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Collagen activates superoxide anion production by human polymorphonuclear neutrophils

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Human polymorphonuclear neutrophils (PMNs), purified on Ficoll–Hypaque cushions, were incubated for 5 min with calf skin acid-soluble collagen and the released superoxide anions (O_2^-) measured spectrophotometrically by reduction of ferricytochrome *c* or by chemiluminescence analysis. This collagen stimulated the release of O_2^- unless it had been treated with pepsin. The stimulatory activity remained in denatured collagen, was contained only in the α1(I) chain and was present in the α1(I)-CB 6 (CNBr-cleaved) peptide, which is *C*-terminal. The activity was linearly dependent on the collagen concentration up to about 200 μg/ml. In addition, this collagen induced a release of β-glucuronidase and *N*-acetyl-β-glucosaminidase from PMNs.

INTRODUCTION

Native soluble collagen and collagen degradation products are chemotactic for PMNs *in vivo* (Chang & Houck, 1970; Riley *et al.*, 1984). Laskin *et al.* (1986) showed recently that low-M_r synthetic polypeptides (consisting of triplet units of proline, hydroxyproline and glycine), bovine dermal collagen digested with bacterial collagenase and type I calf skin collagen fragments produced with CNBr are all potent chemoattractants for human peripheral-blood neutrophils. As many factors, such as complement components C3a and C5a, arachidonic acid and bacterial polypeptides, are both chemotactic factors and activators for the generation of O_2^- by PMNs, type I collagen itself should induce the formation of oxygen free radicals by PMNs. We report here that bovine acid-soluble collagen and one of the peptides obtained on its digestion with CNBr do trigger superoxide anion (O_2^-) formation by human PMNs *in vitro*. This activation varies with the collagen concentration, is modulated by the extracellular Ca^{2+} concentration and is located in a sequence of the α1(I) chain comprising a helical segment and the *C*-terminal telopeptide.

MATERIALS AND METHODS

Reagents

Ferricytochrome *c* (type VI), FMLP, SOD from bovine erythrocytes, NEM, PMSF and *p*-nitrophenyl β-D-glucuronide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and 3-aminophthalhydrazide (luminol) were bought from Aldrich (Strasbourg, France).

Acetonitrile (h.p.l.c. grade) was obtained from BDH Chemicals (Poole, Dorset, U.K.). Heptafluorobutyric acid (Sequenal grade) was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.) and trifluoroacetic acid (Uvasol grade) was from Merck (Darmstadt, Germany). Deionized water was further purified by filtration through a MilliQ water system (Millipore Corp., Bedford, MA, U.S.A.). All the usual reagents (analytical grade) were obtained from Prolabo (Paris, France) or Merck (Darmstadt, Germany).

PBS (Ca^{2+}-free) and PBS containing 1.3 mM-$CaCl_2$ were prepared as described by Curnutte *et al.* (1979). FMLP was dissolved at a concentration of 1 mg/ml in DMSO. The stock solution was stored at −20 °C and further diluted in PBS before use. Cytochrome *c* was dissolved in PBS (final concn. 1.0 mmol/ml) and SOD was diluted in PBS to a concentration of 0.1 mg/ml, corresponding to an activity of 60 units/ml; units of SOD are defined as described by McCord & Fridovich (1969).

Preparation of collagens

Acid-soluble collagen was obtained from calf skin by extraction with 0.5 M-acetic acid and then precipitation with 0.7 M-NaCl at acidic pH by the method of Piez *et al.* (1963).

The α-chains from calf skin acid-soluble (non-pepsin-treated) collagen were purified by gel-filtration chromatography on Agarose A5M (Bio-Rad, Richmond, CA, U.S.A.) in 0.05 M-Tris/HCl buffer, pH 7.4, containing 1 M-$CaCl_2$. The fraction containing the α-chains was further chromatographed on CM-cellulose under denaturing conditions (Pontz *et al.*, 1973) in order to prepare pure α1(I) and α2(I) collagen chains. The TC^A and TC^B fragments obtained in native form by tadpole-collagenase digestion of bovine type I collagen were a gift from Dr. Y. Nagai (Medical Research Institute, Tokyo, Japan).

