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Bovine lens capsule basement membrane collagen exerts a negative priming on polymorphonuclear neutrophils

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After a 30 min contact between purified bovine lens capsule basement membrane type IV collagen and polymorphonuclear neutrophils, stimulation of these cells by N-formyl-methionyl-leucyl-phenylalanine, PMA or type I collagen releases a decreased amount of superoxide ions (negative priming). The inhibitory activity is located in the NCl domain. On the other hand, after pepsin digestion, the helical part of type IV collagen determines a positive priming of neutrophils.

Neutrophil; Basement membrane; Collagen; fmlp; Phorbol myristate acetate (PMA); Superoxide

1. INTRODUCTION

In a previous paper, we described that polymorphonuclear neutrophils, upon contact with purified mammalian type I collagen or its α (I) polypeptide chain, became activated, changed their shape and started to secrete lytic enzymes and oxygen free radicals such as superoxide ion, that we chose as an index of this activation [1–3]. Activation by collagen I probably takes place when the collagen fibres begin to be fragmented and denatured, during the initial stages of inflammation. The present paper addresses the question does type IV collagen also adhere to neutrophils and act on them? Type IV collagen is the major constituent of basement membranes. It is well known that neutrophils have to cross the vascular basement membrane in order to exert their physiological functions in inflammed areas, therefore interactions between neutrophil and type IV collagen should occur.

2. MATERIAL AND METHODS

2.1. *Reagents*

Ferricytochrome *c* (type VI), *N*-formyl-methionyl-leucyl-phenylalanine (fmlp), superoxide dismutase (SOD) from bovine erythrocytes, bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). All usual reagents (analytical grade) were from Prolabo (Paris) or Merck (Darmstadt, Germany). Purified EHS tumor type IV collagen was a generous gift from Dr. N.A. Kefalides (Philadelphia) and EHS tumor laminin was given to us by Dr. J.M. Tournier (INSERM U 314, Reims).

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2.2. *Neutrophils*

Human neutrophils from freshly drawn heparinized venous blood, were prepared by a single step centrifugation procedure through metrizoate-polyprep gradient (Nycomed, Oslo, Norway) as already described [2].

2.3. *Bovine anterior lens capsule type IV collagen*

Bovine lens obtained from a local slaughterhouse were dissected in the cold. The anterior capsule was extracted overnight in a 0.1 M solution of tartaric acid (100 ml per 220 capsules) [4]. The clear supernatant was fractionated on a DEAE cellulose column equilibrated with 50 mM Tris-HCl buffer, pH 8.3, containing 6 M urea, according to Brinker [5]. The unbound fraction contained a very pure type IV collagen (44 mg obtained from 220 capsules).

A part of this type IV collagen was digested with pepsin at 4°C for 16 h (molar ratio 1/100) and the non-digested fraction purified by dialysis against distilled water. Another part of the collagen was digested with purified bacterial collagenase and the non-collagenous domains fractionated by S-300 Sephacryl gel filtration according to Tsilibary et al. [6].

2.4. *Adhesion experiments*

Polystyrene 96 well-plates (Nunc, Copenhagen) were coated with type IV collagen or its fragments, solubilized in a volume of 100 μl of 0.018 M acetic solution at a concentration of 250 μg/ml and evaporated to dryness at 37°C for 24 h. The coated plates were washed 3 times with 0.9% NaCl solution and dried again at 37°C for 30 min prior to addition of 1.5×10^5 cells in 100 μl of Dulbecco's solution containing 1.3 mM Ca^{2+} and 0.5 mM Mg^{2+}. After a 30-min incubation, supernatant was counted for the number of non-adherent cells in a model S plus Coulter counter (Coultronics, Hialeah, FL). Adhesion of neutrophils was also evaluated as the absorbance of Crystal violet-stained nuclei in the adhering cells according to Kueng [7].

