

# COLLAGEN- AND COLLAGEN PEPTIDE-INDUCED CHEMOTAXIS OF HUMAN BLOOD MONOCYTES\*

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Macrophages and precursor blood monocytes are important effector cells of the host defense and immune systems. Monocytes are attracted to areas of inflammation by one or more chemotactic stimuli. For inflammation mediated by the cellular immune system, a major stimulus appears to be a lymphocyte-derived chemotactic factor (1-4). Endogenous factors, such as C5a (5), kallikrein, and plasminogen activator (6) and other undefined substances found in serum also provide chemotactic stimuli for monocytes (7, 8). In addition, products derived from bacteria (7, 9) and also *N*-formylmethionyl peptides are chemotactic for monocytes (10).

We have found that another endogenous substance, collagen, is chemotactic for human peripheral blood monocytes *in vitro*. Collagen is the most abundant and ubiquitous structural protein in the human body and is intimately involved in tissue remodelling and repair which accompany tissue damage and inflammation. Enzyme systems capable of breaking down collagen have been detected in a wide variety of physiologic and pathologic states (11). In this report, we present data that indicate that native tropocollagen, as well as peptide fragments, derived from the protein by degradation with CNBr, pepsin, or bacterial collagenase are chemotactic for monocytes. In contrast to the monocyte responsiveness, human peripheral blood neutrophils do not recognize collagen or collagen-derived peptides as chemotactic stimuli.

## Materials and Methods

*Monocyte Chemotaxis Assay.* The assay used to measure monocyte chemotaxis is essentially the same as the modified Boyden technique previously described by Snyderman et al. (5). Human mononuclear cells, isolated by isopyknic centrifugation on a Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mo. and Winthrop Laboratories, New York) gradient, were suspended at a concentration of  $5 \times 10^6$  cells per ml in 0.01 M HEPES-buffered Gey's balanced salt solution (H-GBSS)<sup>1</sup> containing 2% bovine serum albumin, pH 7.0, and were loaded into the upper compartments of modified Boyden chemotaxis chambers as test substances were added simultaneously to the lower

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<sup>1</sup> *Abbreviations used in this paper:* GGBS, 0.01 M glycylglycine/0.14 M NaCl, pH 7.2; H-GBSS, HEPES-buffered Gey's balanced salt solution; MNL/OIF, mononuclear leukocytes per oil immersion field; PMN/OIF, polymorphonuclear leukocytes migrating per oil immersion field.

compartments. The compartments of each chamber were separated from each other by a polycarbonate filter with 5- $\mu\text{m}$  pores (Wallabs, Inc., San Rafael, Calif.). Substances being assayed for chemotactic activity were dissolved in 0.01 M glycylglycine/0.14 M NaCl, pH 7.2 (GGBS). Four parts of this solution were mixed with 3.5 parts of fresh H-GBSS and added to modified Boyden chambers.

Loaded chambers were incubated in a humidified atmosphere at 37°C for 80 min. At the end of the incubation period, the filters were removed from the chambers, stained with hematoxylin, and mounted on glass slides. The chemotactic activity was quantitated by counting in 20 oil immersion fields (1,000  $\times$  magnification), the number of monocytes that migrated and adhered to the lower surface of each filter. The results are expressed as the number of mononuclear leukocytes per oil immersion field (MNL/OIF). All substances were assayed in triplicate and values represent the mean  $\pm$  1 standard error of the mean.

*Neutrophil Chemotaxis Assay.* The leukocyte-rich plasma obtained from dextran sedimentation of heparinized human peripheral blood was mixed with an equal volume of normal saline and centrifuged at 200 *g* for 10 min at 4°C. The cell pellet was exposed to 10 ml of a 0.2% NaCl solution for 20 s to lyse contaminating erythrocytes. The mixture was then made isotonic by the addition of 10 ml of a 1.6% NaCl solution. 20 ml of H-GBSS, pH 7.2, was added to the cell suspension. The cells were pelleted by centrifugation, as described above, and resuspended at a concentration of  $1 \times 10^6$  cells/ml in H-GBSS. The remainder of the assay was similar to that described to measure monocyte chemotaxis with the following differences: (a) polycarbonate filters with 3- $\mu\text{m}$  pores (Wallabs, Inc.) were used to retard the migration of residual monocytes to the lower filter surface; (b) chambers were incubated 45 min; and (c) chemotactic activity was expressed as the number of polymorphonuclear leukocytes migrating per oil immersion field (PMN/OIF).

