Activation of human neutrophils by type I collagen

Requirement of two different sequences

Jean-Claude MONBOISSE,* Georges BELLON,* Alain RANDOUX,* Jean DUFER† and Jacques-Paul BOREL*

* Laboratory of Biochemistry, CNRS URA 610, UFR Medicine, 51 rue Cognacq-Jay, F51095 Reims Cedex, and

† Laboratory of Hematology, Institut Jean Godinot, 45 rue Cognacq-Jay, F51062 Reims Cedex, France

Contact between type I collagen purified from several species and human polymorphonuclear neutrophils (PMNs) triggers the production of $O_2^{\cdot-}$ by these cells. The activity of collagen is located in the $\alpha_1(I)$ -CB6 cyanogen bromide-cleaved (CB)-peptide, which is the C-terminal CB-peptide of the $\alpha_1(I)$ chain. Experiments based on the competitive inhibition of $O_2^{\cdot-}$ production by simultaneous incubation of PMNs with type I collagen and synthetic peptides identical to the conserved sequences of this collagen demonstrated that the binding of collagen to PMNs and the subsequent activation of these cells depend on the simultaneous presence of two sequences: Arg-Gly-Asp [residues 915, 916 and 917 of the complete $\alpha_1(I)$ chain, located in the helical part] Asp-Gly-Gly-Arg-Tyr-Tyr (residues 1034–1039, located in the C-terminal non-helical telopeptide).

INTRODUCTION

The activation of human polymorphonuclear neutrophils (PMNs) by particulate or soluble stimuli constitutes the first stage of phagocytosis. This activation is marked by the secretion of degradative enzymes, either free or contained in granulations, by alterations in the shape and motility of the cells and by the secretion of oxygen free radicals ($O_2^{\cdot-}$) that actively participate in the defensive catabolic phenomena of inflammation (Babior *et al.*, 1973). A number of stimuli may operate in this process.

In a previous paper (Monboisse *et al.*, 1987) we demonstrated that type I collagen when not treated by pepsin can trigger the secretion of $O_2^{\cdot-}$ by PMN to an extent comparable with that induced by bacterial polypeptides such as *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe). The activating part of this collagen is located at the C-terminal end of the $\alpha_1(I)$ chain. Type I collagen is the main form of collagen, existing in broad-diameter fibrils in all interstitial connective tissues, such as skin, tendon, bone, etc. The structural formula of type I collagen is $[\alpha_1(I)]_2[\alpha_2(I)]$. Only the $\alpha_1(I)$ chain, and more precisely the C-terminal peptide $[\alpha_1(I)$ -CB6] separated after cyanogen bromide cleavage, were found to be active on PMNs (Monboisse *et al.*, 1987).

The present paper reports inhibition experiments with synthetic peptides, which demonstrate that the activation of PMNs depends on two distinct peptide sequences at the C-terminal end of the $\alpha_1(I)$ chain: the classical adhesion sequence Arg-Gly-Asp and the sequence Asp-Gly-Gly-Arg-Tyr-Tyr.

MATERIALS AND METHODS

Reagents

Ferricytochrome *c* (type VI), fMet-Leu-Phe, superoxide dismutase (SOD) from bovine erythrocytes and the synthetic peptide Gly-Arg-Gly-Asp were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The other peptides Ser-Asp-Gly-Arg, Arg-Phe-Asp-Ser, Asp-Gly-Gly-Arg, Asp-Gly-Gly-Arg-Tyr-Tyr and Pro-Gln-Pro-Pro-Gln-Glu were prepared specially by Neosystem Laboratories (Strasbourg, France). Acetonitrile

(h.p.l.c. grade) was obtained from BDH Chemicals (Poole, Dorset, U.K.). Trifluoroacetic acid (Uvasol grade) was from Merck (Darmstadt, Germany). All the usual reagents (analytical grade) were purchased from Prolabo (Paris, France) or Merck (Darmstadt, Germany).

Collagens and collagen fractions

Acid-soluble collagen samples were prepared from calf, rabbit, human, rat or chicken skin by extraction with 0.5 M-acetic acid followed by precipitation with 0.7 M-NaCl at pH 4.5 according to the method of Piez *et al.* (1963).

The α -chains from acid-soluble (non-pepsin-treated) collagens were purified by gel-filtration chromatography on Agarose A5M from Bio-Rad (Richmond, CA, U.S.A.) in 0.05 M-Tris/HCl buffer, pH 7.4, containing 1 M-CaCl₂. The fraction containing the α -chains was further chromatographed on CM-cellulose under denaturing conditions (Pontz *et al.*, 1973) in order to prepare pure $\alpha_1(I)$ collagen chains.

