Title
The role of bacteria in tissue destructive enzyme production and latent human leukocyte collagenase activation

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THE ROLE OF BACTERIA IN TISSUE DESTRUCTIVE ENZYME PRODUCTION AND LATENT HUMAN LEUKOCYTE COLLAGENASE ACTIVATION

by

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THESIS

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INTRODUCTION

Collagen destruction has been measured in a variety of lesions such as corneal ulceration (1), rheumatoid arthritis (2), epidermolysis bullosa (3) and cholesteatoma (4). The mechanism of collagen degradation includes an initial specific collagenase action followed by further degradation by other proteinases. Connective tissue cells and leukocytes have been shown to produce specific collagenases and certain bacteria produce potent collagenases. In infected, necrotizing lesions, gross loss of tissue mass is observed but the mechanism of collagen destruction has not been investigated. In this work, the role of bacteria in tissue destructive enzyme production and latent human leukocyte collagenase activation will be studied.

Metchnikoff suggested in 1887 that polymorphonuclear neutrophils entering an acute inflammatory site are potentially injurious to tissues, in addition to serving a beneficial role of phagocytizing debris and invasive microorganisms (5). In recent years, many investigators have studied the role of polymorphonuclear neutrophils in the promotion of tissue damage ranging from minor derangements, such as local changes in vessel permeability, to frank destruction of tissue during acute inflammatory reactions (6-11). There are more than a dozen potentially tissue-damaging substances localized in the polymorphonuclear neutrophil, which can be grouped into two separate categories, non-enzymatic and enzymatic. Non-enzymatic substances include histamine, cationic proteins and slow-reacting substances associated with anaphylaxis, such as bradykinin and prostaglandins. These substances cause vasodilation, increased vascular permeability, bactericidal activities, degranulation of mast cells,

induction of fever, and stimulation of smooth muscle. The enzymatic components include proteolytic enzymes such as leukoprotease, collagenase, elastase, kininogenases, and plasminogen. They are responsible for attacking vascular basement membrane, degradation of collagen and elastin in tissues, generation of kinins and the digestion of fibrin. Although the individual characteristics of these components have been well studied, the interrelation of these substances in the mechanism of tissue damage has yet to be established.

The possible involvement of leukocyte enzymes in tissue damage has been suggested in the pathology of rheumatoid arthritis. This disease is characterized by a chronic and progressive inflammatory reaction of articular surfaces and by atrophy and rarefaction of bones and muscles. In the early stages of the disease there is a marked proliferation of the synovial lining and the stromal cells. Pathologic studies reveal hyperemia, edema and inflammation in the synovial and subsynovial layers. The first inflammatory cell infiltration, consisting of lymphoid cells, plasma cells, mononuclear neutrophils and many polymorphonuclear neutrophils is diffuse and evenly distributed (12,13). The granule fraction of human granulocytes contains an active collagenase and a collagenase precursor (14). Furthermore, collagenolytic activity has been detected in the synovial fluid of rheumatoid arthritis patients (15,16). Immune complexes of the IgG or IgM type against IgG have been found in synovial fluid and as cytoplasmic inclusions in leukocytes (17,18). Using human leukocytes prepared by dextran sedimentation of peripheral blood, Oronsky et al. investigated the phagocytic process in relation to the production of collagenase from these cells (14). Aggregated human gamma globulin was exposed to human leukocytes in culture. After phagocytosis

of the aggregate and a period of subsequent incubation, the whole cell lysate and the supernatant from the leukocytes were tested for collagenase activity. Neither showed much activity, but their collagenolytic ability was sharply increased after activation with trypsin or rheumatoid synovial fluids. Furthermore, Oronsky was able to establish that this proenzyme of collagenase came mainly from neutrophils. Only a small amount was present in lymphocytes. In view of these findings, it is possible that phagocytosis triggers the phagocytic cells to release their intracellular enzyme constituents which, when activated by various intrinsic factors, may destroy joint tissue.

It has also been proposed that granulocyte enzymes participate in tissue destruction in chronic periodontal disease. Periodontal tissue contains much fibrous protein and collagen. It is unclear, at present, whether the loss of collagen in periodontal disease is due to decreased production of collagen by fibroblasts or increased collagen degradation. However, there is substantial evidence that collagenase does participate in the destructive process (19). Collagenase has been found in the gingival crevicular fluid from healthy individuals as well as from individuals suffering from periodontal disease (20). However, it has been shown that collagenase activity in such gingival fluid increases with the severity of the periodontal disease (21). Oral flora, such as Bacteroides melaninogenicus and Clostridium histolyticum produce free collagenase which could easily account for loss of gingival collagen. Nevertheless, the mechanism appears to be more complex. Both the tissue and granulocytic sources of collagenase may be responsible for the gingival tissue destruction (22). During acute inflammation, leukocytes infiltrate the tissue site, phagocytize microorganisms as well as their

products and release lysosomal enzymes into the surrounding tissue.

When bacterial plaque accumulates at the junction between teeth and gingiva, a series of inflammatory reactions are initiated which often result in the gradual destruction of periodontal tissue. Since gingival tissue is largely made up of collagen, the role of leukocyte collagenase in periodontal disease was investigated. Uitto et al. studied collagenase activity in human leukocytes, gingival crevicular fluid and bacterial plaque (23). Gingival fluid from individuals with healthy gingivae and from those with periodontal disease was assayed for collagenolytic activity. This activity was approximately seven times as high in inflammed as in healthy gingival samples. Furthermore, the addition of plaque or trypsin to the gingival fluid increased its collagenolytic activity. The collagenase present in the gingival fluid of healthy individuals existed mostly in inactive form. In addition, Oronsky applied bacterial plaque to human leukocyte cultures and showed that plaque stimulated the lymphocytes to release collagenase and activate the latent enzyme.

Collagenase is an enzyme capable of degrading native collagen in a milieu found in vivo (24). In the human, collagenase has been isolated from various tissues and cells including skin, bone, synovium, synovial fluid, gingiva, cornea, stomach, embryonal skin, cholesteatoma, tumors, granulocytes, macrophages, fibroblasts, synovial cells and platelets (22). All of these collagenases are capable of cleaving native soluble collagen or reconstituted collagen fibrils at a single peptide bond along the helical portion of the collagen molecule when incubated at physiologic pH and ionic strength and at temperatures below the denaturation point of the reaction products (25). The collagen molecule consists of three polypetide chains, each of approximately 95,000 daltons molecular weight

and containing about 1,000 amino acids. Glycine is present in every third position of the amino acid sequence. Collagen can be differentiated into three types according to source. Type I collagen is derived from skin and bone; type II, from cartilage; and type III, from blood vessels and dermis. They differ in the components of their peptide chains (26). Collagenase initiates degradation of the collagen molecule which results in two peptides of uneven sizes. The large one accounts for approximately 75% of the collagen molecule, the smaller one 25%. Although other non-specific proteases are able to degrade collagen, they can only function after collagenase has initiated the process (27-29). More specifically, these non-specific proteases work on the clevage products of the collagen-collagenase reaction and further degrade the molecules into dialyzable sizes.