2.5. *Activation experiments*

The superoxide anion (O_2^-) production was measured as the SOD-inhibitable reduction of ferricytochrome *c* in test-tubes described by Monboisse [1]. The neutrophil suspension (100 μl containing 10^6 cells) was added to glass tubes containing 0.85 ml of Dulbecco's solution and 0.1 ml of 1 mM cytochrome *c* solution. O_2^- release by neutrophils was triggered by adding 0.1 ml of each of the activation agent solution to give final concentrations as follows: either 0.5 μM fmlp or 0.8 μM

PMA or 0.3 μM type I collagen; every other protein to be tested (BSA, EHS tumor type IV collagen, bovine lens capsule type IV collagen, pepsin-digested bovine lens capsule type IV collagen; EHS tumor laminin) at 100 μg/ml. Absorbance was measured at 550 nm.

2.6. *Preincubation followed by activation experiments*

Several preliminary experiments showed that type IV collagen (100 μg/ml) had no direct effects on neutrophil activation but that it induced the neutrophils to a state of insensitivity to any other stimuli. In order to study this 'negative priming' (see section 4), the following experiments were devised. A neutrophil suspension (1.2 ml containing 5×10^6 cells/ml) in Dulbecco's solution was incubated at test tubes with type IV collagen or its fractions that resisted the proteolytic digestion. All the fractions to be tested were used at 100 μg/ml. After this first 30-min incubation, neutrophils were collected by centrifugation, washed twice with Dulbecco's solution and resuspended in 1.2 ml of Dulbecco's solution. Aliquots of 0.1 ml of this suspension were added in tubes containing 0.85 ml of Dulbecco's solution, 0.1 ml of 1 mM ferricytochrome *c* solution and the activating agent (either 0.5 μM fmlp or 0.8 μM PMA or 0.3 μM type I collagen at final concentration). Production of O_2^- was measured by ferricytochrome *c* reduction.

2.7. *Kinetics of the 'negative priming'*

In order to measure the duration of the inhibition period after a single contact onto type IV collagen, we made initial 30-min pre-incubations of neutrophils (5×10^6 cells/ml) with NCI domain from bovine lens capsule type IV collagen (25 μg/ml). After this first 30-min incubation, neutrophils were collected by centrifugation, washed twice with Dulbecco's solution and resuspended in 1.2 ml of Dulbecco's solution. Aliquots of 0.1 ml of this suspension were added to glass tubes containing 0.85 ml of Dulbecco's solution, 0.1 ml of 1 mM ferricytochrome *c* solution and the activating agent (either 0.5 μM fmlp or 0.8 μM PMA or 0.3 μM type I collagen) 0, 30, 60, 90, 120 and 150 min after the end of the first period of incubation. This second incubation lasted for 15 min and the extent of inhibition of O_2^- production was checked by the ferricytochrome *c* reduction method.

3. RESULTS

3.1. *Preparation of type IV collagen from bovine anterior lens capsules*

We found that tartaric acid extraction yielded 4-times more type IV collagen than acetic acid extraction. The purity, checked by SDS-PAGE and amino acid composition, was more than 98% (data not shown). The NCI fraction was obtained in good yield (Fig. 1).

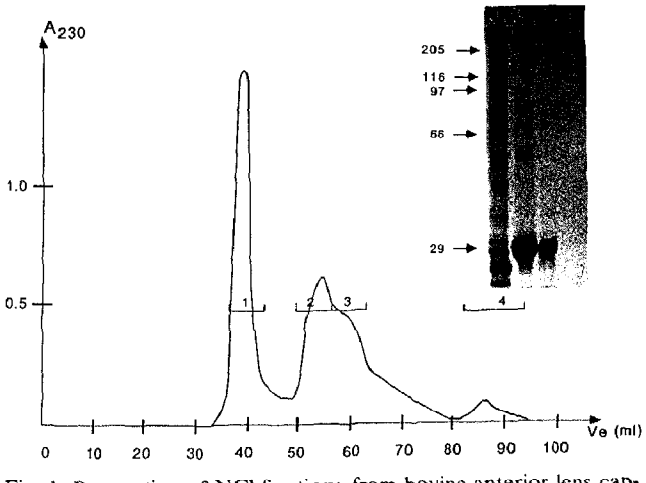

Fig. 1. Preparation of NCI fractions from bovine anterior lens capsules. Fraction 3 was used for incubation experiments.