Collagen concentrations are expressed in α_1 chain concentrations calculated on the basis of a molecular mass of 100 kDa for one α_1 chain.

C-Terminal CB-peptide of rabbit-skin acid-soluble collagen

Acid-soluble rabbit-skin collagen was treated by CNBr, in accordance with the method of Epstein *et al.* (1971), in 70% (v/v) formic acid under an N₂ atmosphere for 4 h. The resulting CNBr-cleaved peptides (CB-peptides) were evaporated under N₂ and solubilized in 0.1 M-acetic acid. This solution was ultrafiltered under N₂ on YM 10 Diaflo membranes (Grace, Danvers, MA, U.S.A.). The ultrafiltrate was submitted to gel-filtration chromatography on a Trisacryl GF 05 column (2.5 cm × 90 cm) in 0.1 M-acetic acid (Trisacryl GF 05 was from IBF, Paris, France).

The fraction containing the C-terminal CB-peptide was then chromatographed by reverse-phase h.p.l.c. on an Aquapore RP 300 C18 column from Brownlee Laboratory (Santa Clara, CA, U.S.A.). Elution was performed with a linear gradient of acetonitrile (16–40% in 60 min) as previously described (Van der Rest & Fietzek, 1982).

Abbreviations used: CB-peptide, cyanogen bromide-cleaved peptide; fMet-Leu-Phe, *N*-formyl-methionyl-leucyl-phenylalanine; PBS, phosphate-buffered saline (135 mM-NaCl/5 mM-KCl/10 mM-potassium phosphate, pH 7.4); PMN, polymorphonuclear neutrophil; SOD, superoxide dismutase.

Neutrophil suspension

Neutrophils were purified from freshly drawn human blood by centrifugation through the PMN-separation medium Polyprep, prepared by Nycomed (Oslo, Norway) (Ferrante & Thong, 1980). Volumes of 3.5–5.0 ml of whole blood drawn on heparin were layered over 3.5 ml of Polyprep. The samples were centrifuged at 500 *g* for 30 min, and the lower band of leucocytes containing the PMNs were harvested. The cells were rinsed twice with phosphate-buffered saline (PBS) and were resuspended at 10⁷ cells/ml in PBS. PMNs constituted 96 ± 4 % of the cells in the suspension.

Measurement of O₂^{•-} released by PMNs

O₂^{•-} release was measured from the SOD-inhibitable reduction of ferricytochrome *c* according to the method of English *et al.* (1981), as described (Monboisse *et al.*, 1987). The PMN suspension (100 µl containing 10⁶ cells), prewarmed at 37 °C for 5 min, was transferred to 13 mm × 100 mm glass tubes containing 0.85 ml of PBS and 0.1 ml of 1 mM-cytochrome *c* solution. O₂^{•-} release by PMNs was triggered by adding 0.1 ml of 7.7 µM acid-soluble collagen or 7.7 µM-α₁(I) chain solution. In all experiments, the effect of 0.1 ml of 6 µM-fMet-Leu-Phe solution was also tested, to standardize the reactivity of the PMN preparations. Absorbance was monitored at 550 nm for 10 min and the linear portion of the curve was determined. As a control in every experimental category, SOD (50 µl, corresponding to 50 units) was added to one of the test tubes before the addition of cells.

For the inhibition experiments, PMN suspensions were preincubated for 5 min with the synthetic peptides (Gly-Arg-Gly-Asp, Ser-Asp-Gly-Arg, Arg-Phe-Asp-Ser, Asp-Gly-Gly-Arg, Asp-Gly-Gly-Arg-Tyr-Tyr or Pro-Gln-Pro-Pro-Gln-Glu) at 37 °C, and then acid-soluble collagen was added. Incubations and measurements were carried out as just described.

RESULTS

Activation of PMNs by α₁(I) collagen chain

We did not observe any significant differences between PMN O₂^{•-} production triggered by the collagen α₁ chains from various species (Table 1).

The α₁(I) chains purified from calf-skin acid-soluble collagen triggered a dose-dependent activation of O₂^{•-} production by PMNs (Fig. 1), with a maximal effect at 1 µmol/l. At higher α₁(I) concentrations, fibrillation of collagen was observed.

