The Chemotactic Properties of the Products of Collagenolysis[1] (35834)

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Over a decade ago, we found chemical evidence that collagenolysis was a concomitant finding of tissue necrosis (1, 2). This collagenolysis of a normally metabolically "inert" insoluble fiber, we felt, had to proceed *via* the release of a collagenolytic enzyme into the extracellular space of the dermis where the collagen is located. We found that there existed in the extracellular space of rat skin an inhibitor-collagenase complex which could be activated by limited proteolysis of the inhibitor (3, 4). Recently, we have isolated and purified this enzyme after trypsin activation over 450-fold (5, 6).

During the course of our studies of the chemical kinetics of necrotic wounds, we often noticed that tissue collagenolysis preceded the infiltration of the wound by polymorphonuclear leukocytes (PMN). This observation is of some significance in view of the recent report by Lazarus *et al.* (7), of the collagenolytic activity of granulocytes. Further, the early studies of Spector (8), suggested that peptides produced by the proteolytic degradation of various proteins all produced leukocyte emigration if they were between 5 to 14 amino acid residues in size. Therefore, it occurred to us that possibly the degradation products of collagen that were most probably released during collagenolysis from the necrotic wound might also be involved in the PMN infiltration so characteristic of inflammation.

We therefore developed an *in vivo* modification (9) of the *in vitro* Boyden chamber technique used by Ward (10) to study chemotaxis. Using this method, we then demonstrated that soluble collagen itself was chemotactic and that this chemotaxis of soluble collagen was destroyed by gelatinization or by incubation with bacterial collagenase (9), but was increased by incubation with a purified cutaneous collagenase (5). This paper demonstrates the *in vivo* leukotactic properties of these product peptides released by the activity of this cutaneous collagenase from native soluble collagen.

Materials and Methods. Isotonic saline extracts of rat skin were prepared via the extraction in the cold of 100 g of minced skin with 1000 ml of 0.15 *M* NaCl in a Lourdes blender for 45 min. After standing in the cold to swell for 16 hr, the supernatant was collected by centrifugation at 12,000*g* for 2 hr. This supernatant was dialyzed exhaustively against water, recentrifuged, and lyophilized. This lyophilized skin extract (S_1) was activated with 1 mg of crystalline trypsin/10 mg of S_1 at pH 5.5 and 22° for 20 min. At this time, 1.1 mg of soybean trypsin inhibitor was added/mg of trypsin; and the solution was made up to 65% with ammonium sulfate in the cold. After centrifuging, the supernatant was dialyzed against water until free of the ammonium ion and lyophilized. The lyophilizate was reconstituted in 0.15 *M* NaCl and eluted from Bio-Rad P-100 columns (Cal Biochem.). All the collagenolytic activity was found to be excluded from the column just behind the voided volume. This active peak was dialyzed, lyophilized, and redissolved in sucrose and subjected to isoelectric focusing in an LKB device. All the collagenolytic activity was found at pH 5.2; and this

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fraction contained only a double band upon acrylamide gel electrophoresis. This material could both solubilize native insoluble collagen and release dialyzable hydroxyproline containing peptides from native soluble collagen (5, 6).

Native soluble collagen was prepared and purified from 0.5 *M* NaCl extracts of rat skin according to Piez *et al.* (11). The intrinsic viscosity of this collagen was about 14 dl/g; its specific optical rotation (at the sodium D line) was —355°.

The abdominal area of male Sprague-Dawley rats, weighing 150–200 g was shaved and cleaned with pHisoHex. The epidermis of the sides of the abdomen of these rats were scraped away using a sterile scalpel, avoiding gross bleeding. Sterile Millipore filters (pore size 450 mμ) were placed (grid side up) upon the area abraded free of epidermis. On top of this filter was placed a 13-mm diameter filter disc (S&S Co. No. 470). The disc was then moistened with 0.1 ml of the sample fluid, *i.e.*, collagen incubation mixture, etc., and covered with a piece of parchment paper. The whole "sandwich" of filter, disc, and paper was then covered with a piece of adhesive tape. Four of these "sandwiches" could be applied, two to a side, to the abdominal area of a single rat. One sandwich always contained a buffer control, another a standardized sample of chemotactically active bacterial toxin, and the remaining two contained duplicate experimental samples. All determinations were done using at least three rats each, as described previously (9). Controls never showed any cells had migrated through the Millipore filter; 5–15 cells/field was termed ±; 20–60 cells was +; and over 100 cells/field was ++.