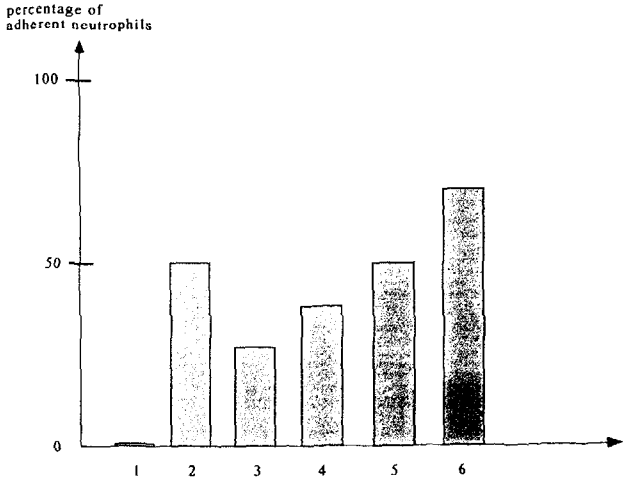

Fig. 2. Adhesion of human neutrophils to various substrates evaluated in percentage of the initial number of 5×10^5 neutrophils per well suspended in Dulbecco's medium. (1) BSA; (2) type I collagen; (3) EHS-tumor type IV collagen; (4) bovine lens capsule type IV collagen; (5) the same treated by pepsin; (6) EHS-tumor laminin. All the proteins were at 25 μg/well.

3.2. *Adhesion of neutrophils*

Fig. 2 shows that neutrophils adhere to the various samples of type IV collagen studied. The adhesion onto laminin is always high whereas there is no adhesion to BSA.

3.3. *Activation of neutrophils*

Fig. 3 shows that none of the samples of type IV collagen or laminin are able to directly stimulate neutrophils. As already demonstrated, type I collagen directly activates O_2^- production.

3.4. *Negative priming*

Neutrophils pre-incubated with bovine lens type IV

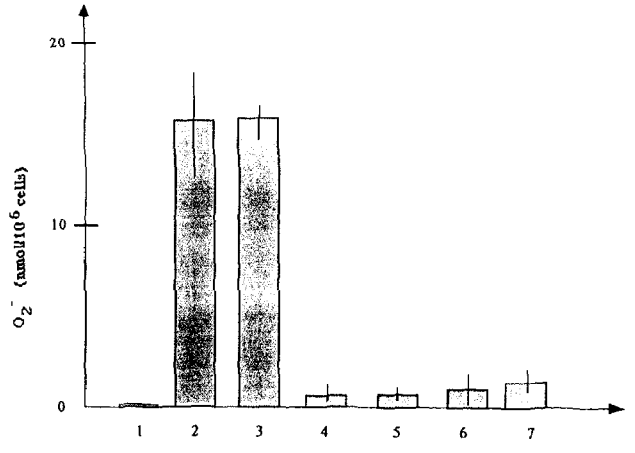

Fig. 3. Formation of O_2^- by neutrophils as evaluated by measuring the increase in absorbance at 550 nm produced by ferricytochrome *c* reduction in test tubes (for techniques, see text). (1) BSA; (2) fmlp; (3) bovine type I collagen; (4) EHS-tumor type IV collagen; (5) bovine lens capsule type IV collagen; (6) pepsin-digested bovine lens capsule type IV collagen; (7) EHS tumor laminin. All the proteins were at the concentration 100 μg/ml.