CNBr cleavage of α1(I) chains prepared from calf skin acid-soluble collagen was done by the method of Epstein

Abbreviations used: CM-cellulose, carboxymethylcellulose; CB-peptide, CNBr-cleaved peptide; DMSO, dimethyl sulphoxide; NEM, *N*-ethylmaleimide; FMLP, *N*-formylmethionyl-leucylphenylalanine; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline (135 mM-NaCl/5 mM-KCl/10 mM-potassium phosphate, pH 7.4); PMN, polymorphonuclear neutrophil; PAGE, polyacrylamide-gel electrophoresis; SOD, superoxide dismutase; O_2^-, superoxide anion; TC^A and TC^B, tadpole-collagenase fragments A and B.

et al. (1971) in 70% (v/v) formic acid under an N_2 atmosphere for 4 h. The resulting CB-peptides were thoroughly dialysed against distilled water. The remaining non-diffusible peptides were separated by reverse-phase h.p.l.c. in a system comprising two pumps (Altex A 110), a gradient programming system controlled by an Apple IIe computer and a Rheodyne 7125 injection valve, all bought from Touzart et Matignon (Paris, France). The column was an Aquapore RP 300 C_{18} (particle size 10 μm) (4.6 mm × 250 mm) from Brownlee Laboratories (Santa Clara, CA, U.S.A.). The eluate was monitored at 214 nm by a model SPD-2A spectrophotometer (Shimadzu, Kyoto, Japan). The method was a modification of that described by Van der Rest & Fietzek (1982).

The purity of collagen fractions and α-chains was checked by PAGE performed by the method of Laemmli (1970) in slab gels containing 5% (w/v) polyacrylamide. The CB-peptides were characterized by the same technique, with the percentage of polyacrylamide raised to 12.5 (w/v).

Acid-soluble collagen was submitted to limited pepsin digestion, at an enzyme/substrate ratio of 1:1000 (w/w) in 0.5 M-acetic acid at 4 °C for 18 h, by the method of Chung *et al.* (1974). The pepsin was then inactivated by dialysis against PBS. Denatured acid-soluble collagen was obtained by heating the collagen solution at 60 °C for 45 min. Several preparations of acid-soluble collagen were also delipidated by extraction with chloroform/methanol (2:1, v/v) at 4 °C for three periods of 24 h each.

Acid-soluble collagen was degraded with *Clostridium histolyticum* collagenase (CLSPA; Worthington, Freehold, NJ, U.S.A.) purified by the method of Peterkofsky & Diegelman (1971) and treated with proteinase inhibitors (NEM and PMSF). Collagenase was then removed by ultrafiltration with microcollodion bags (Sartorius, Göttingen, Germany). The amounts of collagen hydrolysates added to test tubes were calculated so as to yield the same hydroxyproline concentration as in whole collagen.

Preparation of the neutrophil suspension

Human PMNs were purified by the method of Markert *et al.* (1984), with minor modifications. As described by Homan-Müller *et al.* (1975), the contaminating erythrocytes were lysed by a hypo-osmotic buffer (10 mM-$KHCO_3$, 15 mM-NH_4Cl, 0.14 mM-EDTA, pH 7.2). A suspension of 10^7 PMNs/ml in balanced Hanks solution was used for the experiments. It contained 96±4% PMNs, with a viability greater than 95% as assessed by Trypan Blue exclusion. The contaminating cells were lymphocytes.