Necrotizing fasciitis in humans is characterized by the presence of edema and necrosis, often with partial liquefaction of subcutaneous fat and adjacent deep fascia. Progressive stages often involve cyanosis and gangrene of the skin as well as systemic toxicity with fever. Microscopic examination of debrided tissue shows intense polymorphonuclear cell infiltrate, focal necrosis and microabscesses in the fascia and subcutaneous tissue (30,31). Giuliano, Lewis, Hadley and Blaisdell have recently reported the bacteriologic etiology of necrotizing fasciitis (30). They showed that in some cases, group A Streptococcus was the only bacterial species isolated, while in other cases, a mixture of various streptococci, Enterobacteriaceae, Pseudomonas aeruginosa and anaerobic bacteria were isolated. These results confirm the findings of Melenley in 1924 that a streptococcus was invariably found in such lesions (31).

Collagenase activity in homogenates of polymorphonuclear leukocytes

was first reported by Lazarus et al. in 1968 (28). Only a small amount of activity was found with a relatively large amount of cell protein. Later, it was discovered that the amount of procollagenase in the leukocytes is about three times greater than that of free collagenase (14,32,33). It has been postulated that the zymogen of collagenase is bound to an inhibitor of the enzyme and is contained in the azurophilic granules of the polymorphonuclear leukocytes (34). Thus, the proenzyme can be activated by a substance that would either displace, destroy or cleave the inhibitor from the zymogen.

The role of bacterial products in collagenolysis and procollagenase activation is the subject of these studies. In particular, Streptococcus pyogenes (Group A Streptococcus) and its products have been studied for these activities since it is the sole bacterial isolate in some collagen destructive lesions.

METHODS

Labeling of collagen:

Acetylation of collagen was done according to the method of Gisslow and McBridge (35). Purified calf skin collagen was purchased from Sigma (catalogue #C3511) and ^{3}H acetic anhydrid from Amersham (catalogue #TRA. 381). The lyophilized acid-soluble collagen was dissolved in 0.01% acetic acid by stirring at 4°C overnight. The concentration of collagen was 2 mg/ml of distilled water and 250 mg of collagen was used for each labeling experiment. Immediately prior to addition of the label, the pH of the collagen solution was brought to 8.9 by the addition of 1 M K_2HPO_4. The acetylating agent, ^{3}H acetic anhydride (5 mCi in 1.5 ml of benezene), was added dropwise over a period of two hours. The reaction was carried out on ice and the pH was maintained at 8.0 with the addition of 1N NaOH using a pH meter. The reaction mixture was left to stir for one hour on ice after all the ^{3}H acetic anhydride had been added. The pH of the mixture was then adjusted to 4.0 with glacial acetic acid and the benzene removed. The acetylated collagen was dialyzed at 4°C against distilled water with many changes to remove label that was not incorporated and ^{3}H acetic acid which formed as a by-product of the reaction. Dialysis was continued until no further ^{3}H was detected in the dialysate. The final product was lyophylized and stored at -20°C. The product had 0.5 uCi/mg collagen.

Preparation of leukocyte enzyme:

The leukocyte enzyme was prepared according to Uitto's method (23), with a few modifications. White cells were obtained from fresh whole

blood taken from normal adult donors or purchased from Irwin Memorial Blood Bank as the by-product of preparation of leukocyte-poor blood units. The blood was haparinized (20 units/ml of blood) and mixed with 6% dextran solution at ratio of 5:1 by volume. Blood units from Irwin Memorial Blood Bank contained 15% by volume of citrate-phosphate-dextrose solution (1.64 gm dextrose, 206 gm sodium citrate and 140 mg sodium biphosphate/ 100 ml water). After allowing the red cells to settle at room temperature for one hour, the leukocyte-rich supernatant was decanted and centrifuged at 1000 rmp in a Sorval-2 centrifuge at 4°C. The white cell pellet was resuspended in saline at 4°C and washed several times. Then, a white cell count was done and the leukocytes were sonicated in an ice bath. This whole cell lysate was centrifuged at 25,000 g in an ultracentrifuge for 30 minutes. The volume of the resultant supernatant was adjusted with Tyrode's solution to give a preparation derived from 1×10^8 cells per ml. The extract was made from leukocytes within 24 hours after blood collection. This crude enzyme was frozen and stored at -20°C. Since this preparation consisted predominantly of polymorphonuclear neutrophiles (PMN) (28), the extract is referred to as PMN extract in this thesis.

Collagenase Assay (Trichloroacetic acid precipitation method):

Assay for collagenase was done according to Z. Werb's method (36). ^3H or ^{14}C labeled collagen in solution with a minimal activity of 6000 cpm per 200 ul aliquot was used as substrate. 100 ul of leukocyte sonicate and 200 ul of collagen substrate as well as 300 ul of assay buffer were added to a 1.5 ml microfuge tube. The mixture was incubated at 37°C for 12-15 hours. At the end of the incubation period, 50 ul of a 0.5% bovine serum albumin in 0.115 M NaCl was added to the reaction mixture and was held for 30 minutes at 4°C until complete precipitation

occurred. The mixture was centrifuged at 9,000 g for 2 minutes in a Beckman Microfuge B. 200 ul aliquot portion of the supernatant was added to 10 ml Aquasol for counting. Counting was performed by a Packard 3330 counter at 55% counting efficiency.

For activation experiments, 100 ul of leukocyte sonicate was mixed with 100 ul of a possible activator and the mixture was incubated at 37°C for 15 minutes before the addition of 200 ul of collagen substrate and 200 ul of assay buffer. The rest of the procedure was carried out as mentioned above. A negative control of collagen alone was set up to monitor self degradation. A trypsin control (.01 mg/ml) was included to determine the non-specific collagenolytic activity of a proteinase other than collagenase. Various bacterial culture supernatants were tested for their activation ability. They were prepared by ultracentrifuging (25,000g) 24-hour cultures of the organisms in Tood-Hewitt Broth.

Collagenase Assay [dioxane precipitation method (37)]:

The reaction mixture as well as the subsequent incubation was set up the same way as the trichloroacetic acid-tannic acid precipitation method. At the end of the incubation period, 0.6 ml of dioxane was added to the reaction mixture instead of trichloroacetic acid-tannic acid mixture, and the resultant mixture was centrifuged at 9,000g for 5 minutes in the Beckman Microfuge B. 200 ul aliquot portion of the supernatant was added to 10 ml aquasol (New England Nuclear Corp., liquid scintillation counting mixture, NEF-934) for counting.

Screening of bacterial isolates for collagenase production:

Screening of bacterial isolates for collagenase production was done according to Levenson's collagenase film microassay method (39). A

0.05 ml aliquot of an 18 hour culture growing in brain heart infusion (BHI - Difco B37A, Difco laboratories, Detroit, Michigan) broth was placed as a spot on a collagen coated glass slide. A Clostridium histolyticum culture was used as a positive control. The slides were incubated at 37°C in 100% humidity for 18 hours. At the end of the incubation period, the slides were washed gently with distilled water and subsequently stained by a few drops of 2% coomassie blue (in 10% acetic acid). The dye was washed off after 15 seconds. Collagenolysis was indicated by clearing of the collagen layer where the sample was spotted on the slide.