Soluble collagen was made up in 0.05 *M* acetate buffer adjusted to a pH of 5.5. The final concentration of the collagen was about 0.7 mg/ml. This substrate solution was then incubated for 16 hr at 22° and pH 5.5 with 20 μg/ml of a cutaneous collagenase solution which had been sterilized via Millipore filtration. Occasionally, these incubation mixtures were tested after 16 hr for bacteriological contamination on blood agar plates. Little contamination could ever be demonstrated.

Experimental Results. The nature of the products of collagenolysis produced by 20 μg/ml of cutaneous collagenase when incubated with soluble collagen is visualized via acrylamide gel electrophoresis (12) as in Fig. 1. Figure 1 shows the electrophoretic pattern of the gelatins obtained from heating soluble collagen alone to 45° at pH 4.0 (gel 1). The electrophoretic patterns of these gelatins after prior incubation at pH 5.5 and 22° with 20 μg of boiled collagenase (gel 2) or native collagenase (gel 3) for 16 hr indicated that active collagenase released a wide variety of products from the soluble collagen substrate while trypsin (20 μg/ml) at pH 7.5 had no significant effect upon the soluble collagen substrate (gels 4 and 5).

These variously sized products of collagenolysis could be separated from intact soluble collagen (mol wt 300,000) and collagenase [mol wt 100,000 (5, 6)], by gel sieving using Bio-Rad P-100 columns. The incubation mixture of cutaneous collagenase with soluble collagen substrate was therefore eluted from these columns with 0.05 *M* acetate buffer (pH 5.5) and the effluent was monitored at 220 mμ ("end absorption region") because of the lack of aromatic amino acid residues (capable of absorbing energy at 280 mμ) in the primary structure of collagen. All of the material held back more than 1.5 void volumes from the solvent front (mol wt $<$100,000) on these columns was pooled and subjected to sequential membrane fractionation using Diaflo membranes (Amicon Corp.), according to the method of Zipilivan *et al.* (13). In this fashion, the products of collagenolysis were molecularly sieved into three different sized fractions, *i.e.*, 50,000–30,000; 30,000–10,000; and 10,000–1,000. After reaching elution equilibrium, these fractions were all concentrated via ultrafiltration using the 500 mol wt Diaflo filter under nitrogen pressure (13), to equal volumes of about 8 ml. Aliquots of all four fractions were read in a Beckman DB at 220 mμ and tested chemotactically. The results are shown in Table I and indicate that most of the chemotactic activity of these collagenolysis products resided in products weighing 30,000 to 10,000 and 10,00 to 1000 daltons.

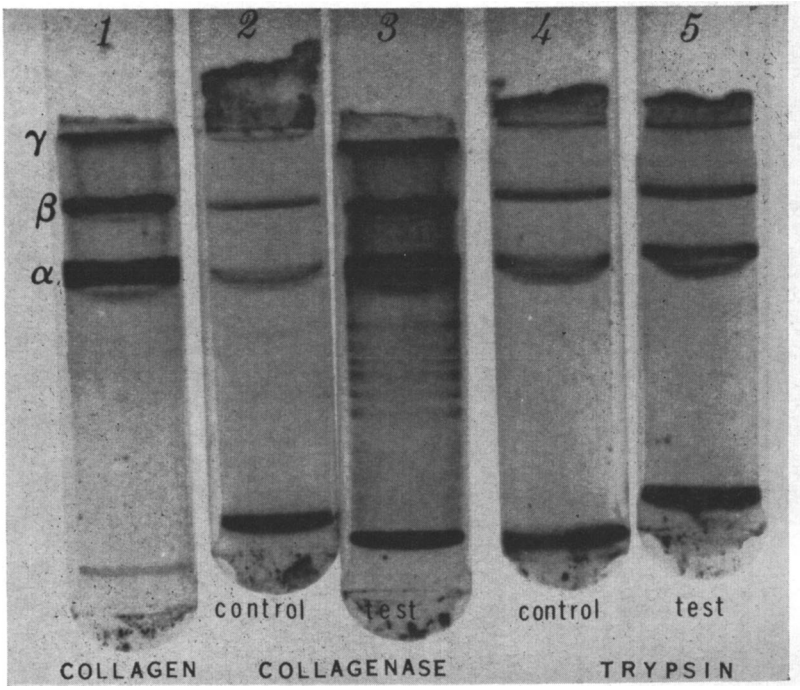

FIG. 1. Polyacrylamide gel-electrophoretic patterns of the incubation mixtures (16 hr at 22° and pH 5.5) of cutaneous collagenase with soluble collagen: (1), soluble collagen alone; (2) soluble collagen after incubation with boiled collagenase; (3) soluble collagen after incubation with native collagenases; (4 and 5) soluble collagen after incubation with thermally denatured and native trypsin at pH 7.5.