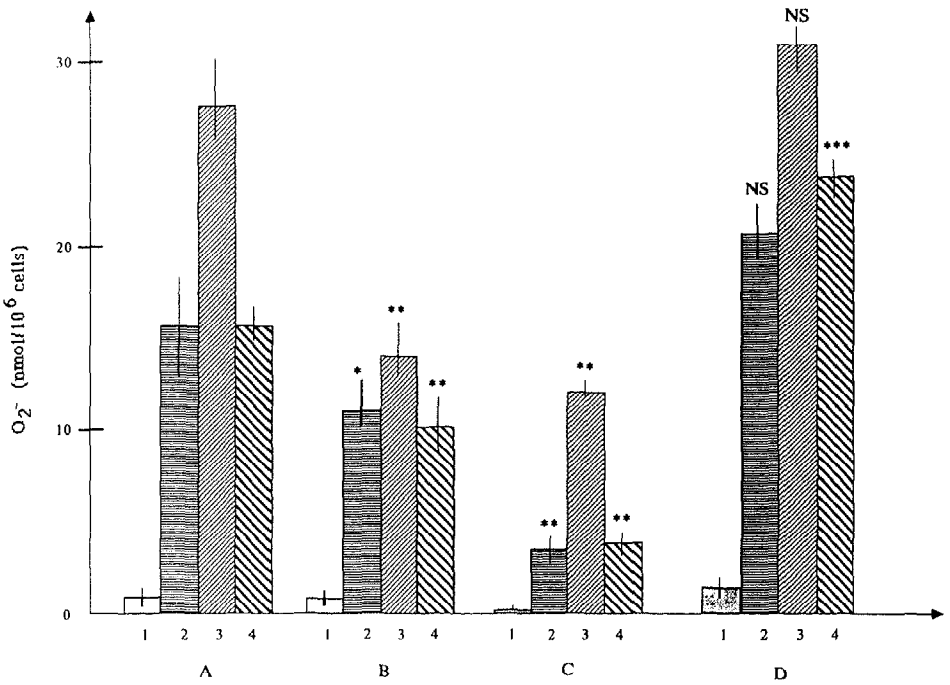

Fig. 4. Effect of pretreatment of neutrophils by various fractions of type IV collagen (for description of experiments, see text). Pretreatment with: (A) saline; (B) bovine lens type IV collagen (100 μg/ml); (C) NCl domain from bovine type IV collagen (100 μg/ml); (D) pepsinized bovine type IV collagen (100 μg/ml). Second incubation with: (1) sline; (2) 0.5 μM fmlp; (3) 0.8 μM PMA; (4) 0.3 μM type I collagen. *Decrease significant at $P < 0.05$; **decrease significant at $P < 0.001$; ***increase significant at $P < 0.05$.

collagen react with an evidently decreased intensity upon fmlp, PMA or type I collagen stimulation (Fig. 4B). NCl preparations, denatured NCl preparations or reduced NCl preparations produce the same negative effect on the fmlp, PMA or type I collagen stimulation of neutrophils (Fig. 4C).

3.5. *Positive priming by pepsin treated type IV collagen*

In contrast to complete type IV collagen, the helical domain remaining after pepsin digestion elicits a positive priming effect (Fig. 4D).

3.6. *Dose effect relationship and duration of the effect*

Fig. 5A shows that the relationship between the amount of type IV collagen used in the pre-incubation and the intensity of negative priming is dose-dependent in the range of concentrations studied. Fig. 5B shows that the effect continues for 45–60 min after the end of the preincubation period.

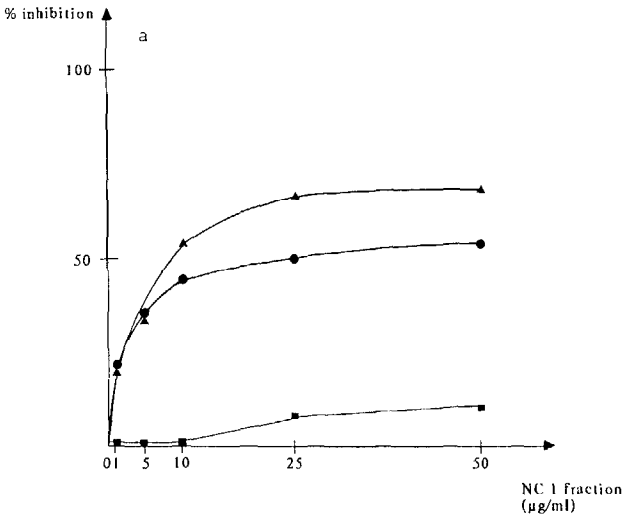

Fig. 5A. Dose-dependent inhibition elicited by pretreatment with increasing amounts of NCl domain followed by treatment with either 0.5 μM fmlp (●), 0.8 μM PMA (■) or 0.3 μM type I collagen (▲).