Screening of bacterial isolates for hyaluronidase production:

Screening for hyaluronidase production was done according to Ibrahim and Streitfeld's method (40). Isolates were grown in 5 ml of veal infusion broth (Difco) for 18 hours. The culture suspension was subsequently spun down and 0.1 ml of the supernatant was mixed with 1.1 ml of hyaluronic acid at 800 ug per ml in a test tube. The mixture was incubated at 37°C for 30 minutes followed by chilling in an ice bath for 15 minutes. 1 ml of the reaction mixture was then drawn into a capillary tube followed by an equal volume of acidified bovine serum albumin (10% BSA in 0.85% saline acidified with glacial acetic acid to .57N). The turbidity was scored. Absence of turbidity indicated that the hyaluronic acid was degraded, and thus constituted a positive reaction.

Screening of bacterial isolates for elastase production:

Screening for elastase production was done according to Bieth et al.'s method (41), using Brain Heart Infusion agar (BHIA) containing 0.3% elastin. Isolates were stabbed into this agar and incubated at 37°C. Plates were examined for 7 days for clearing around the original stabs

RESULTS

Patients were identified as having necrotizing faciitis by presence of general signs of fever (100° or greater) and marked toxicity, and the anatomic evidence of necrosis involving fascia, subcutaneous tissues and skin (to some degree) but not having major muscle necrosis.

Portions of the skin, subcutaneous adipose tissue and fascia were obtained at the time of therapeutic, radical, surgical debridement of this tissue from the patient. 3cm cubes of tissue were obtained from the central portion of the necrotic lesion and from the surgical margin. When the surgical margin of non-necrotic well vascularized tissue was narrow, smaller cubes were obtained from this location.

Tissue was obtained from the operating room as soon as the debridement was completed and refrigerated at 4°C or frozen -70°C within 15 minutes. The tissue blocks were homogenized by grinding with a mortar and pestle. Aliquots were taken from enzyme testing and culture. Most of the specimens were refrigerated and tested within 6 hours after removal from the patient. Two specimens (patients JB and DG) were frozen at -70°C for up to 4 weeks before testing.

Table I: Collagenase Test on Tissues

0.1 ml of tissue homogenate or culture supernatant was spotted on the collagen layer.

PATIENT	LAB NUMBER	COLLAGENASE ASSAY	
		CENTRAL	MARGIN
DG	15804 (78)	±	-
JW	28468 (78)	+	-
RT	1150 (79)	++	-
JB	3406 (79)	++	±
WH	7411 (79)	+	±

Extent of Reaction in Collagen Layer on Slide

\+ 5 mm or less

++ Greater than 5 mm

± Incomplete clearing

\- No clearing

Several viridans streptococci isolated from cases of necrotizing faciitis along with stock cultures of various microaerophillic viridans streptococci were tested for collagenase activity by Levenson's film micro assay method (39), hyaluronidase activity by Ibrahhim and Streitfeld's capillary tube method (40), and elastase activity by Birth's plate method (41). The results are summarized in table II.

Table II: Screening of Viridans Streptococcal Species for Collagenase, Hyaluronidase and Elastase activities

Streptococcal Species	No. of Strains Tested	No. of Collagenase Positive Strains	No. of Hyaluronidase Positive Strains	No. of Elastase Positive Strains
Laboratory Strains:				
S. mutans	6	0	0	0
S. constellatus	15	0	11	0
S. mitis	10	0	1	0
S. intermedius	33	0	11	0
S. sanguis I	7	0	0	0
S. sanguis II	16	0	3	0
S. parvulus	2	0	1	0
S. micros	3	0	1	0
S. morbillorum	2	0	0	0
S. salivarius	2	0	0	0
Clinical Isolates from Necrotizing Fasciitis:				
S. intermedius	3	0	2	0
S. sanguis I	1	0	0	0
S. sanguis II	1	0	0	0
Positive Control:				
Cl. histolyticum	1	1	0	0

None of the viridans streptococcal strains isolated from cases of necrotizing faciitis showed collagenase or elastase activity while 2 out of 3 Streptococcus intermedius strains isolated from such lesions showed positive hyaluronidase activity.

No collagenase or elastase activity was found in any of the 96 stock cultures of viridans streptococci. With the exception of S. mutans, S. morbillorum and S. salivarius, hyaluronidase activity was detected in representatives of each strain of viridans streptococci tested.

Various organisms isolated from cases of necrotizing faciitis, burn and other traumatic wounds were tested for their ability to produce collagenase as well as activate latent human leukocyte collagenase. The supernatants following ultra centrifugation of broth cultures of these organisms were used as the source of their extracellular enzymes. The collagenase assay using tritiated calf skin collagen as substrate was used in these experiments. The results are summarized in the following table IIIA and figure I. Statistical analyses are summarized in table IIIB. Detail calculations are shown in appendix I.

Table IIIA: Test of Wound, Burn and Necrotizing Fasciitis Isolates for their Abilities to Produce Collagenase and Activate Latent Leukocyte Collagenase

	Mean CPM	Standard Deviation	Percentage Degradation
Total collagen	10753	106	0
Collagen control	3498	27	32.5
Broth control	3481	22	60.9
PMN ext. alone	6548	47	60.9
PMN ext. + broth	6343	99	58.9
E. coli + PMN ext.	6227	29	57.9
E. coli alone	3435	14	31.9
Enterococci + PMN ext.	6660	31	61.9
Enterococci alone	3412	15	31.7
S. epidermidis + PMN ext.	6162	55	57.3
S. epidermidis alone	3436	12	31.8
S. sanguis (286)* + PMN ext.	6344	41	58.9
S. sanguis (286)* alone	3375	20	31.3
S. aureus + PMN ext.	9405	23	87.5
S. aureus alone	3513	16	32.7

	Mean CPM	Standard Deviation	Percentage Degradation
S. intermedius (7)* + PMN ext.	9031	15	84.0
S. intermedius (7)* alone	4332	19	40.2
S. pyogenes (256)* + PMN ext.	6570	35	61.1
S. pyogenes (256)* alone	3523	29	32.7
S. pyogenes (298)* + PMN ext.	9738	38	90.6
S. pyogenes (298)* alone	4051	17	37.6
S. pyogenes (30370)* + PMN ext.	8902	30	82.8
S. pyogenes (30370)* alone	3526	11	32.7
S. pyogenes (291)* + PMN ext.	8511	13	79.2
P. aeruginosa (290)* + PMN ext.	9245	34	85.9
P. aeruginosa (290)* alone	9258	23	86.1
P. aeruginosa (D)* + PMN ext.	10430	50	96.9
P. aeruginosa (D)* alone	9594	55	89.2
P. aeruginosa (B)* + PMN ext.	9596	74	89.2
P. aeruginosa (B)* alone	9325	19	86.7
C. histolyticum + PMN ext.	10216	7	95.0
C. histolyticum alone	9295	10	86.4

	Mean CPM	Standard Deviation	Percentage Degradation
Trypsin + PMN ext.	20793	12	100
Trypsin alone	4234	8	39.3

*strain of the particular bacterial species

Table IIIB: Summary of Statistical Analysis on Activation of Latent Leukocyte Collagenase by Bacterial Products