*Collagen.* Type I collagen was extracted and purified from the skin of 3-wk-old lathyritic chickens as previously described (12). The details of these procedures, as well as the criteria of its purity, were also described (12). Human collagen was similarly obtained from the skin of extremities previously amputated to treat peripheral vascular disease. Purified chick and human skin collagens were stored at room temperature in a lyophilized state until used.

Lyophilized collagen was prepared for chemotaxis studies by dissolving aliquots in 0.5 M acetic acid and stirring overnight (4°C). The acetic acid solution of collagen was dialyzed extensively against large volume of 0.01 M phosphate/0.14 M NaCl, pH 7.4, for 24 h and then against GGBS for 24 h at 4°C. In all experiments, the concentration of collagen or collagen-derived peptides (see below) was determined by hydroxyproline assay (13) on suitable aliquots.

*$\alpha$ -Chains.* A portion of lathyritic chick skin collagen was dissolved in 0.06 M sodium acetate, pH 4.8, and denatured by heating the solution for 15 min at 45°C. Purified  $\alpha$ 1- and  $\alpha$ 2-chains were obtained by ion-exchange chromatography of heat-denatured collagen on carboxymethylcellulose, as previously described (12). The  $\alpha$ -chains were desalted, lyophilized, and stored at room temperature.  $\alpha$ -chains were prepared for use in chemotaxis studies by dissolving aliquots in 0.5 M acetic acid and extensively dialyzing the solubilized  $\alpha$ -chains against large volumes of GGBS at room temperature for 24 h.

*Degradation of  $\alpha$ -Chains by Cyanogen Bromide and Pepsin.*  $\alpha$ 1- and  $\alpha$ 2-chains obtained from lathyritic chick skin collagen were degraded by incubation with CNBr using the procedures reported previously (14, 15). The reaction was terminated and the excess CNBr removed by lyophilization of the incubation mixtures.

In a separate experiment,  $\alpha$ 1- and  $\alpha$ 2-chains (5 mg each) were digested with pepsin (50  $\mu\text{g}$ ) in 0.01 M HCl at 37°C for 16 h. The digestion products from each experiment were separately lyophilized. The peptic or the CNBr peptides were then prepared for chemotaxis experiments by dialyzing them with GGBS in an ultrafiltration cell containing a UM 2 membrane (Amicon Corp., Lexington, Mass.).

*Digestion of Collagen by Collagenase.* Solubilized chick skin collagen (60 mg in 23 ml) was digested for 18 h with purified bacterial collagenase (600  $\mu\text{g}$ ), (CLSPA, Worthington Biochemical Corp., Freehold, N. J.) at 37°C in GGBS containing 0.005 M  $\text{CaCl}_2$ . Aliquots of the digest were directly assayed for chemotactic activity for human monocytes and neutrophils. Collagen solution incubated without the added bacterial collagenase and collagenase incubated alone served as controls.