Assessment of O_2^- released by ferricytochrome *c* reduction

O_2^- release was measured by the method of English *et al.* (1981). The cell suspension (100 μl) was prewarmed to 37 °C for 5 min and transferrred to 13 mm × 100 mm glass tubes containing 0.85 ml of PBS and 0.1 ml of cytochrome *c* solution. Activation was started by adding 0.1 ml of solution of the stimulating agent, either FMLP (final concn. 0.5 μM) or collagen (final concn. 12.5–400 μg/ml). For multiple determinations, the required numbers of replicates (five) were prepared, containing identical quantities of every reagent. As a control in each experimental category, SOD (50 μl) was added to one of the test tubes before the addition of cells. This was utilized as the spectrophotometric blank. At 5 min after the start of incubation, SOD (50 μl) was added to stop the reduction of cytochrome *c* by O_2^- in all except the control tubes. The tubes were then cooled to 4 °C and centrifuged. Reduced cytochrome *c* was assayed in supernatants by measuring the $A_{549.5}$ in a Beckman DU 40 spectrophotometer against the SOD-inhibited blank. O_2^- concentrations were calculated by using an ϵ_{mM} value of 15.5 $mM^{-1} \cdot cm^{-1}$ (ferrocytochrome *c* minus ferricytochrome *c*) according to the method of Margoliash & Frohwirt (1959) and expressed in mmol of O_2^-/5 min per 10^6 cells.

Assessment of O_2^- release by luminol-dependent chemiluminescence analysis

PMN chemiluminescence assays were performed by the method of Tenner & Cooper (1982). Portions of PMN suspension (0.1 ml) containing 10^6 cells, 0.1 ml of collagen suspension and 0.02 ml of 1 mM-luminol solution in 0.1 M-NaOH were added to 0.78 ml of PBS/0.02 M-Hepes/0.25% bovine serum albumin in 6 ml polypropylene Picovials (Packard, Rungis, Paris, France). Chemiluminescence was monitored every 0.5 min for 30 min in a Packard 4430 liquid-scintillation counter. Chemiluminescence values were expressed as c.p.m. per 10^6 cells after subtraction of background chemiluminescence.

Enzyme release from PMNs

Portions (0.1 ml) of acid-soluble collagen solution were added to 0.2 ml of PMN suspension containing 10^6 cells in PBS supplemented with 1.3 mM-$CaCl_2$ and incubated for 20 min at 37 °C. The tubes were rapidly cooled, centrifuged at 800 *g* for 10 min, and the enzyme activities measured in supernatant and in the remaining cell pellet, repeatedly frozen and thawed.

Lactate dehydrogenase was measured by the method described by Buhl *et al.* (1978), β-glucuronidase and *N*-acetyl-β-glucosaminidase respectively with *p*-nitrophenyl β-D-glucuronide and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside as substrates (Troost *et al.*, 1976). Results (means of four determinations) were expressed as percentages of the total (cell + supernatant) enzymic activity.

RESULTS

Acid-soluble collagen stimulation of PMNs

The contact of PMNs with acid-soluble collagen molecules induced the production of O_2^-, which, monitored by the ferricytochrome *c* reaction, was linear for 8 min (Fig. 1). The amount of O_2^- formed in response to acid-soluble collagen at 0.3 μmol/l was comparable with that elicited by 0.5 μM-FMLP (11.85±0.85 as against 12.55±0.57 nmol of O_2^-/5 min per 10^6 cells). Pepsin-treated collagen was devoid of activity.

The generation of O_2^- by PMNs increased with the concentration of non-pepsin-treated collagen up to a maximum at 200 μg/ml. Heat-denatured collagen had the same effect (Table 1). At concentrations over 200 μg/ml the collagen molecules began to precipitate

into fibrils. Activation was more efficient when Ca^{2+} at 1.3 mmol/l was added to the solution.

The luminol-dependent chemiluminescent response of PMNs in the presence of 0.3 μM acid-soluble collagen is shown in Fig. 2.

Enzyme release from PMN under collagen stimulation

The percentage of activity of lactate dehydrogenase released in supernatant was unchanged after stimulation by acid-soluble collagen, whereas percentages of β-glucuronidase and *N*-acetylglucosaminidase were significantly increased (Table 2).

Determination of the active fragment of acid-soluble collagen

Heat-denatured and delipidated calf skin acid-soluble collagens were active. They triggered roughly the same amounts of O_2^- as did the non-pepsin-treated calf skin collagen. Both pepsin digestion and bacterial-collagenase treatment suppressed the induction of O_2^- production. When the fragments TC^A and TC^B prepared from type I collagen by the action of tadpole collagenase were tested, the TC^B fragment was active, whereas the TC^A fragment was not. The α1 chain purified from acid-soluble collagen was active, and the α2 chain was not. The non-diffusible CB-peptides induced the production of even more O_2^- than did the collagen itself (19.65 ± 2.09 as against 11.98 ± 0.85 nmol of O_2^-/5 min per 10^6 cells).