	Significant Difference from PMN ext. alone? ($p < .01$)
E. coli + PMN ext.	YES
Enterococci + PMN ext.	NO
S. epidermidis + PMN ext.	YES
S. sanguis + PMN ext.	YES
S. aureus + PMN ext.	YES
S. intermedius + PMN ext.	YES
S. pyogenes (256) + PMN ext.	NO
S. pyogenes (298) + PMN ext.	YES
S. pyogenes (30370) + PMN ext.	YES
S. pyogenes (291) + PMN ext.	YES
P. aeruginosa (290) + PMN ext.	YES
P. aeruginosa (D) + PMN ext.	YES
P. aeruginosa (B) + PMN ext.	YES
C. histolyticum + PMN ext.	YES
Trypsin + PMN ext.	YES

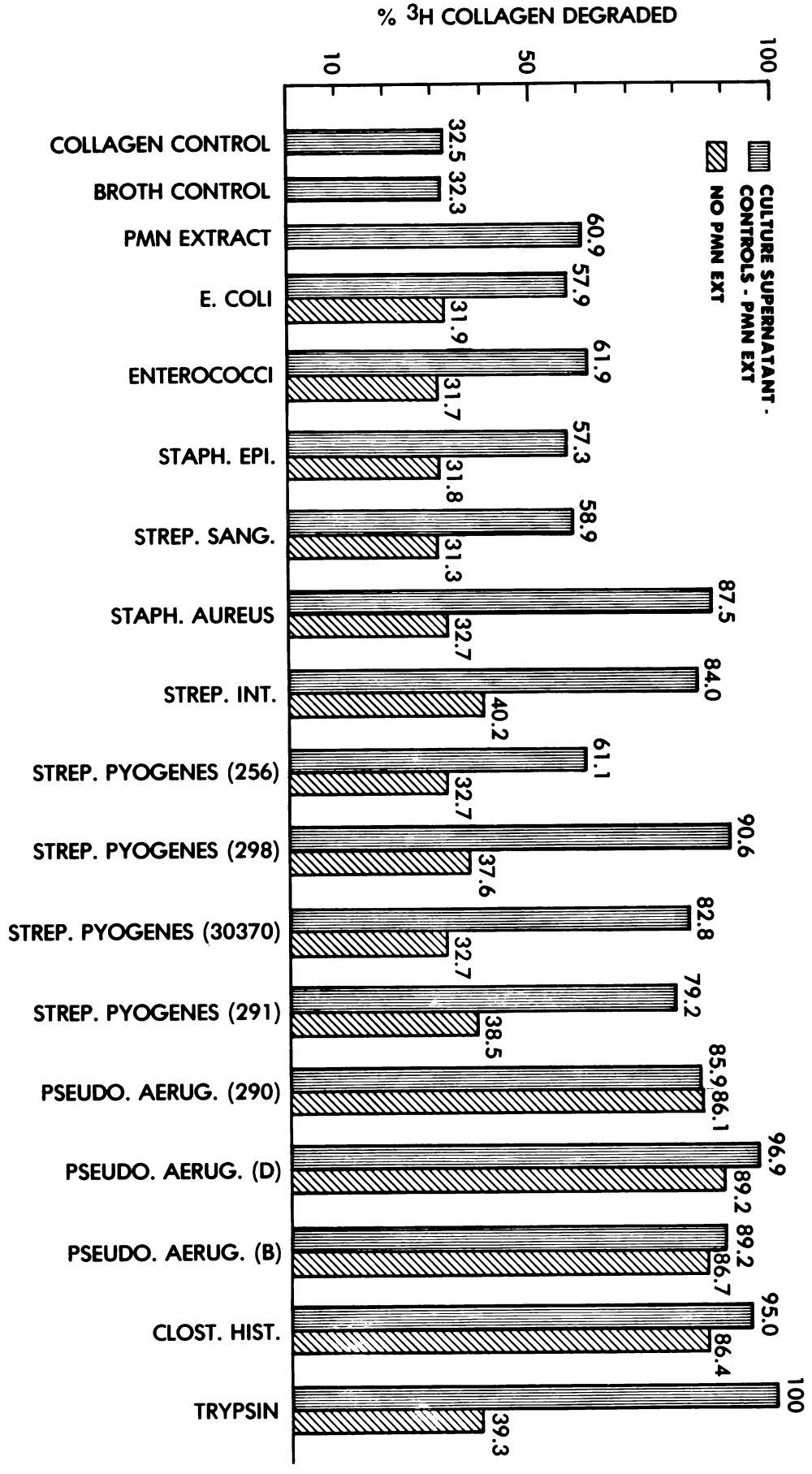

Figure I: ACTIVATION OF LATENT LEUKOCYTE COLLAGENASE BY SOLUBLE PRODUCTS FROM VARIOUS MICROORGANISMS

Various streptococcal enzymes were tested for their ability to activate latent leukocyte collagenase. Enzymes (1 mg/ml) tested include streptolysin O (Beckmen 252527), deoxyribonuclease B (Beckmen 252527), hyaluronidase (Difco 093533) and streptokinase (Sigma SO255). Werb's collagenase assay, using tritiated calf skin collagen as substrate and dioxane precipitation was used in these experiments. The results are summarized in table IVA and figure II. Statistical analysis is summarized in table IVB. Detail calculcations are shown in appendix II.

Table IVA: Ability of Streptococcal Enzymes to Activate Latent Leukocyte Collagenase - Using ^{3}H Collagen as Substrate and Dioxane Precipitation Method.

	Mean CPM	Standard Deviation	Percentage Degradation
Total collagen	11053	69	0
Collagen control	4333	116	39.2
PMN extract	5920	83	53.6
Streptolysin O + PMN ext.	5032	95	45.5
Streptolysin O alone	3447	30	31.2
DNAase B + PMN ext.	6236	87	56.4
DNAase B alone	5888	87	53.2
Hyaluronidase + PMN ext.	6190	131	56
Hyaluronidase alone	5134	85	46.5
Streptokinase + PMN ext.	10539	151	95.4
Streptokinase alone	4339	109	39.3

Table IVB: Summary of Statistical Analysis on the Ability of Streptococcal Enzymes to Activate Latent Leukocyte Collagenase - Using ^{3}H Collagen as Substrate and Dioxane Precipitation Method

	Significant Difference from PMN Ext. Alone? ($P < .01$)
Stretolysin O + PMN ext.	YES
DNAase B + PMN ext.	NO
Hyaluronidase + PMN ext.	YES
Streptokinase + PMN ext.	YES