*Gel Filtration of Collagenase-Degraded Collagen.* Peptides generated by collagenase digestion of collagen were fractionated by gel filtration on Sephadex G-50, (Pharmacia Fine Chemicals,

TABLE I  
*The Chemotactic Responses of Monocytes and Polymorphonuclear Leukocytes to Collagen and  $\alpha$ -Chains\**

Substance tested	Concentration	Chemotactic activity (mean $\pm$ SEM)	
		$\mu M$	<i>MNL's/OIF</i>
Human skin collagen	0.63	72 $\pm$ 7	5 $\pm$ 1
	0.10	37 $\pm$ 1	7 $\pm$ 1
Chick skin collagen	2.14	84 $\pm$ 8	8 $\pm$ 2
	0.31	47 $\pm$ 7	5 $\pm$ 2
$\alpha$ 1-chain (chick)	10.0	82 $\pm$ 4	8 $\pm$ 2
	0.72	37 $\pm$ 3	6 $\pm$ 2
$\alpha$ 2-chain (chick)	10.7	82 $\pm$ 12	5 $\pm$ 1
	3.05	38 $\pm$ 5	7 $\pm$ 2
Buffer	—	10 $\pm$ 2	6 $\pm$ 1
Human serum control (25%)	—	91 $\pm$ 17	43 $\pm$ 5

\* Chemotaxis assays were performed using a modified Boyden chamber, and the results are expressed as the number of leukocytes migrating across the filter per oil immersion field. See Materials and Methods for details.

Piscataway, N. J.). Aliquots of column fractions were assayed for chemotactic activity for human monocytes and neutrophils. Collagenase incubated alone was processed in an identical manner.

## Results

*Chemotactic Attraction of Monocytes to Collagen and  $\alpha$ -Chains.* Collagen of human or chick skin, as well as the  $\alpha$ 1- and  $\alpha$ 2-chains of chick skin collagen, were each assayed at two different concentrations for monocyte and neutrophil chemotactic activity. Each of the native collagen and  $\alpha$ -chain preparations caused monocytes, but not neutrophils, to migrate through the polycarbonate filters (Table I). Monocyte migration was obliterated to the level obtained with the buffer control when equal concentrations of the collagen preparations were added to the upper and lower chambers, indicating that collagen-induced monocyte migration was chemotactic in nature (Table II).<sup>2</sup>

Moreover, the chemotactic response of monocytes was concentration dependent, increasing concentrations of collagen or  $\alpha$ -chains producing greater chemotactic responses (Fig. 1). Native collagen, however, was a more potent chemotactic stimulus than the denatured  $\alpha$ 1- or  $\alpha$ 2-chains, the latter requiring approximately 10-fold greater concentrations to produce a chemotactic response similar to that of native collagen. These results indicate that the tertiary structure of collagen is important for its chemotactic activity, but in addition that the covalent structure of collagen also plays a role in monocyte chemotaxis.

<sup>2</sup> The chemotactic responses to chick skin collagen and  $\alpha$ -chains presented in Table II are reduced when compared to values given in Table I. This is due to the fact that in experiments summarized in Table II, the cells were suspended in a 4:3.5 (vol/vol) mixture of GGBS and H-GBSS, a condition less optimal for monocyte migration than undiluted H-GBSS used in experiments summarized in Table I.

TABLE II  
*Effect of Neutralization of Concentration Gradient on  
 Chemotactic Response of Monocytes to Collagen and  $\alpha$ -Chains\**

Substance tested	Concentration gradient <sup>‡</sup>	Chemotactic activity (mean $\pm$ SEM)
		<i>MNL's/OIF</i>
Collagen (0.69 $\mu$ M)	Present	20 $\pm$ 4
	Absent	3 $\pm$ 1
$\alpha$ 1-chain (12.4 $\mu$ M)	Present	16 $\pm$ 2
	Absent	4 $\pm$ 1
$\alpha$ 2-chain (19.3 $\mu$ M)	Present	11 $\pm$ 1
	Absent	2 $\pm$ 2
Buffer		4 $\pm$ 1

\* Chemotaxis assays were performed as described in the Materials and Methods section, except that monocytes in the upper compartment of the modified Boyden chamber were suspended in a 4:3.5 (vol/vol) mixture of GGBS and H-GBSS.

<sup>‡</sup> "Present" indicates that the test substance was added to the lower compartment of chemotaxis chambers only. "Absent" indicates that the test substance was added in equal concentrations to both the upper and the lower compartments of chemotaxis chambers.