Inhibitory effect of Gly-Arg-Gly-Asp on the activation of PMNs by acid-soluble collagen

When the PMN suspensions had been preincubated for 5 min with Gly-Arg-Gly-Asp before the addition of acid-soluble collagen, O₂^{•-} production was strongly inhibited (Table 2). This peptide at 250 µmol/l inhibited by up to 90 % the O₂^{•-} production triggered by 0.64 µM acid-soluble collagen. The peptide had no significant effect on O₂^{•-} production by non-activated PMNs or by PMNs previously activated with fMet-Leu-Phe. Even at higher concentrations (1 mmol/l), the peptides Ser-Asp-Gly-Arg and Arg-Phe-Asp-Ser had no inhibitory effects on O₂^{•-} production by collagen-activated PMNs.

The Gly-Arg-Gly-Asp-induced inhibition of O₂^{•-} production triggered by collagen was dose-dependent. The peptide concentration giving a 50 % inhibition was 10 µmol/l for 0.64 µM-collagen (Fig. 2). When the data were expressed in a Dixon diagram, the straight lines obtained for different collagen concentrations converged to a single point of negative abscissa and positive ordinate, indicating a competitive inhibition process. The apparent inhibition constant *K*_i was 22 ± 6 µmol/l (Fig. 3).

Table 1. O₂^{•-} production by PMNs activated by acid-soluble non-pepsin-treated collagens from various species

The α₁(I) chain concentration for each collagen was 0.64 µmol/l. Values are means ± S.D. (*n* = 4). * Significantly different from O₂^{•-} production activated by calf-skin collagen (*P* < 0.05).

Activator	O ₂ ^{•-} production (nmol/5 min per 10 ⁶ cells)
Unactivated cells	1.12 ± 0.41*
0.5 µM-fMet-Leu-Phe	9.06 ± 0.85*
Calf skin collagen I†	10.63 ± 0.91
Chicken skin collagen I	9.79 ± 0.76
Human skin collagen I	11.18 ± 1.16
Rabbit skin collagen I	9.53 ± 0.83
Rat skin collagen I	9.86 ± 0.70
Human α ₁ chain	9.15 ± 0.64
Human α ₂ chain	1.05 ± 0.15*

† Result recorded for the sake of comparison; already published in Monboisse *et al.* (1987).

Fig. 1. O₂^{•-} production by PMNs activated by α₁(I) chains of calf-skin acid-soluble collagen

Fibrillation of collagen occurred at chain concentrations greater than 1 µmol/l. Values are means ± S.D. (*n* = 4).

Table 2. Effects of synthetic peptides on O₂^{•-} production by PMNs activated with acid-soluble collagen or fMet-Leu-Phe

PMNs were preincubated for 5 min with the synthetic peptides before the addition of acid-soluble collagen. Values are means ± S.D. (*n* = 4). Significance of differences from O₂^{•-} production by PMNs not preincubated with the synthetic peptides: * *P* < 0.001. Peptides are given in one-letter code for convenience.

Peptide	O ₂ ^{•-} production (nmol/5 min per 10 ⁶ cells)		
	Unactivated PMNs	fMet-Leu-Phe (0.5 µM)	Acid-soluble collagen (0.64 µM)
None (control)	1.12 ± 0.41	9.06 ± 0.85	10.22 ± 0.73
250 µM-GRGD	2.60 ± 0.57	9.52 ± 0.48	2.23 ± 0.74*
250 µM-SDGR	2.35 ± 0.32	11.32 ± 0.94	9.63 ± 0.58
250 µM-RFDS	0.95 ± 0.31	9.20 ± 2.44	11.22 ± 1.20
None	0.78 ± 0.42	13.22 ± 0.68	13.14 ± 0.95
200 µM-DGGR	0.72 ± 0.20	13.40 ± 0.64	6.32 ± 0.35*
200 µM-DGGRYY	1.60 ± 0.55	12.91 ± 0.93	3.50 ± 0.55*
200 µM-PQPPQE	0.48 ± 0.29	13.72 ± 3.08	14.97 ± 1.22

Fig. 2. Effect of the addition of Gly-Arg-Gly-Asp on $O_2^{\cdot-}$ production by PMNs activated by acid-soluble collagen

The $\alpha_1(I)$ chain concentration was $0.64 \mu\text{mol/l}$. Values are means $\pm$ S.D. ($n = 4$).

Effect of the C-terminal CB-peptide of the $\alpha_1(I)$ rabbit collagen chain on $O_2^{\cdot-}$ production by collagen-activated PMNs

The rabbit collagen C-terminal CB-peptide at the concentration $50 \mu\text{g/ml}$ ($25 \mu\text{M}$) inhibited $O_2^{\cdot-}$ production by collagen-activated PMNs (6.75 ± 0.35 versus 10.63 ± 0.91 nmol of $O_2^{\cdot-}/5$ min per 10^6 cells). This peptide incubated with PMNs alone was devoid of effect.