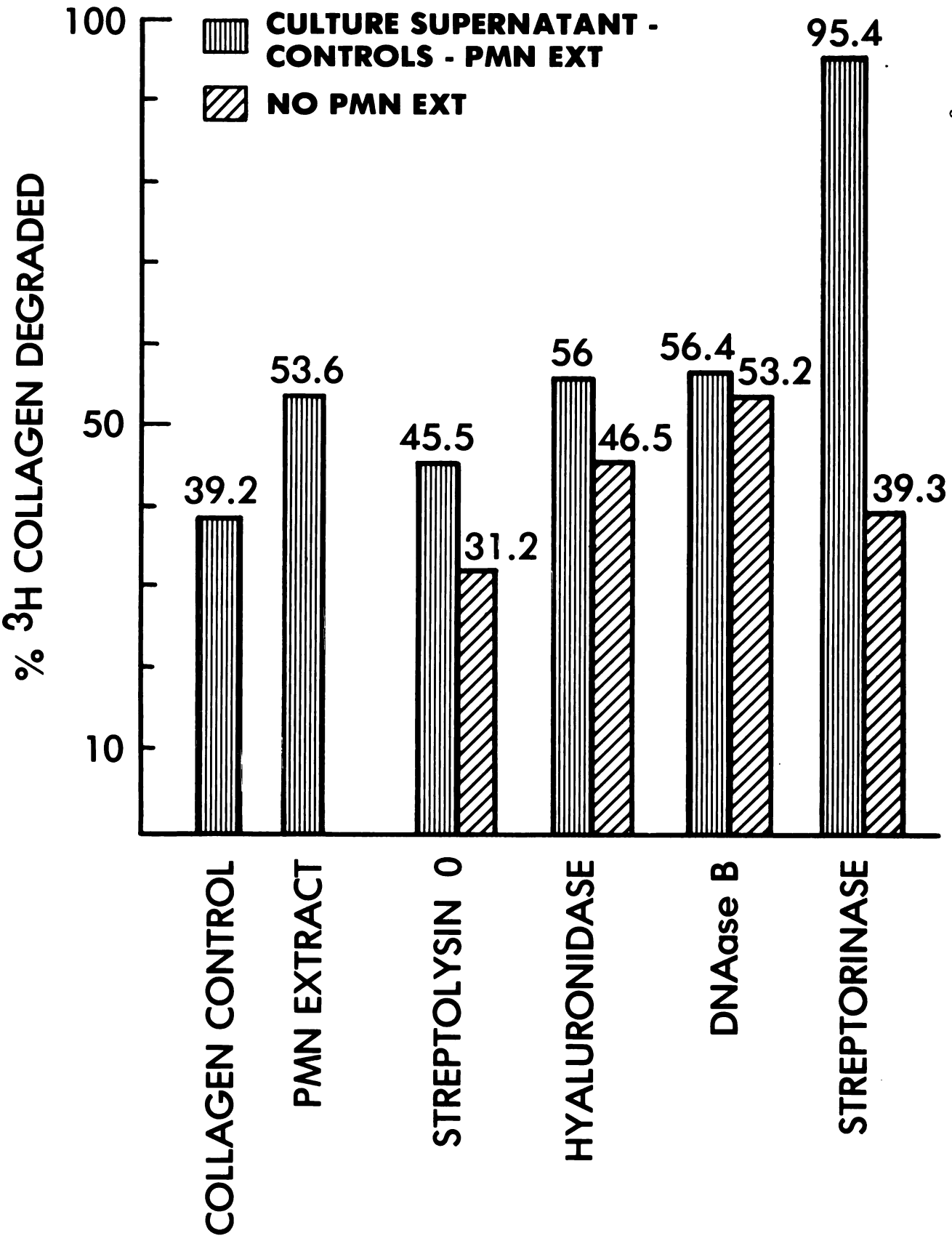

Figure II: ACTIVATION OF LATENT LEUKOCYTE COLLAGENASE BY VARIOUS STREPTOCOCCAL PRODUCTS USING ^{3}H COLLAGEN AS SUBSTRATE AND DIOXANE FOR PRECIPITATION

The various streptococcal enzymes were tested again for their ability to activate latent leukocyte collagenase, using ^{14}C rat tail collagen as substrate. Trichloroacetic acid preparation method was used in the collagenase assay. Results are summarized in table VA and figure III. Statistical analysis is summarized in table VB. Detail calculations are shown in appendix III.

Table VA: Testing of Various Streptococcal Enzymes for their Ability to Activate Latent Leukocyte Collagenase - Using ^{14}C Collagen as Substrate and TCA Precipitation Method

	Mean CPM	Standard Deviation	Percentage Degradation
Total collagen	4624	31	0
Negative Control	172	12	3.7
PMN extract alone	3652	24	78
Streptolysin - + PMN ext.	2356	24	51
Streptolysin 0 alone	173	11	3.74
Hyaluronidase + PMN ext.	3235	37	70
Hyaluronidase alone	178	7	3.8
DNAase B + PMN ext.	3771	18	81
DNAase B alone	172	18	3.72
Trypsin + PMN ext.	5686	108	100+
Trypsin alone	268	16	5.8
Streptokinase + PMN ext.	4320	33	93.4
Streptokinase alone	146	19	3.2

Table VB: Summary of Statistical Analysis on the Ability of Streptococcal Enzymes in Activating Latent Leukocyte Collagenase - Using ^{14}C as Substrate and TCA Precipitation Method

	Significant Difference from PMN Ext. Alone? ($p < .01$)
Streptolysin O + PMN ext.	YES
Hyaluronidase + PMN ext.	YES
DNAase B + PMN ext.	YES
Streptokinase + PMN ext.	YES