*Collagen Degradation Products.* To determine whether the large size of the intact  $\alpha$ -chains (mol wt 100,000 daltons) was essential for chemotaxis or whether monocytes could respond to smaller fragments of collagen, collagen was degraded separately by digestion with bacterial collagenase, CNBr, and pepsin. Degradation of purified collagen by bacterial collagenase did not destroy its chemotactic property (Table III). Analysis of fractions obtained by gel filtration on Sephadex G-50 of collagenase-digested collagen indicated that peptides of various sizes, ranging from small oligopeptides to polypeptides of the size of ovalbumin, were generated (Fig. 2). Virtually all fractions tested, except those eluting before the void volume of the column, contained chemotactic activity for human monocytes (Fig. 2). In contrast, none of these same column fractions contained chemotactic activity for neutrophils (Fig. 3). As a control, collagenase incubated in an identical manner, but without collagen was chromatographed on the same column. This did not yield any chemotactically active fractions (data not shown).

The  $\alpha$ -chains of chick skin collagen were also degraded by digestion with CNBr and pepsin and tested for chemotaxis. The results are summarized in Table IV. The peptides so generated retained chemotactic activity for monocytes, but were not chemotactic for neutrophils.

These results indicate that the minimum collagen-peptide sequence(s) recognized by monocytes as a chemotactic stimulus is not very large, since small collagenase-generated peptides eluting near the column volume of Sephadex G-50 (Fig. 2) with estimated sizes in the range of tri to decapeptides are chemotac-

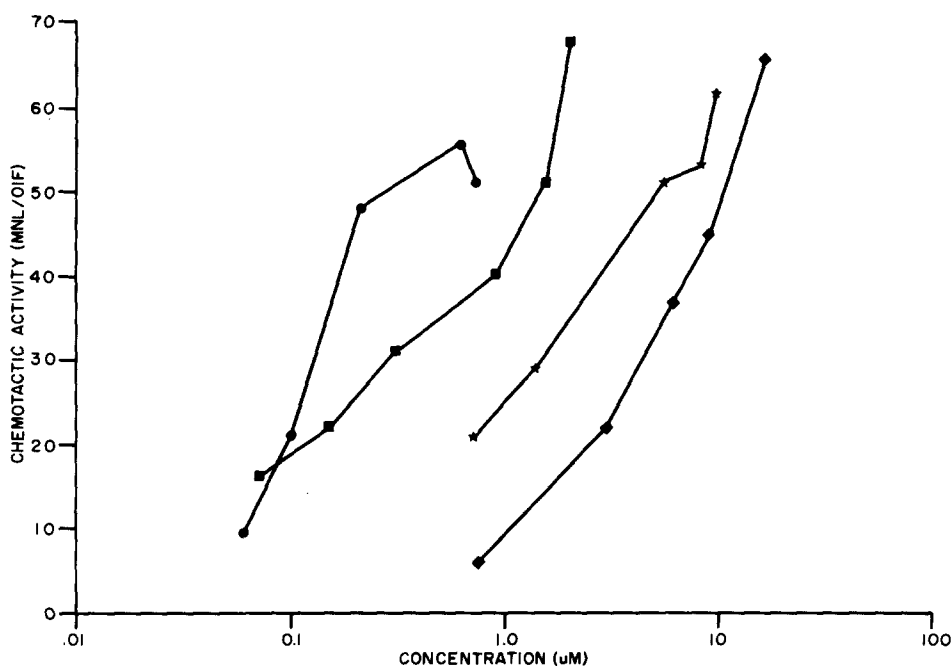


FIG. 1. Chemotactic response of human monocytes to human skin collagen (●—●), chick skin collagen (■—■), the  $\alpha$ 1-chain (★—★), and the  $\alpha$ 2-chain (◆—◆) of chick skin is concentration dependent. Points represent the observed chemotactic activity minus the activity of buffer control ( $9 \pm 2$ ). Standard error for each original value was less than 15%.