Separate experiments indicated that this chemotactic activity was lost when these extracts were pretreated with either purified (from G-200 columns) bacterial collagenase or by gelatinization at 45° for 1 hr.

The two chemotactically active fractions resulting from membrane ultrafiltration of the collagenase-soluble collagen incubation mixtures were further resolved into various peptide fractions by cation exchange chromatography using Aminex 50W-X2 ion exchange resin columns. A stepwise elution with 120 ml of 0.2 *N* citrate buffer (pH 3.1) and 120 ml of 0.25 *N* citrate acetate buffer (pH 4.5) were used. A typical elution pattern is shown in Fig. 2 for the products of soluble collagen hydrolysis weighing 30,000 to 10,000 daltons. The break in the abscissa indicates the buffer change from pH 3.1 to 4.5; the arrows indicate the pooled collection tubes (*i.e.*, nos. 5–11 are peak A; 45–47 are peak B; and 47–51 are peak C., etc.). Each of these pools was dialyzed against distilled water using Diaflo membranes retentive of molecules in excess of 500 mol wt and concentrated by ultrafiltration using the same

TABLE I. The Chemotactically Active Molecular Fractions of the Products of the Collagenolysis of Soluble Collagen.

Fraction	Mol wt (daltons)	Peptide conc OD (220 mμ)	Chemotactic activity (4 hr application)
1	50,000–30,000	0.930	±
2	30,000–10,000	0.280	++
3	10,000–1000	0.100	+

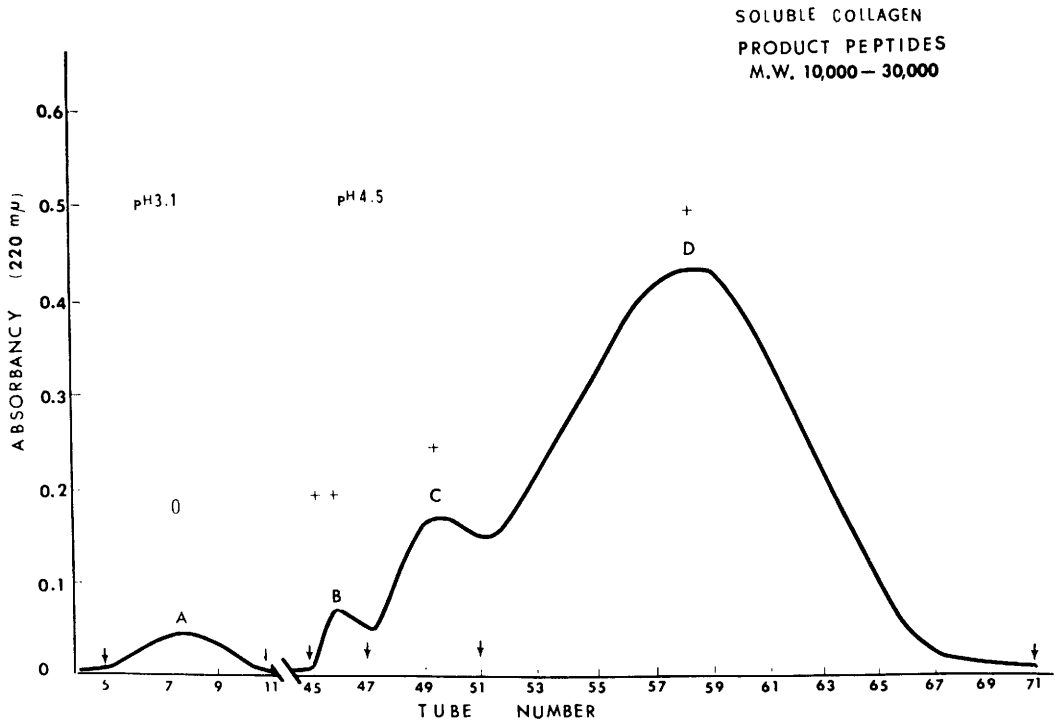

FIG. 2. Cation exchange chromatography of soluble collagen peptide products (10,000 to 30,000 mol. wt) on Aminex 50W-X2 resin: The chemotactic activity of each pooled fraction is indicated as 0, +, + +, above each peak.

membrane. Aliquots of each pool were assayed for chemotaxis and the results are also reported in Fig. 2.

Three chemotactically active fractions were resolved from the 30,000 to 10,000 mol wt sized products of the collagenolysis of soluble collagen. Similar results were obtained using the 10,000 to 1000 dalton sized products saved in that four chemotactically active fractions were obtained upon cation exchange chromatography, as shown in Fig. 3.