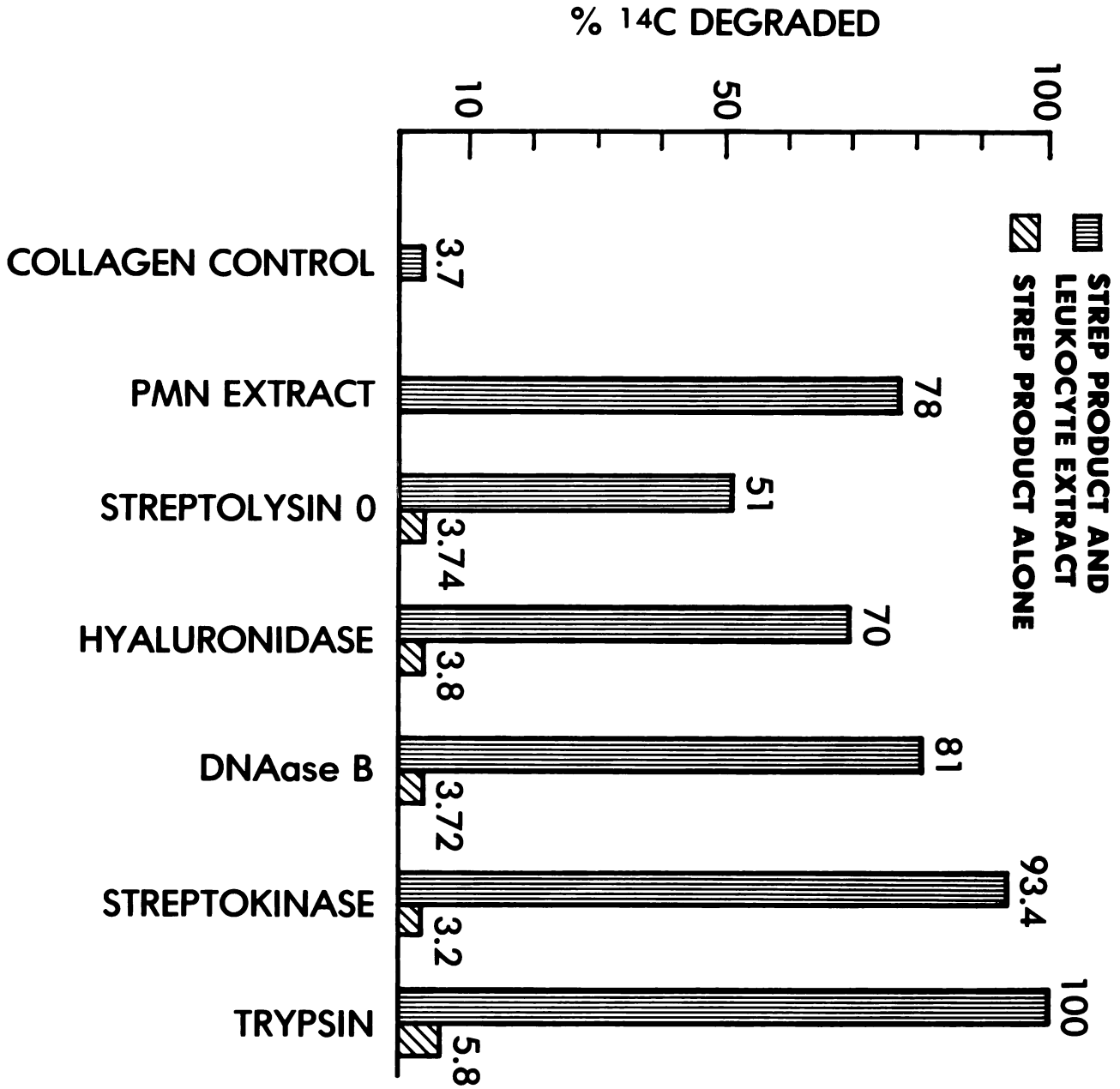

Figure III: ACTIVATION OF LATENT LEUKOCYTE COLLAGENASE BY VARIOUS STREPTOCOCCAL ENZYMES USING ^{14}C COLLAGEN AS SUBSTRATE AND TCA FOR PRECIPITATION

Various soluble enzymes prepared from four strains of Streptococcus pyogenes were subjected to alkaline inactivation (4z). With the addition of 0.1N NaOH, the pH of these enzyme preparations was raised to 9.5 for an hour at room temperature. Then, the pH was brought back to 7.3 with 0.1N HCl. Streptokinase was used as control. After the pH treatment, these streptococcal enzyme preparations were tested for their ability to activate latent leukocyte collagenase. Werb's collagenase assay using tritiated calf skin collagen and tricholoacetic acid precipitation method was employed in these experiments. The results are summarized in table VI. Statistical analysis is summarized in appendix IV.

Table VI: Testing of the Ability of Streptococcus pyogenes Culture Supernatant to Activate Latent Leukocyte Collagenase After pH Treatment

	Mean CPM	Standard Deviation	Percentage Degradation	Significant Difference from PMN ext. alone ($P < .01$)
Total collagen	6469	59	0	
Collagen control	1113	26	17	
PMN ext. alone	5078	41	79	
PMN ext. + treated #291*	5080	70	79	NO
PMN ext. + treated #268*	5255	74	81.2	NO
PMN ext. + treated #298*	5098	24	79	NO
PMN ext. + treated #250*	4830	100	75	NO
PMN ext. + treated Streptokinase	5060	58	78	YES

*Streptococcus pyogenes strains

Serial 10-fold dilution of a 1 mg/ml solution of streptokinase was made and each dillution was tested for its ability to activate latent human leukocyte collagenase. Werb's collagenase assay using tritiated calf skin collagen and trichloroacetic acid precipitation method was employed in these experiments. The results are summarized in table VII.

Table VII: Titration of the Activation Effect of Streptokinase on Latent Leukocyte Collagenase

	Mean CPM	Standard Deviation	Percentage Degradation
Total collagen	7800	34	
Collagen control	804	18	10.3
PMN extract alone	5001	30	64.1
PMN ext. + 10^{-1} streptokinase	7109	44	91.2
PMN ext. + 10^{-2} streptokinase	6104	23	78.3
PMN ext. + 10^{-3} streptokinase	5039	34	64.5
PMN ext. + 10^{-4} streptokinase	4822	30	61.8
PMN ext. + 10^{-5} streptokinase	4887	33.9	62.6
PMN ext. + 10^{-6} streptokinase	4793	34.1	61.5

DISCUSSION

The purpose of this project was to examine the role of bacterial products; particularly, those of Streptococcus pyogenes, in collagenolysis and human leukocyte procollagenase activation. While none of the bacterial products tested showed any collagenolytic ability, some did exhibit the ability to activate latent collagenase. Among this group of organisms, Streptococcus pyogenes was further studied. A few of its soluble enzymes, namely, streptokinase, hyaluronidase, streptolysin 0, and deoxyribonuclease B were tested individually for their ability to activate latent collagenase. Only streptokinase caused activation.

The data from experiments involving assay of collagenolytic activity in tissues obtained from patients with necrotizing fasciitis as summarized in table I confirmed the presence of collagenase in the center of the lesion. The margin of the lesion, while serving as a normal control, showed zero or decreased collagenolytic activity when compared to the center. Two out of five tissue specimens obtained from the margin showed slight collagenolytic activity. The absence of a sharp, gross demarcation of the lesion made difficult the determination of whether the margin of debrided tissue was normal or abnormal. The selection of tissues to be debrided was based entirely on the surgeon's judgement of which tissues should be removed for the best therapeutic result. Thus, these two factors can account for the slight collagenolytic activity in the marginal tissue specimens. These results demonstrated that collagenase is present in the central portion of the nacrotizing fasciitis lesion and that this collagenolytic activity is greatly decreased or becomes non-detectable in normal tissues surrounding the center of the lesion. The collagenase

found in such lesions is probably not derived from the host connective tissue cells unless traumatic processes stimulate the fibroblasts to increase their collagenase production. At present, there is no experimental data to prove or disprove this speculation.

Assuming that the collagenase found came either from the invading bacteria or the host's polymorphonuclear leukocytes, experiments were conducted to test the various viridans streptococci isolated from such lesions for their ability to produce collagenase. Results are summarized in table II. Of the 101 strains tested, none showed any collagenase activity. Presence of hyaluronidase as well as elastase activities were also tested. While none of the strains showed any elastase activity, 30 out of the 101 strains tested showed positive hyaluronidase activity. There was no apparent difference between laboratory strains and isolates from cases of necrotizing fasciitis. While hyaluronidase cannot initiate the degradation of collagen in tissue, it can contribute to the pathogenesis of necrotizing fasciitis by changing the viscosity of the deep fascia which contains large amounts of hyaluronic acid.

The role of various bacteria isolated from traumatic wounds, burns and necrotizing fasciitis in activating latent leukocyte collagenase was studied. ^{3}H acetylated collagen and Werb's dioxan precipitation method (36) were used to test various soluble products from these bacteria for their ability to degrade collagen and to activate latent collagenase in the extracts of human polymorphonuclear leukocytes. The degradation, as reflected from counts per minute, due to the leukocyte extract alone was used as the baseline. An increase in counts per minute in these experiments suggests activation of the latent collagenase while a decrease in counts suggests interference with the collagen-

collagenase reaction. Results are summarized in table III and figure I. With the exception of products from the control bacteria, Pseudomonas aeruginosa and Clostridium histolyticum, which are known to contain collagenase (43), none of the bacterial products alone showed any collagenolytic activity. However, when combined with the leukocyte extracts, various bacterial culture supernatants enzymes showed a significantly higher cpm than that of the leukocyte extracts alone, suggesting activation of the latent collagenase. These included preparations from Staphlococcus aureus, Streptococcus intermedius, Streptococcus pyogenes, Pseudomonas aeruginosa as well as Clostridium histolyticum. At present, there is no single theory on the nature of the latent enzymes. If the enzyme-inhibitor theory is true, bacterial enzymes may activate the latent enzyme by means of cleaving off the inhibitor from the active enzymes.