TABLE III  
The Effect of Bacterial Collagenase Treatment of Collagen on the Chemotactic Property\*

Substance tested	Chemotactic activity (mean $\pm$ SEM)
	<i>MNL's/OIF</i>
Collagen (280 $\mu$ g/ml)	66 $\pm$ 3
Collagenase-digested collagen (280 $\mu$ g/ml)	61 $\pm$ 7
Collagenase control	4 $\pm$ 1
Buffer	3 $\pm$ 1
Human serum control (25%)	77 $\pm$ 9

\* See Materials and Methods for conditions of collagenase digestion.

tically active. The data also suggest that such sequence(s) must be common to several regions of the collagen chains, since peptides of varying sizes are chemotactically active.

**Amino Acids.** Inasmuch as chemotactic activity was retained by small peptides obtained from  $\alpha$ -chains (see Fig. 2), it was of interest to determine whether individual amino acids unique or common to collagen were chemotactic for monocytes. *l*-Lysine, *l*-histidine, *l*-tryptophan, *d,l*-4-hydroxyproline, *d,l*-allohydroxylysine, *l*-glycine, *l*-glutamine, and *l*-proline were individually as-

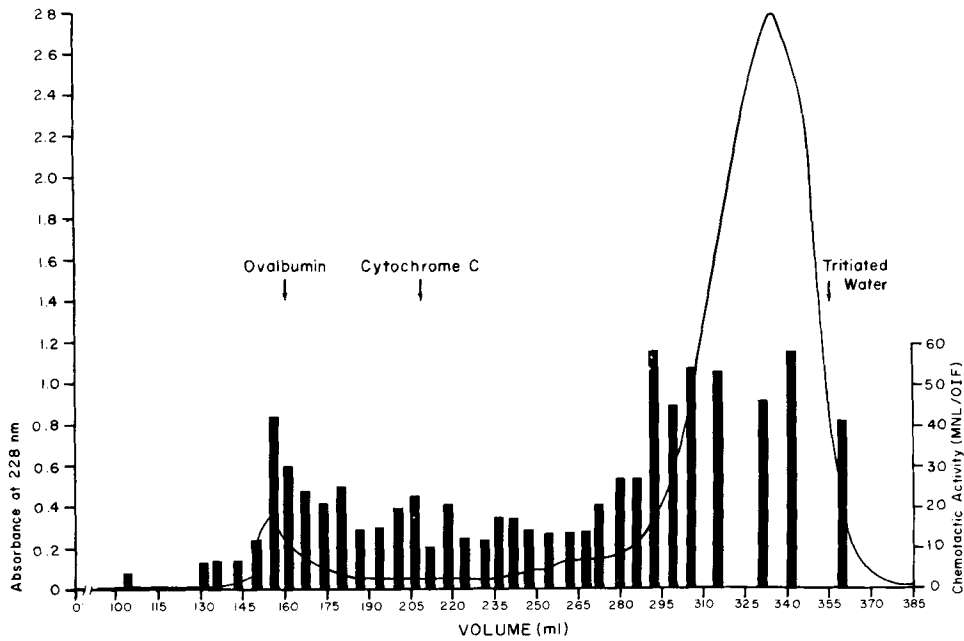


FIG. 2. Lathyritic chick skin collagen (60 mg) dissolved in 23 ml GGBS, pH 7.2, containing 0.001 M  $\text{CaCl}_2$  was digested with bacterial collagenase (600  $\mu\text{g}$ ), lyophilized, and redissolved in 3.5 ml of the same buffer and applied to a  $2.5 \times 100\text{-cm}$  column of Sephadex G-50. Fractions of 5.2 ml were collected, and aliquots were assayed for chemotactic activity for human monocytes. The solid line indicates the absorbance at 228 nm, and the bars indicate the chemotactic activity per milliliter aliquots. The vertical arrows mark the elution positions of the substances indicated. Activity was present in virtually all fractions eluting after the ovalbumin marker.