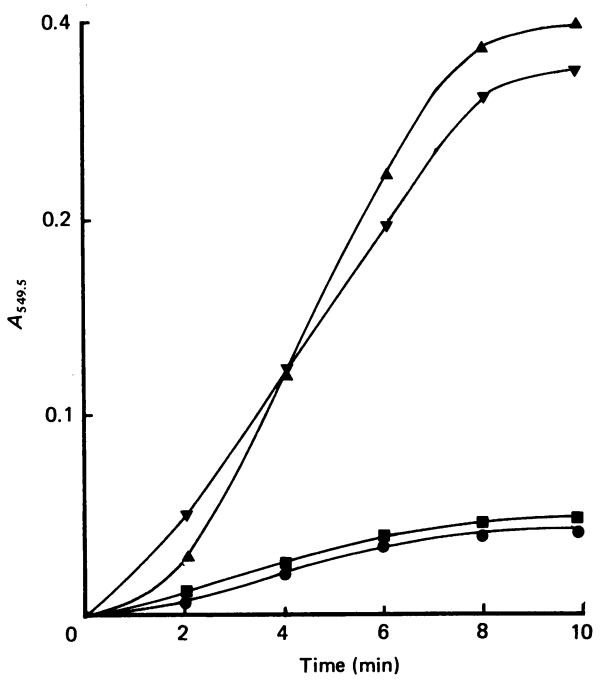

Fig. 1. Continuous monitoring of O_2^- production by collagen-activated PMNs evaluated by ferricytochrome *c* reduction

■, Non-activated PMNs; ●, pepsin-treated acid-soluble collagen (0.3 μM); ▼, acid-soluble collagen (0.3 μM); ▲, FMLP (0.5 μM). Collagen or FMLP solution (0.1 ml) was added to the incubation medium. The reduction of ferricytochrome *c* was monitored at 549.5 nm against a spectrophotometric blank supplemented with SOD before the addition of the cell suspension.

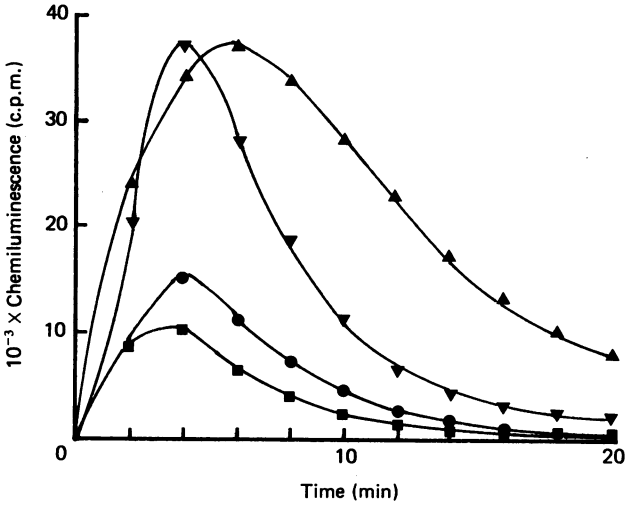

Fig. 2. Luminol-dependent chemiluminescent response of PMNs activated by collagen

■, Non-activated PMNs; ●, pepsin-treated acid-soluble collagen (0.3 μM); ▼, acid-soluble collagen (0.3 μM); ▲, FMLP (0.5 μM).

Table 1. Dose-dependent O_2^- generation by neutrophils activated by acid-soluble collagen in the presence or in the absence of extracellular calcium

Results are means ± 1 S.D. (n = 4).