The various fractions were all concentrated and aliquots were analyzed for nitrogen by a Kjeldahl digestion, Nesslerization procedure (2). Assuming that these collagen peptides contained 18.6% nitrogen, the concentration (μg of peptide) of each eluted fraction was determined. Each fraction was then diluted and assayed for chemotactic activity. The results of the one inactive and seven active fractions from the collagenolysis of soluble collagen are presented in Table II and show that as little as 0.3 ng of fraction C (30,000–10,000 mol wt) was chemotactic. Assuming an average molecular weight of 20,000 for the polypeptide, this means that peptide C is chemotactic at a concentration of 1.5×10^{-10} *M*. Similarly, 1.4 ng of peptide H (mol wt 10,000–1000) was still chemotactic. Assuming an average mol wt of 5000 this becomes 0.3×10^{-10} *M*. Thus, the various peptides from the collagenolysis of soluble collagen are chemotactically active at a concentration of as little as $0.3\text{–}1.5 \times 10^{-10}$ *M*.

Discussion. The kinetics of the necrotic processes within wounds suggests that the infiltration of PMN into the wound might be in chemotactic response to both the activation of complement (10) and to the establishment of a concentration gradient of the hydrolysis products formed during the proteolysis of the proteins of the injured tissue (6). Collagen constitutes about 70% of the total protein of the skin (14) and quantitatively thus would appear to be more important than noncollagenous protein in terms of

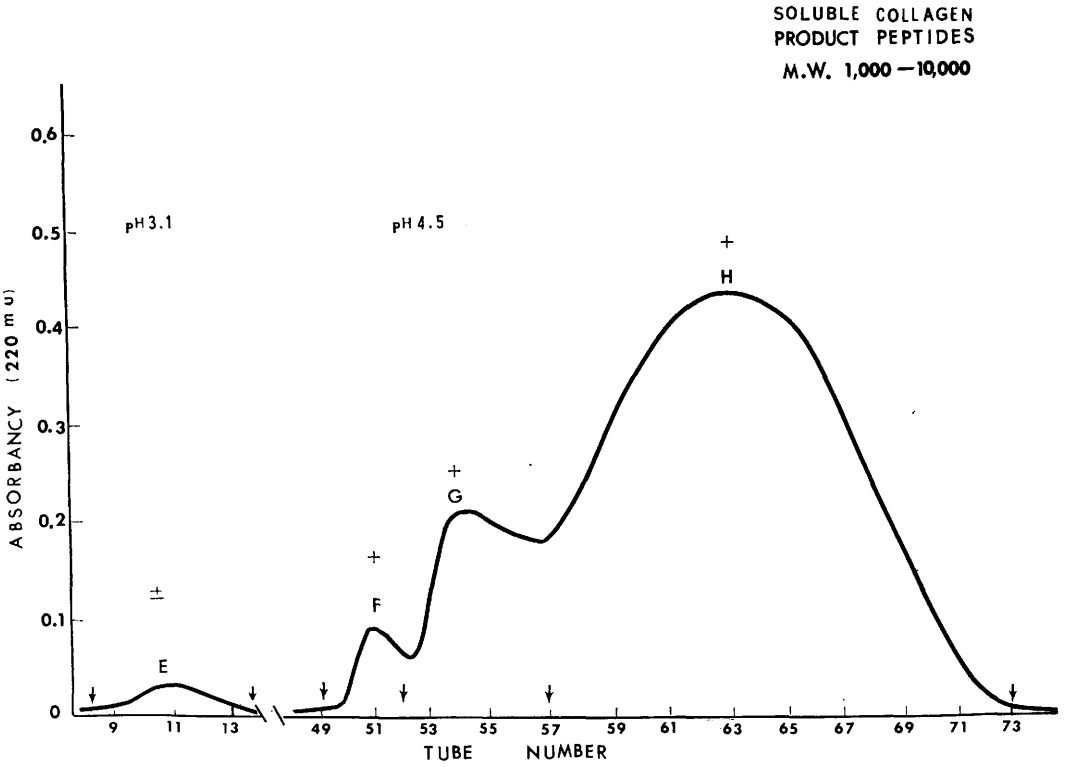

FIG. 3. Cation exchange chromatography of soluble collagen peptide products (1000 to 10,000 mol wt) on Aminex 50W-X2 resin: The chemotactic activity of each pooled fraction is indicated (±, +) above each peak.

providing hydrolysis products for this purpose.