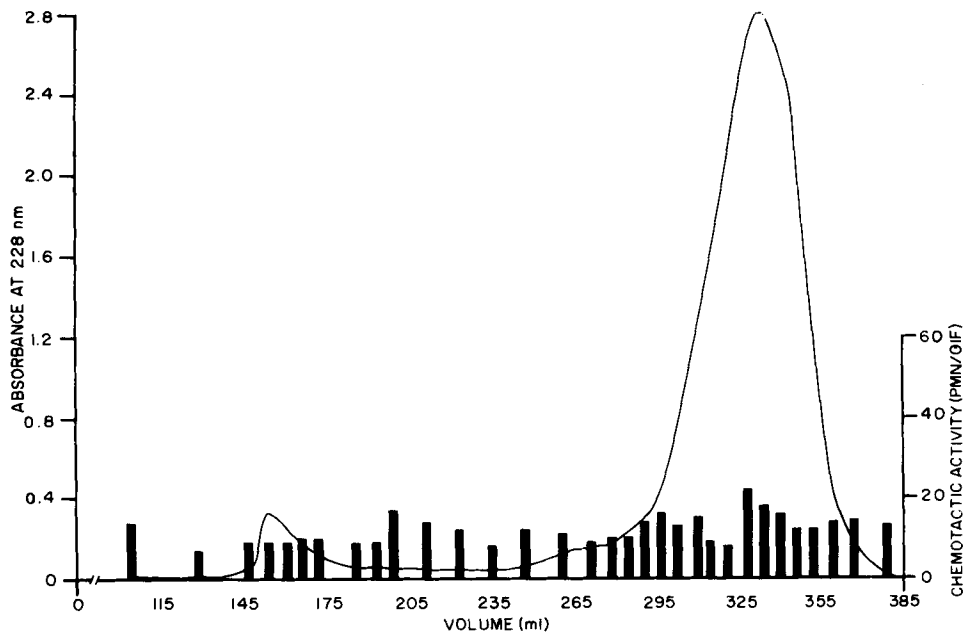


FIG. 3. The effluent fractions from the same experiment presented in Fig. 2 were also assayed for chemotactic activity for human neutrophils. No significant activity above that of buffer control was observed in any of the fractions. A positive control for the experiment (25% normal human serum) was  $52 \pm 8$ .

TABLE IV  
Chemotactic Assay of Degraded  $\alpha$ -Chains

Substance tested	Concentration	Chemotactic activity (mean $\pm$ SEM)	
		Monocyte	Neutrophil
	$\mu\text{g/ml}$	<i>MNL's/OIF</i>	<i>PMN's/OIF</i>
$\alpha 1$ CNBr	1.33	46 $\pm$ 2	7 $\pm$ 1
	0.67	31 $\pm$ 1	8 $\pm$ 1
$\alpha 2$ CNBr	1.33	31 $\pm$ 4	7 $\pm$ 1
	0.67	22 $\pm$ 2	7 $\pm$ 2
$\alpha 1$ Pepsin	1.84	65 $\pm$ 3	9 $\pm$ 2
	0.92	30 $\pm$ 2	6 $\pm$ 1
$\alpha 2$ Pepsin	1.84	49 $\pm$ 5	7 $\pm$ 1
	0.92	19 $\pm$ 1	5 $\pm$ 1
Pepsin control		7 $\pm$ 1	8 $\pm$ 1
CNBr control ( $\alpha 1$ )		6 $\pm$ 2	7 $\pm$ 1
CNBr control ( $\alpha 2$ )		7 $\pm$ 2	9 $\pm$ 1
Buffer control		8 $\pm$ 1	6 $\pm$ 2
Human serum control (25%)		83 $\pm$ 11	46 $\pm$ 6

sayed for monocyte chemotactic activity at concentrations of 100 and 10  $\mu\text{M}$ . None of these amino acids were chemotactic for monocytes (data not shown), indicating that the chemotactic property of collagen peptides is not ascribable to any single amino acid residue of collagen, but rather to some unique covalent sequences contained in the protein.