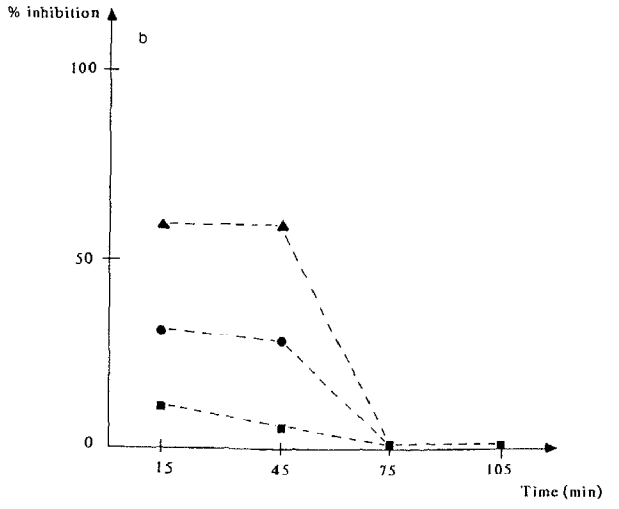

Fig. 5B. Duration of the negative priming effect after pre-incubation with 25 μg/ml NCl domain followed by tentative activation by 0.5 μM fmlp (●). 0.8 μM PMA (■) or 0.3 μM type I collagen (▲).

4. DISCUSSION

Neutrophils permanently circulate in blood. When they are chemo-attracted towards an inflammed area, they have to move through the vascular wall [8]. During this move they enter into contact with basement membrane molecules, particularly type IV collagen. As we had demonstrated previously that type I collagen could activate neutrophils [1–3], were were interested in verifying whether other types of collagens such as type IV could interfere with these cells. Any premature activation of neutrophils during the crossing of the vascular wall would exert deleterious effects on these tissues.

Bovine anterior lens capsule type IV collagen was choosen for this study because it is easy to prepare in relatively large amounts without pepsin digestion (N.A. Kefalides, personal communication). We improved the extraction by the use of tartaric acid in the place of acetic acid. Neutrophils adhere to this type IV collagen with a lower affinity than on laminin but they are not activated at all. This adhesion is not surprising because it is known that these cells have to adhere temporarily to solid tissues in order to move. It is satisfying that they are not activated for the sake of protection of capillaries.

The important fact evidenced in this study is that after neutrophils have been pre-incubated for 30 min with type IV collagen, they more or less lose their capacity to release superoxide ion when stimulated by fmlp, PMA or type I collagen. The inhibition is significant for all stimuli tested, dose-dependent and lasts for more than 60 min. We propose to term this effect "negative priming" by analogy with the positive priming that has been described in the case of molecules such as TNF α [9] or endotoxin [10]. The molecular mechanisms of these interactions are far from being understood.

We found that the NCI domain, isolated from type IV collagen by bacterial collagenase digestion and chromatography, was even more active than the complete type IV collagen molecule in eliciting this negative priming. Its activity was not diminished by heat denaturation or reduction and alkylation, suggesting that it depends on a definite sequence of this domain. On th other hand, when type IV collagen has been digested b pepsin, the helical domain exerts a positive priming o neutrophils, making them more susceptible to stimula tion by fmlp, PMA or type I collagen.

These apparent contradictory properties are actuall relevant to two different physiological situations. Whe neutrophils cross the vascular wall, they must adher temporarily in order to move but it is of primary im portance that they remain inactivated for some time, i order to be prevented from secreting proteolytic en zymes and oxygen free radicals that would degrade th capillaries. An inhibiting effect of the subendothelia extracellular matrix on neutrophils was alread suggested by Matzner et al. [11]. On the other hand, i case of degradation of the basement membrane, th helical domain probably resists for a longer time tha the NCI domain, so that it participates in the activatio of neutrophils, realizing the detersion of the inflammec area.

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