periodontitis patients	19	0	0
2) Normal subjects	10	0	0

* A total of 15 specimens tested in 4 experiments.

† Three or more specimens tested in each of 3 experiments.

‡ Includes cases whose tissues were retested after freezing and thawing.

TABLE II. Relation of Inflammatory Reactions and Gamma Globulin Localization in Gingival Tissue to Collagenolytic Reactions of the Tissue.

Case No.	Inflammatory foci*	IgG localization*	Collagen lysis
1	+	+	+‡
2†	+	+	+ and —
3†	+	+	+‡
4†	+	+ and —	+ and —
5†	+ and —	+ and —	+ and —
6	—	+	—
7	—	+	+ and —
8	—	—	+ and —
9	—	—	+ and —
10	—	—	—
11	—	—	—
12	—	—	—

* Hematoxylin and eosin strained tissue sections were examined for inflammatory foci. Frozen sections stained with a fluorescein labelled rabbit antiserum to human IgG (gamma 2 immunoglobulin) were examined for localization of human gamma globulin.

† In each of the first 5 cases, 2 or more specimens were examined by each of the 3 methods. Only single specimens were available for some of the studies in the remaining 9 cases. Case numbers have been assigned arbitrarily for convenience of presentation.

‡ Cases in which 3 of 4 specimens examined for collagen lysis were positive.

To examine these relationships in greater detail, gingival tissues of 13 cases were examined histologically for inflammatory reactions and by immunofluorescence for the localization of immunoglobulins as well as by the collagen gel method for lytic activity. Readings of the histologic preparations for the presence of inflammatory foci as well as readings of frozen sections for gamma globulin localization were made and recorded without knowledge of the collagenolytic activity of the tissue. Deposits of gamma globulin in the connective tissue (particularly subjacent to the epithelium) were rated as + or ++. For this summary all such reactions are recorded simply as +. The association between collagenase reactions on the one hand and inflammatory foci and immunoglobulin localization on the other hand in 12 cases subjected to detailed studies are summarized in the following Table II.

As indicated by the findings summarized in Table II, inflammatory foci and gamma globulin localization in gingival tissue from patients with periodontitis or gingivitis tended to be associated with the presence of collagenolytic activity in the inflamed tissue. Considerable variation in reactivities of different specimens from single patients. Thus, some sampling errors might be expected. Indeed, the 4 cases in which histologic, immunologic and enzymatic studies yield divergent results (cases 6, 7, 8 and 9) only single specimens were available for histologic and immunofluorescent studies.

Discussion. After completion of these studies another similar study came to the writers' attention(7). The available information suggests that this simultaneous and independent investigation yielded essentially comparable findings to those reported here with reference to the presence of collagenolytic activity in both viable and frozen and thawed gingival tissue from patients with periodontal disease as well as the association of this activity with the presence of inflammatory cells.

These findings suggest that a collagenase(s) emanating from inflammatory cells may be responsible for the massive destruction of collagen fibers in periodontitis. The histologic observations of destruction of collagen fibers in the vicinity of inflammatory cells is consistent with this observation. These findings are highly suggestive with the following reservations:

1) The relation between observed collagenase activity and *in vivo* destruction of collagen remains to be proven.

2) While the tissue collagenase may contribute to the breakdown of collagen, it may be, as suggested by others(3), that bacterial collagenases also play a role.

3) While addition of antibiotics to the collagen gel suppressed bacterial growth, the possibility of a bacterial collagenase being responsible for the observed lytic activity has not been eliminated.

If indeed, we assume that the breakdown of collagen in periodontal tissue is catalyzed by an endogenous collagenase emanating from inflammatory cells, we are faced with the question of what causes the formation of inflammatory foci in the periodontium. The writers believe the most probable explanation is that an allergic reaction to the antigens of the microbial flora of subgingival calculus

is responsible. Evidence supporting this view will be reported later.

Summary. Collagenase activity was demonstrated by lysis of precipitated collagen gel. Such collagenase activity was found to occur in both viable gingival explants (14 of 41 cases) and in frozen and thawed specimens (14 of 46 cases). This collagenase activity appeared to be associated with the presence of inflammatory foci and the accumulation of gamma globulin in the tissue specimens in most of the cases examined. Scrapings of teeth adjacent to excised gingivae failed to yield lytic activity on collagen gel. On the basis of these findings, it is concluded that the observed collagenolytic activity is produced either by: 1) the inflamed gingival tissue itself, 2) the gingival tissue in response to microbial or other exogenous stimuli or 3) tissue plus microbial collagenases.

1. MacDonald, J. B., Socransky, S. S., Gibbons, R. J., J. Dent. Res., 1963, v42, 529.

2. Schultz-Haudt, S. D., Scherp, H. W., Proc. Soc. Exp. Biol. and Med., 1965, v89, 697.

3. Mergenhagen, S. E., Scott, D. B., Scherp, H. W., ibid., 1959, v103, 227.

4. Lapiere, C. M., Gross, J., Mechanisms of hard tissue destruction, Publ. 75 of Am. Assn. for Advancement of Science, Wash., D. C., 1963, 663-694.

5. Triftshauser, C., Ph.D. Thesis, SUNY at Buffalo, Schools of Medicine and Dentistry, 1965.

6. Triftshauser, C., Beutner, E. H., Hazen, S. P., in preparation.

7. Bennick, A., Master's Thesis, School of Graduate Studies, Univ. of Toronto, 1965.

Received November 10, 1965. P.S.E.B.M., 1966, v121.

The Different Effects of Vinblastine Sulfate and Nitrogen Mustard Upon Neutrophil Kinetics in the Dog.* (30973)

D. R. BOGGS,† J. W. ATHENS, G. E. CARTWRIGHT AND M. M. WINTROBE
Department of Medicine, University of Utah College of Medicine, Salt Lake City, Utah

It is generally assumed that an antitumor drug induces neutropenia simply by interrupting production of new neutrophils by its destructive effect upon the mitotic pool of neutrophil precursors in the bone marrow. Neutropenia should develop when the initially undamaged post-mitotic maturation and storage pool of the bone marrow [marrow granulocyte reserve, MGR(1)] is exhausted.

This hypothesis proved sufficient to explain observations of the effect of vinblastine sulfate (VLB) upon the morphology(2) and kinetics(3) of canine neutrophils. Within one day of administering 0.2 mg/kg of VLB, more than 90% of morphologically identifiable, potentially mitotic, neutrophil precursors had disappeared from the bone marrow. No direct effect of VLB upon either blood neutrophils or post-mitotic marrow neutrophils of the MGR was apparent. However, since production was interrupted, the size of the MGR gradually decreased as few new cells were added to it and cells continued to feed out into the blood. The rate at which cells entered the blood from the MGR remained normal until the compartment was exhausted. Therefore blood neutrophil concentration remained normal until the MGR was exhausted and then declined abruptly. The abrupt decline usually occurred on the fourth day after VLB, which suggests that the MGR of the dog normally contains enough cells to supply the blood for slightly less than 4 days. Independent measures of the size of this compartment in the dog, utilizing either radioactive diisopropylfluorophosphate (DFP^{32})(3), tritiated thymidine(4), radioactive phosphorus(5), or radioactive sulfate(6) as isotopic labels for neutrophils are in agreement with this figure.

Certain reports(7,8) suggest that nitrogen mustard (HN2) might have a qualitatively different effect upon neutrophils from VLB. After HN2 administration to the dog, the

* This investigation was supported by a research grant AM-04489 from Nat. Inst. of Arthritis and Metab. Dis., Bethesda, Md.

† Faculty Research Associate of Am. Cancer Soc.