*Noncollagenous Proteins and Peptides.* To determine whether the chemotactic response of monocytes to collagen was specific or shared by other proteins and peptides, several well characterized proteins including bovine serum albumin, ovalbumin, and cytochrome C were tested for chemotaxis before and after degradation with pepsin. None of the proteins or their peptic degradation products was chemotactic for human monocytes (data not shown).

### Discussion

In this report, data are presented that indicate that type I collagen from human and chick skin is chemotactic for human peripheral blood monocytes *in vitro*. Type I is the most abundant collagen in most connective tissues of man. It appears that the tertiary structure of collagen is important for its chemotactic property, since native collagen is more potent than the denatured  $\alpha$ -chains. In addition, however, the covalent structure of the protein also plays a role since isolated  $\alpha$ -chains, as well as smaller peptides derived from degradation of collagen by digestion with CNBr, pepsin, or bacterial collagenase, are also

chemotactic for monocytes. In particular, the finding that peptides of various sizes obtained by bacterial collagenase treatment of collagen are chemotactic for monocytes suggests that many regions of the collagen chains contain amino acid sequences that are capable of eliciting a monocyte chemotactic response.

Our results that neither native collagen nor peptides derived from it possess chemotactic activity *in vitro* for neutrophils are in agreement with *in vitro* and *in vivo* chemotaxis studies reported by Stecher (16). However, neither our findings nor those reported by Stecher support an earlier report by Chang and Houck (17) that rat collagen was chemotactic for rat neutrophils *in vivo*. The reasons for the discrepant results are not clear.

In normal connective tissues, collagen fibrils are tightly packed into larger bundles or fibers. Mucopolysaccharides are closely associated with collagen fibers. Thus, in normal situations, collagen may be effectively "shielded" from monocytes and apparently does not present a chemotactic stimulus to monocytes. However, at sites of tissue damage and inflammation, the collagen fibers may become "unshielded" by the action of various lysosomal glycosidases and hydrolases and be degraded by specific collagenases. Such lysosomal enzymes capable of degrading mucopolysaccharides, as well as proteins, have been demonstrated in human neutrophils (18), and specific collagenases capable of digesting native tropocollagen molecules and collagen fibrils have been isolated from neutrophils and several inflamed tissues (11, 19). In addition it has recently been found that a lymphokine can cause macrophages to release a collagenase (20). Once collagen is initially cleaved by a specific collagenase, the physicochemical properties of the digestion products are such that they denature under the physiologic conditions of body temperature, pH, and ionic strength, and the products can be degraded further by the action of nonspecific proteases (21). Our results obtained in this study suggest that such collagen degradation products might serve as chemotactic stimuli for peripheral blood monocytes *in vivo*.

The relative importance of collagen-derived peptides as chemotactic stimuli for monocytes as compared with C5a generated in complement-mediated inflammation, the lymphocyte-derived chemotactic factor liberated in cell-mediated immune reactions, or other factors in various types of inflammation is not established. However, collagen-derived peptides might act in concert with these factors to promote the influx of monocytes into an area of inflammation.

### Summary

The ability of collagen and collagen-derived peptides to act as chemotactic stimuli was investigated by *in vitro* chemotaxis assays. Native human and chick skin collagen (type I) and  $\alpha$ -chains obtained from purified chick skin collagen were each chemotactic for human peripheral blood monocytes. In addition, smaller peptides obtained either by digesting native collagen with bacterial collagenase or by degrading purified  $\alpha$ -chains with cyanogen bromide or pepsin were also chemotactic for monocytes. In contrast, native collagen,  $\alpha$ -chains, and smaller collagen-derived peptides were not chemotactic for human neutrophils. Since collagen is degraded at sites of tissue damage and inflammation, our findings suggest the possibility that such collagen-derived degradation products might directly serve as chemotactic stimuli for human peripheral blood monocytes *in vivo*.



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