In a few cases of necrotizing fasciitis observed in San Francisco General Hospital, Streptococcus pyogenes was the only etiologic agent isolated. Knowing the soluble products from Streptococcus pyogenes can activate latent leukocyte collagenase, a few of their enzymes, namely, streptolysin 0, hyaluronidase, deoxyribonuclease B and streptokinase, were tested for their ability to activate the latent enzymes. Commercial preparations of highest purity available were used. Initially, ^{3}H acetylated collagen and Werb's dioxane precipitation method (36) were used to test these products. As seen in the summarized results in table IV and figure II, Streptokinase showed ability to activate latent collagenase, increasing the percentage degradation from 53.6% (leukocyte extract alone) to 95.4% (leukocyte extract + streptokinase). Although the addition of streptolysin 0 as well as hyaluronidase to the leukocyte

extract also resulted in a significant difference (at $P < .01$) in the percentage degradation when compared to the effect of leukocyte enzyme alone, these differences were negative differences which suggest possible inhibition of the leukocyte enzyme by these two streptococcal enzymes. Furthermore, numerically, the difference in percent degradation initiated by the effect of streptolysin 0 (7%) and hyaluronidase (2%) on the leukocyte enzyme was much smaller than that due to streptokinase (43%). Thus, the slight "inhibitory" effect showed by streptolysin 0 as well as hyaluronidase may well be experimental random errors.

Table V and figure III summarized the results of the same experiments with the use of ^{14}C methylated collagen and tricholoroacetic acid precipitation (37). The background collagen count was reduced to 3.7% and once again, streptokinase exhibits ability to activate latent leukocyte collagenase. Although the addition of other streptococcal enzymes to the leucolyte extract also results in significant changes in collagenolytic ability of the leukocyte extract, these differences were either of "inhibitory nature", (streptolysin 0 and hyaluronidase) or the differences were once again, very small, (3% as in the case of Deoxyribonuclease B).

In order to further establish the role of streptokinase in the activation of leukocyte procollagenase, experiments were set up to show the disappearance of the activation ability with the inactivation of streptokinase. A specific enzymatic inhibitor for streptokinase is not known. A non-specific alkaline inactivation procedure was employed. Preparations of streptokinase enzyme as well as soluble products from the Streptococcus pyogenes strains which had previously been shown to be able to activate latent collagenase were subjected to the alkaline

treatment (42). After neutralization, they were tested for ability to activate the latent leukocyte collagenase. As summarized in table VI, there was no substantial increase in collagen degradation after the leukocyte extracts were exposted to the streptokinase or Streptococcus pyogenes supernatants subjected to the alkaline inactivation procedure. However, it must be pointed out that this alkaline inactivation process is non-specific for streptokinase and that it might also inactivate other soluble products present in the supernatants of these Streptococcus pyogenes cultures which may be able to activate latent collagenase.

The last series of experiments involved the titration of streptokinase with subsequent study on its activation ability at various dilutions. Serial 10-fold dilution of a 1 mg/ml solution of streptokinase was made. As summarized in table VII, the ability to activate latent collagenase decreased as the dilution went up and finally vanished between the 10^{-2} and 10^{-3} dilution. These results suggest that the activation process is an enzymatic reaction.

CONCLUSION

These series of experiments confirm the interaction of bacterial products and latent collagenase in human leukocyte extract. Streptokinase, one of the soluble products of Streptococcus pyogenes, was shown to activate latent collagenase. None of the bacterial strains isolated from cases of necrotizing fasciitis was found to produce collagenase. Therefore, it is very likely that these microorganisms take part in tissue destruction by activating latent endogenous collagenase of the host. Furthermore, some viridan streptococci were found to produce hyaluronidase which may be an important enzyme in breaking down tissue in vivo.

APPENDIX AND BIBLIOGRAPHY

Appendix I: STATISTICAL ANALYSIS OF DATA FROM TABLE IIIA

Enzyme	CPM	$\bar{X}$	$\bar{X}_{test} - \bar{X}_{PMN}$	Significant difference ($P<.01$)
PMN ext.	6495,6568,6568	6548	0	-
E. coli + PMN ext.	6193,6238,6249	6227	-321	Yes
P. aeruginosa (B)* + PMN ext.	9680,9572,9538	9596	+3048	Yes
S. aureus + PMN ext.	9396,9387,9430	9405	+2902	Yes
P. aeruginosa (D)* + PMN ext.	10372,10463,10455	10430	+3882	Yes
Enterococci + PMN ext.	6658,6693,6631	6660	-112	No
S. epidermidis + PMN ext.	6093,6172,6198	6162	-386	Yes
S. pyogenes (256)* + PMN ext.	6530,6586,6594	6570	+22	No
S. sanguis + PMN ext.	6357,6298,6377	6344	-204	Yes
P. aeruginosa (290)* + PMN ext.	9253,9208,9274	9245	+2697	Yes
S. pyogenes (298)* + PMN ext.	9695,9753,9766	9738	+3190	Yes
S. pyogenes (30370)* + PMN ext.	8936,8895,8877	8902	+2354	Yes
S. pyogenes (291)* + PMN ext.	8496,8518,8520	8511	+1963	Yes
S. intermedius + PMN ext.	9032,9017,9046	9031	+2483	Yes
C. histolyticus + PMN ext.	10209,10216,10222	10216	+3668	Yes
Trypsin + PMN ext.	10803,10780,10796	10793	+4245	Yes

Appendix I

$$\text{Sum of square (factor)} = \frac{(19646)^2+(18680)^2+\ldots(32379)^2}{3} - \frac{(19646+18680\ldots+32379)^2}{48}$$

$$= 3513146939 - 3385502540$$

$$= 127644399$$

$$\text{Sum of square (error)} = (6495)^2+(6568)^2+(6583)^2+\ldots(10796)^2-3513146939$$

$$= 3513192052 - 3513146939$$

$$= 45113$$

Source	Sum of square	Degree of freedom	Mean square
Factor	127644399	15	8509626.6
Error	45113	32	1409.8

$$F = \frac{8509626.6}{1409.8} = 6036.1$$

H_o: $\bar{X}_{PMN} = \bar{X}(PMN + E.\ coli) = \bar{X}(PMN + Pseudo) = \ldots.\bar{X}(PMN + Trypsin)$

$F_{(15,32,0.05)} = 1.97$ ---------Reject H_o

$Q_{(16,32,0.99)} = 6.18$

H'_o: $\bar{X}_{PMN} = \bar{X}_{test}$

$\therefore$ if $\dfrac{(\bar{X}_{PMN} - \bar{X}_{test})}{\sqrt{S_p^2 / n}} > 6.18$ ----------Reject H'_o