Concn. of collagen or gelatin ($\mu g \cdot ml^{-1}$)	Production of O_2^- (nmol/5 min per 10^6 cells)			
	Acid-soluble collagen without Ca^{2+}	Gelatin without Ca^{2+}	Acid-soluble collagen with Ca^{2+}, 1.3 mM	Gelatin with Ca^{2+}, 1.3 mM
0	1.27 ± 0.73	1.27 ± 0.73	1.74 ± 0.52	1.74 ± 0.52
12.5	1.76 ± 0.24	1.57 ± 0.25	2.40 ± 0.21	2.10 ± 0.47
25	2.63 ± 0.19	1.86 ± 0.19	3.79 ± 0.23	2.76 ± 0.10
50	5.01 ± 0.25	3.06 ± 0.15	6.15 ± 0.10	5.55 ± 0.34
100	8.63 ± 0.34	7.30 ± 0.53	10.51 ± 0.50	10.54 ± 0.58
200	10.23 ± 0.22	9.18 ± 0.32	15.12 ± 0.25	15.48 ± 0.56
300	2.05 ± 0.41	8.23 ± 0.63	4.45 ± 0.26	12.69 ± 0.44
400	1.84 ± 0.31	8.31 ± 0.33	2.56 ± 0.39	11.90 ± 0.96

Table 2. Enzyme activities released in the supernatant after activation of PMNs by collagen, expressed as percentages of total (cell + supernatant) activity

Differences from non-activated PMNs significant for: $*P < 0.01$; $**P < 0.001$. NS, not significant.

	Lactate dehydrogenase	β-Glucuronidase	N-Acetyl-β-glucosaminidase
Non-activated PMN	3.12±1.15	3.80±2.27	5.98±0.45
FMLP (0.5 μM)	4.82±0.92 NS	12.75±1.63*	11.31±0.83**
Acid-soluble collagen (0.3 μM)	2.25±1.90 NS	17.50±3.11*	16.34±1.15**
Pepsin-treated acid-soluble collagen (0.3 μM)	5.13±0.77 NS	2.75±1.20 NS	5.46±0.63 NS
CB-peptides from acid-soluble collagen (100 μg/ml)	2.67±1.19 NS	16.35±0.35**	12.79±0.87**

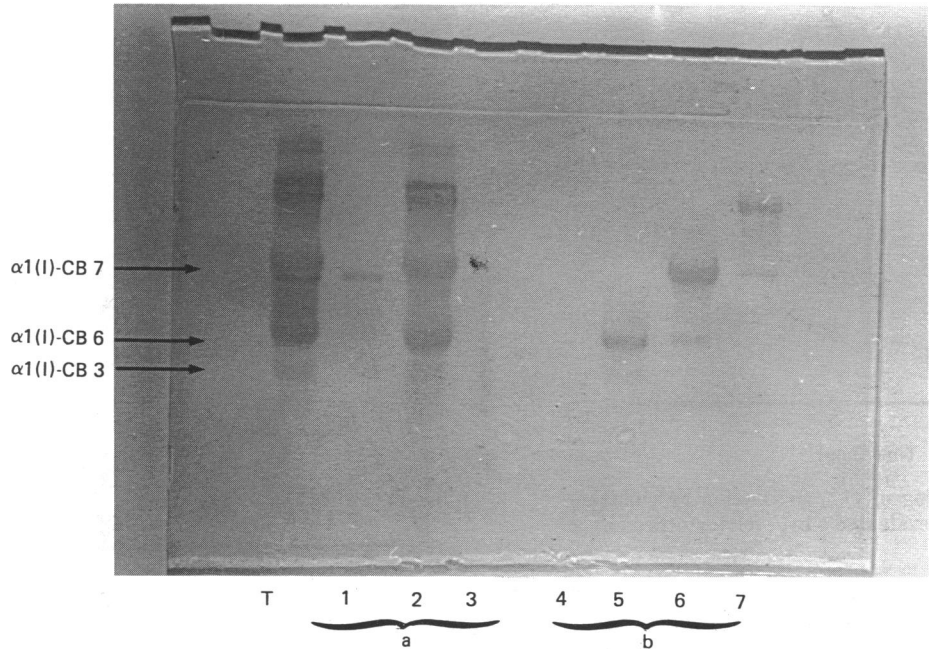

Fig. 3. SDS/PAGE of the non-diffusible CB-peptides from α1(I) chain: separation by two successive reverse-phase h.p.l.c. steps

T, non-diffusible CB-peptides of the collagen α1(I) chain; a, lanes 1, 2 and 3, material from the three peaks of the first h.p.l.c. step: b, lanes 4, 5, 6 and 7, material from the four peaks of the second h.p.l.c. step. The fractions in lanes 2 and 5 activated O_2^- production by PMNs. Lane 6 induces a very slight activation, owing to contamination.