Menkin (15, 16), a generation ago, proposed that a small polypeptide released from the degradation of intracellular noncollagenous protein was both chemotactic and capable of increasing capillary permeability. Duthie and Chain (17) reported that the proteolysis of various noncollagenous proteins released peptides effecting both the permeability of the microcirculation and the infiltration of PMN. More recent studies by Spector (7) further confirmed and enlarged upon these reports.

TABLE II. The Chemotactic Response to Various Concentrations of the Peptide Products from the Collagenolysis of Soluble Collagen (mol wt 1000–30,000).

Mol wt	Peptide no.	Conc of collagen peptide applied (μg/0.1 ml)	Chemotactic activity; dilution					
			0	1:10	1:100	$1:10^3$	$1:10^4$	$1:10^5$
30,000	A	1.43	0	0	0	0	0	0
to	B	3.16	++	+	±	±	0	0
10,000	C	2.70	+	+	+	±	±	0
	D	2.10	+	0	0	0	0	0
10,000	E	1.43	±	±	0	0	0	0
to	F	2.10	+	±	±	0	0	0
1000	G	2.33	+	±	±	0	0	0
	H	1.43	+	++	±	±	0	0

The major criticism of these reports has been rigorously summarized by Harris (18). He points out that the methods for the determination of chemotactic activity used by most investigators were not adequate because they did not exclude either the mechanical sequestration of randomly moving cells within the injured tissue or the artifactual environmental gradients of temperature, pH, specific gravity, etc., which could lead to a biologically spurious accumulation of PMN within an experimental system.

This criticism could not be valid for studies in which cells *in vivo* are required to crawl through and into pores whose diameter is less than 1/20 of their normal diameter. Obviously, from a knowledge of the physical chemistry and energetics of surfaces, such cells must expend huge amounts of energy to increase their surface area so markedly by such a profound distortion of their normal spherical shape. Thus, the finding that collagen from the skin was rendered considerably more chemotactic by collagenolysis would appear to be a valid one in regard to biologically meaningful chemotaxis in the rat (9).

Quantitatively, the increased chemotactic activity of the products of collagenysis might be because soluble collagen is extremely viscous and hence very slow to diffuse. Thus, collagenolysis would increase the rate of diffusion of the chemotactically active portions of the collagen molecules and hence permit a concentration gradient of these products to be more rapidly established.

Figure 1 shows clearly that our cutaneous collagenase released a wide variety of sizes of products from purified and native soluble collagen. Those products, presumably weighing less than 100,000 (held back on Bio-Rad P-100 columns), were in turn sieved through Amicon Diaflo membrane filters and if the absorbance at 220 mμ is meaningful quantitatively, the bulk of the products from the collagenolysis of soluble collagen were larger than 30,000 daltons in mol wt. The most active chemotactically of these products were less than 30,000 and larger than 1000 daltons in mol wt (see Table I), however.

The peptide elution patterns of both sizes of collagenolysis products released from soluble collagen were very similar (Figs. 2 and 3). Three major chemotactically active peaks were eluted from each sized product of collagenolysis beginning with the pH 4.5 gradient. Quantitatively, the most chemotactically active peptide peak (Peak C) was obtained from the products weighing between 30,000 and 10,000 which had been released from soluble collagen during collagenolysis. The finding that these collagenolysis peptide products from soluble collagen were still chemotactic at concentrations of about 10^{-10} M, suggests that these peptides have a high degree of specificity for chemotactically attracting PMN. The chemotactic activity of the various polypeptides of complement can be roughly calculated from the data of Ward (10) to be in the range of 10^{-6} M.

Finally, separate experiments indicated that neither gelatin (from the thermal denaturation of soluble collagen) nor the products of bacterial collagenase activity were chemotactic to any significant degree (9). The destruction of the chemotactic properties of these partially purified product-peptides by the action of purified bacterial collagenase is consonant both with these results and the fact that the active peptides were derived from the peptide backbone of collagen.

Why collagen, which is insoluble *in vivo*, should be so strongly and surprisingly chemotactic for PMN is unknown. The purity and chemical composition of these collagenolysis peptide products remains unknown. The mechanism of their chemotactic action upon PMN is unknown. Their importance, relative to other chemotactic polypeptides, in explaining the infiltration of PMN into injured tissue is suggested largely by their extraordinary potency and the large concentration of the parent molecule, collagen, which is known to be degraded during necrotic inflammation.

Summary and Conclusions. The chemotactically active peptide products from the action of cutaneous collagenase upon purified soluble and native collagen were isolated and partially purified. These leukotactic collagenolysis products were made up of at least eight polypeptides weighing between 30,000

and 1000 daltons and seven of these peptides were leukotactic *in vivo* at a concentration of 10^{-10} *M*.

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