$(\bar{X}_{PMN} = \bar{X}_{test}) > 6.18\sqrt{1409.8 / 3}$ ----------Reject H'_o

i.e. When $(\bar{X}_{PMN} - \bar{X}_{test}) > 134$ ----------Reject H'_o

Appendix II: STATISTICAL ANALYSIS ON DATA FROM TABLE IVA

Enzyme	CPM	Sum	Mean ($\bar{X}$)	$\bar{X}_{test} - \bar{X}_{PMN}$	Significiant difference ($P<.01$)
PMN ext.	5997,5832,5933	17762	5920.7	0	-
PMN ext. + Streptolysin 0	5011,5104,4982	15097	5032	-888.7	Yes
PMN ext. + DNAase B	6335,6171,6204	18710	6236.6	+315.9	No
PMN ext. + Hyaluronidase	5144,5066,5193	15403	5134	-786.7	Yes
PMN ext. + Streptokinase	10596,10369,10654	31619	10539	+4618.3	Yes

$$\text{Sum of square (factor)} = \frac{(17762)^2+(15097)^2+(18710)^2+(15403)^2+(31619)^2}{3} - \frac{(17762+15097+....31619)^2}{15}$$

$$= 710161907.7 - 648012352.1$$

$$= 62149555.6$$

$$\text{Sum of square (error)} = (5997)^2+(5832)^2+(5933)^2+....(10654)^2-710161907.7$$

$$= 710252499 - 710161907.7$$

$$90591.3$$

Appendix II

Source	Sum of square	Degree of freedom	Mean square
Factor	62149555.6	5.1 = 4	15537388.9
Error	90591.3	10	9059.13

$$F = \frac{15537388.9}{9059.13} = 1715$$

H_o: $\overline{X}_{PMN} = \overline{X}_{PMN + Streptolysin\ O} = \overline{X}_{DNAase\ B + PMN} \ldots = \overline{X}_{PMN + Streptokinase}$

$F_{(1,10,0.05)} = 3.48$ ----------Reject H_o

$Q_{(5,10,0.99)} = 6.14$

H'_o: $\overline{X}_{PMN} = \overline{X}_{test}$

$\therefore$ When $\dfrac{(\overline{X}_{PMN} - \overline{X}_{test})}{\sqrt{S_p^2 / n}} > 6.14$ ----------Reject H'_o

$\therefore$ $(\overline{X}_{PMN} - \overline{X}_{test}) > 6.14\sqrt{9059.13 / 3}$ ----------Reject H'_o

i.e. When $(\overline{X}_{test} - \overline{X}_{PMN}) > 338.1$ ----------Reject H'_o

Appendix III: STATISTICAL ANALYSIS OF DATA FROM TABLE VA

Enzymes	CPM	Sum	Mean CPM ($\bar{X}$)	$\bar{X}$ test - $\bar{X}$ PMN	Significant difference ($P<.01$)
PMN ext.	3660,3620,3671	10951	3650	0	-
PMN ext. + Streptolysin O	2359,2330,2378	7067	2355	-1295	Yes
PMN ext. + Hyaluronidase	3271,3238,3198	9707	3235	-415	Yes
PMN ext. + DNAase B	3751,3774,3787	11312	3770	+120	Yes
PMN ext. + Streptokinase	4326,4285,4350	12961	4320	+670	Yes
PMN ext. + Trypsin	5705,5673,5682	17060	5686	+2036	Yes

$$\text{Sum of square (factor)} = \frac{(10951)^2+(7067)^2+(9707)^2+....(17060)^2}{3} - \frac{(10951+7067+9707+....17060)^2}{18}$$

$$= 283695068 - 264944853.6$$

$$= 18750214.4$$

$$\text{Sum of square (error)} = (3660)^2+(3620)^2+(3671)^2+....(5682)^2 - 283695068$$

$$= 283703720 - 283695068$$

$$= 8652$$

Appendix III

Source	Sum of square	Degree of freedom	Mean square
Factor	18750214.4	6 - 1 = 5	3750042.8
Error	8652	12	721

$$F=\frac{3750042.8}{721}=5201.2$$

H_o: $\overline{X}_{PMN} = \overline{X}(PMN + Streptolysin\ O) = \overline{X}(PMN+DNAase)=....\overline{X}(PMN+Trypsin)$

$F_{(5,12,0.05)} = 3.11$ ----------Reject H_o

$Q_{(6,12,0.99)} = 6.1$

H_o': $\overline{X}_{PMN} = \overline{X}_{test}$

$\therefore$ When $\dfrac{(\overline{X}_{PMN} - \overline{X}_{test})}{\sqrt{S_p^2 / n}} > 6.1$ ----------Reject H_o'

$(\overline{X}_{PMN} - \overline{X}_{test}) > 6.1\sqrt{721 / 3}$ ----------Reject H_o'

i.e. When $(\overline{X}_{test} - \overline{X}_{PMN}) > 94.6$ ----------Reject H_o'

Appendix IV: STATISTICAL ANALYSIS OF DATA FROM TABLE VI.

Enzymes	CPM	Sum	Mean CPM ($\bar{X}$)	$\bar{X}$ test - $\bar{X}$ PMN	Significant difference ($P<.01$)
PMN ext.	5120,5038,5076,	15234	5078	0	-
PMN ext. + 291*	5086,5147,5008	15241	5080	+2	No
PMN ext. + 268*	5336,5240,5190	15766	5255	+177	No
PMN ext. + 298*	5124,5078,5093	15295	5098	+20	No
PMN ext. + 250*	4716,4873,4903	14492	4830	-248	Yes
PMN ext. + Streptokinase	5018,5126,5038	15182	5060.6	+17.4	No

$$\text{Sum of square (factor)} = \frac{(15234)^2+(15241)^2+(15766)^2+....(15182)^2}{3} - \frac{(15234+15241+....15182)^2}{18}$$

$$= 462459268.7 - 462181338.9$$

$$= 277929.7$$

$$\text{Sum of square (error)} = (5120)^2+(5038)^2+(5076)^2+(5086)^2+....(5038)^2 - 462459268.7$$

$$= 462511232 - 462459268.7$$

$$= 51964$$

*Streptococcus Pyogenes Strains

Appendix IV.

Source	Sum of square	Degree of freedom	Mean square
Factor	177929.7	5	55585.9
Error	51964	12	4330.3

$$F=\frac{55585.9}{4330.3} = 12.84$$

$$H_o: \overline{X}_{PMN} = \overline{X}_{(PMN + 291^*)} = \overline{X}_{(PMN + 268^*)} + \cdots \overline{X}_{(PMN + Streptokinase)}$$

$$F_{(5,12,0.05)} = 3.11$$

$\therefore$ Reject H_o

$$Q_{(6,12,0.99)} = 6.1$$

$$H_o': \overline{X}_{PMN} = \overline{X}_{test}$$

$\therefore$ When $\dfrac{(\overline{X}_{PMN} - \overline{X}_{test})}{\sqrt{S_p^2 / n}} > 6.1$ ----------Reject H_o'

$$(\overline{X}_{PMN} - \overline{X}_{test}) > 6.1 \sqrt{4330.3 / 3}$$ ----------Reject H_o'

i.e. When $(\overline{X}_{test} - \overline{X}_{PMN}) > 231.75$ ----------Reject H_o'

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