We isolated the active CB-peptide by reverse-phase h.p.l.c. of the non-diffusible CB-peptides. The first step, in trifluoroacetic acid, separated three peaks; the activity was contained in the second one. SDS/PAGE showed that this peak contained peptides α1(I)-CB 6 and α1(I)-CB 7 plus some higher-M_r components (Fig. 3, a). The active peak was rechromatographed by reverse-phase h.p.l.c. in the same column, but this time in hepta-fluorobutyric acid, four fractions being separated. The O_2^--stimulating activity was found in the second fraction, which contained the peptide α1(I)-CB 6 (Fig. 3, b).

DISCUSSION

The activation of PMNs results in several distinct morphological and metabolic events, all leading to phagocytosis and degradation of foreign substances. Among them is the formation and secretion of oxygen free radicals contemporaneous with a sudden rise in the consumption of oxygen, known as the 'respiratory burst' and triggered by many stimuli. The liberation of O_2^- is easily monitored by a technique using ferricytochrome *c* and by measurement of chemiluminescence.

The family of collagen molecules, particularly of the interstitial type such as type I, is known for its chemoattractant properties (Chang & Houck, 1970; Riley *et al.*, 1984; Laskin *et al.*, 1986). Most other chemoattractant substances, such as FMLP, also stimulate the activation of PMNs. Some authors (Hoffstein *et al.*, 1985) have suggested that any effect of collagen as trigger of the PMN respiratory burst could be ruled out on the basis of experiments conducted with pepsin-treated collagen. However, we show here that acid-soluble collagen, or its main constituent, type I

collagen, when purified without the use of pepsin, is actually as efficient an activator as FMLP. This stimulatory effect depends on the presence of the telopeptides of collagen, as demonstrated by its disappearance when acid-soluble collagen had been treated with pepsin. The effect is dose-dependent up to a limit which appears to correspond to the collagen concentration above which the fibrils precipitate spontaneously. In addition to this precipitation, large amounts of collagen serve as scavengers for O_2^- (Monboisse *et al.*, 1986).

It was found that only the α1(I) chain of collagen was responsible for the effect. Denatured collagen was still active, suggesting that the primary structure of the molecule was responsible for the activity. The telopeptide sequence liberated by treatment of collagen by purified bacterial collagenase was not capable of triggering the activation, but the TC^B peptide was. In order to locate the active region more precisely, we fragmented the collagen molecule with CNBr and assayed the resulting CB-peptides for their ability to stimulate PMN. A modified h.p.l.c. method of CB-peptide separation (Van der Rest & Fietzek, 1982) was used to prepare the peptide α1(I)-CB 6 and purify it from the contaminating α1(I)-CB 3 and α1(I)-CB 7 peptides. α1(I)-CB 6 was the only active CB-peptide.

The location of the active region at the *C*-terminal end of the type I collagen molecule may be fairly close to the region that controls platelet binding (Fauvel *et al.*, 1978), but the latter site remained active after pepsin treatment of the collagen. In addition, Morton & Barnes (1986) recently demonstrated the presence of several other platelet-activating regions in the collagen molecule.

How type I collagen activates PMNs is not yet known. It seems to trigger O_2^- and lysosomal enzyme liberation simultaneously, but it remains to be demonstrated whether there is an effect on the formation of eicosanoids.

The physiological significance of this effect may be of interest with regard to inflammation. The contact of PMNs with altered connective tissues would trigger the degradation reactions characteristic of the first stages of any inflammation reaction. It would provide a link between the accumulation of PMNs in inflamed tissues, owing to the chemotactic effect of several molecules (among them products of degradation of collagen) and the cleaning effect of oxygen free radicals produced by these PMNs.

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