Effect of the peptides Asp-Gly-Gly-Arg and Asp-Gly-Gly-Arg-Tyr-Tyr on $O_2^{\cdot-}$ production by collagen-activated PMNs

The synthetic peptides Asp-Gly-Gly-Arg, Asp-Gly-Gly-Arg-Tyr-Tyr and Pro-Gln-Pro-Pro-Gln-Glu, corresponding to the constant sequences of the C-terminal telopeptides in all tested collagen chains, were tested for inhibitory activity on $O_2^{\cdot-}$ production by collagen-activated PMNs. These peptides incubated with PMNs alone had no triggering effect. Furthermore, when they were incubated with fMet-Leu-Phe-activated PMNs, they had no inhibitory effect on $O_2^{\cdot-}$ production.

When the peptides were added to the PMNs before the addition of acid-soluble collagen, the inhibition of $O_2^{\cdot-}$ production was found to be 52 % and 73 % for $200 \mu\text{M}$ -Asp-Gly-Gly-Arg

Fig. 3. Effect of Gly-Arg-Gly-Asp on $O_2^{\cdot-}$ production by PMNs activated by acid-soluble collagen: Dixon diagram

The $\alpha_1(I)$ chain concentrations were: ■, $0.48 \mu\text{M}$; ▲, $0.64 \mu\text{M}$; ●, $0.96 \mu\text{M}$.

Table 3. Amino acid sequences of the C-terminal telopeptides of $\alpha_1(I)$ and $\alpha_2(I)$ collagen chains from various species

The amino acid sequences of C-terminal telopeptides of $\alpha_1(I)$ and $\alpha_2(I)$ collagen chains are from: calf $\alpha_1(I)$, Scott (1986); chicken $\alpha_1(I)$ and $\alpha_2(I)$, Fuller & Boedtke (1981); human $\alpha_1(I)$, Bernard *et al.* (1983); human $\alpha_2(I)$, De Wet *et al.* (1987); rabbit and rat $\alpha_1(I)$, Rauterberg (1973) (all deduced from cDNA sequencing except for rabbit and rat). (+), activating effect on PMNs; (−), without activating effect. Δ = deletion. Underlined sequences are conserved between species.

Calf $\alpha_1(I)$	-S-G-G-Y-D-L-S-F-L-P-Q-P-P-Q-E-Z-K-A-H-D-G-G-R-Y-Y	(+)
Chicken $\alpha_1(I)$	-S-G-G-F-D-F-S-F-L-P-Q-P-P-Q-E-Δ-K-A-H-D-G-G-R-Y-Y-R-A	(+)
Human $\alpha_1(I)$	-S-A-G-F-D-F-S-F-L-P-Q-P-P-Q-E-Δ-K-A-H-D-G-G-R-Y-Y-R-A	(+)
Rabbit $\alpha_1(I)$	-S-G-G-F-D-I-Δ-F-M-P-E-P-P-E-E-G-K-A-Δ-D-G-G-R-Y-Y-R-A	(+)
Rat $\alpha_1(I)$	-S-G-G-Y-D-F-S-F-L-P-E-P-P-E-E-Q-K-S-Q-D-G-G-R-Y-Y	(+)
Human $\alpha_2(I)$	-G-G-G-Y-D-F-G-Y-D-G-D-Δ-Δ-Δ-Δ-Δ-Δ-Δ-Δ-Δ-Δ-F-Y-R-A	(−)
Chicken $\alpha_2(I)$	-G-G-G-Y-E-V-G-F-D-A-E-Δ-Δ-Δ-Δ-Δ-Δ-Δ-Δ-Δ-Δ-Y-Y-R-A	(−)

Fig. 4. Dose-dependent inhibition induced by addition of peptides Asp-Gly-Gly-Arg (●) and Asp-Gly-Gly-Arg-Tyr-Tyr (■) on $O_2^{\cdot-}$ production by PMNs activated by acid-soluble collagen.

The $\alpha_1(I)$ chain concentration was $0.64 \mu M$. Values are means $\pm$ S.D. ($n = 4$).

and $200 \mu M$ -Asp-Gly-Gly-Arg-Tyr-Tyr respectively (Table 2) and this inhibition was dose-dependent. The inhibition triggered by Asp-Gly-Gly-Arg did not exceed 60% even for peptide concentrations as high as $500 \mu mol/l$ (Fig. 4). Asp-Gly-Gly-Arg-Tyr-Tyr induced an inhibition up to 85% at $500 \mu mol/l$. The concentration of this peptide giving 50% inhibition was $100 \mu mol/l$ for a $0.64 \mu M$ - $\alpha_1(I)$ chain concentration. The simultaneous incubation of PMNs with $200 \mu M$ -Asp-Gly-Gly-Arg-Tyr-Tyr and $50 \mu M$ -Gly-Arg-Gly-Asp did not induce any significant $O_2^{\cdot-}$ production (1.15 ± 0.62 versus 1.12 ± 0.41 nmol of $O_2^{\cdot-}/5$ min per 10^6 cells by non-activated cells).

Pro-Gln-Pro-Pro-Gln-Glu did not induce any significant production of $O_2^{\cdot-}$ when incubated with PMNs alone and did not even trigger any inhibiting effect on the production of $O_2^{\cdot-}$ by collagen-activated PMNs.

DISCUSSION

When acid-soluble non-pepsin-treated collagen was added to PMNs in an appropriate medium, these cells were triggered to secrete superoxide radicals, as indicated by the evaluation of SOD-inhibitable ferricytochrome *c* reduction. This type of interaction suggests that the collagen molecule is recognized by a receptor on the PMN membrane. Before attempting to isolate the receptor, we wished to find out whether a particular peptide sequence of the collagen molecule was responsible for the interaction with PMN.

The earlier demonstration (Monboisse *et al.*, 1987) that the stimulus was in the $\alpha_1(I)$ -CB6 peptide, known to be the C-terminal peptide cleaved by CNBr from the α_1 chain, prompted us to evaluate the contribution of the well-known sequence Arg-Gly-Asp which is contained in this peptide [residues 915, 916, and 917 of the helical part of human $\alpha_1(I)$ chain]. As might be expected, the synthetic peptide Gly-Arg-Gly-Asp, added to the system of PMNs plus collagen, competitively inhibited activation, thus establishing the participation of the Arg-Gly-Asp sequence.

Nevertheless, this sequence alone does not account for the interaction of collagen with PMNs, because it is contained in the helical part of the molecule, which resists pepsin digestion. As stated above, pepsin-treated collagen is no longer active towards PMNs. The presence of another interacting sequence in the C-terminal telopeptide, which is sensitive to pepsin, was probable.

As the complete sequences of the α chains of collagens from several species have been determined, it was easy to see that the sequences of the C-terminal telopeptides of the $\alpha_1(I)$ chains that were active on PMN contained two conserved sequences in all species (Table 3). The first sequence Pro-Gln-Pro-Pro-Gln-Glu (PQQPQE), was ruled out, because it was not capable of inducing inhibition of $O_2^{\cdot-}$ production by collagen-activated PMNs. The other conserved sequence was Asp-Gly-Gly-Arg-Tyr-Tyr (DGGRYY), present in all active $\alpha_1(I)$ chains. This peptide, obtained by synthesis, was found to inhibit the activation of PMNs by collagen (but not that induced by fMet-Leu-Phe). The peptide Asp-Gly-Gly-Arg was also inhibitory, but to a lesser extent. The sequence Asp-Gly-Gly-Arg is somewhat related to Arg-Gly-Asp, with an inversion and an intercalation of a second glycine. This seems to be the first time that such a sequence has been detected as a point of interaction between an extracellular matrix protein and a cell membrane.

The necessity of the sequence Asp-Gly-Gly-Arg-Tyr-Tyr for the attachment of the $\alpha_1(I)$ chain of collagen to a PMN explains why the $\alpha_2(I)$ chain is inactive: this chain contains an Arg-Gly-Asp sequence in the helical part but no Asp-Gly-Gly-Arg-Tyr-Tyr in the C-terminal telopeptide. On the other hand, the sequences Arg-Gly-Asp and Asp-Gly-Gly-Arg-Tyr-Tyr must be attached to the same polypeptide in order to activate PMNs. When these synthetic peptides were simultaneously added to PMN, they did not trigger the formation of $O_2^{\cdot-}$.

The two-point attachment of collagen to PMN membranes may be mediated by a double protein receptor. There are now many known examples of receptors being activated by being gathered into the same area of the membrane. For instance receptors of growth factors such as epidermal growth factor seem to function by mutual phosphorylation of their intracytoplasmic domain; in that case, the ligand acts only to induce the geometric coupling of two receptors. The same mechanism could operate for the activation of PMNs by collagen: the binding of each collagen molecule may force two parts of the receptor into close contact, so that they co-operate in the activation process. Further studies on the structure and function of the collagen receptor on PMNs should clarify this process.

We thank INSERM for grant CRE 892004 which made this work possible. We are grateful to Mrs. R. Platzeck and Mrs. C. Leroux for technical assistance and to Mrs. S. Etienne and Mrs. E. Deschamps for typing the manuscript.

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