The Binding of Type I Collagen to Lymphocyte Function-associated Antigen (LFA) 1 Integrin Triggers the Respiratory Burst of Human Polymorphonuclear Neutrophils

ROLE OF CALCIUM SIGNALING AND TYROSINE PHOSPHORYLATION OF LFA 1

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Monoclonal antibodies to the α1β2 integrin inhibit the binding of type I collagen to PMN (polymorphonuclear neutrophil leukocytes) as well as the subsequent stimulation of superoxide production and enzyme secretion elicited by this collagen. Pepsinized collagen still binds PMN but no longer stimulates them. The I domain of the α chain of the integrin is involved in the binding. Two sequences of the α1(I) polypeptide chain of collagen participate in the process. Experiments of competitive inhibition by synthetic peptides showed that the sequence RGD (915–917) is used for binding to the cells and DGGRYY (1034–1039) serves to stimulate PMN. Experiments of radioactive labeling of the cells and affinity chromatography on Sepharose-collagen confirmed the presence in PMN extracts of two proteins, 95 and 185 kDa, respectively, corresponding to the molecular weights of the β2 and α1 chains of the integrin and recognized by their specific monoclonal antibodies.

The transduction pathways depending on the α1β2 integrin do not involve a G protein (ruled out by the use of cholera and pertussis toxins), whereas the cytoskeleton was found to participate in the process, as evidenced by inhibition by cytochalasin B. After collagen stimulation, cytoplasmic inositol trisphosphate and calcium ion increased sharply for less than 2 min. The use of the inhibitors staurosporine and calphostin C demonstrated that protein kinase C was involved. Evaluation of the activity of this enzyme showed that, upon stimulation of PMN with collagen I, it was translocated to plasma membrane.

Acrylamide gel electrophoresis of the protein bands corresponding to the integrin α1β2, followed by immunoblotting using monoclonal antibodies to phosphotyrosine, permitted us to demonstrate that, prior to stimulation of type I collagen, there was no phosphorylation, whereas after stimulation, both α1 and β2 chains were stained by anti-phosphotyrosine antibodies. The adhesion of PMN to pepsinized type I collagen triggered tyrosine phosphorylation of the β2 chain of the integrin, without stimulating O2 production by these cells, whereas their stimulation by complete type I collagen induced the tyrosine phosphorylation of both α1 and β2 subunits. The tyrosine phosphorylation of both integrin subunits during transduction of stimulus is a heretofore undescribed phenomenon that may correspond to a new system of transmembrane communication.

Type I collagen, a major component of the extra cellular matrix, promotes the adhesion of a variety of cells in solid tissues, influencing many processes such as proliferation, differentiation, migration, and cell shape changes (1). Several receptor molecules have been demonstrated as promoting the adhesion to type I collagen. Among these receptors, some belong to the family of integrins, for instance the β1, β3, and β4 chains of the integrin (2). On the other hand, some types of mobile cells also interact with collagens, for instance polymorphonuclear neutrophils (PMN), a variety of leukocytes circulating in blood, capable of crossing the vascular wall in order to invade the inflamed tissues and to participate in defenses against bacteria or foreign molecules through their property of phagocytosis. In several previous papers, we demonstrated that PMN and type I collagen do interact and began to describe their interactions (3–5).

Purified type I collagen is able to bind to PMN in vitro. This binding is followed by the stimulation of some main functions of PMN, such as emission of pseudopods, secretion of lytic enzymes, and liberation of superoxide. We demonstrated that the stimulation of PMN by collagen occurs through two sequences of the α1(I) chain, both located in the C-terminal region of the molecules, an RGD sequence corresponding to residues 915–917, and a DGGRYY sequence corresponding to residues 1034–1039, located at the C-terminal extremity of the chain. The type I collagen molecule, either fibrillar or denatured, is active, whereas pepsinized collagen, lacking the C-terminal telopeptide, is not. The cyanogen-bromide cleaved peptide α1(I)-CB6, which contains the C-terminal residues (823–1039) of the α1 chain, is also active. In contrast, the addition of the peptides RGD and DGGRYY either separately or together induces an inhibition of PMN. Both sequences must be contained in the same peptidic molecule to remain active on PMN.

By using monoclonal antibodies, we were able to demonstrate that the β2 chain of the integrins is involved in the process of binding of PMN onto type I collagen. The main

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1 The abbreviations used are: PMN, polymorphonuclear neutrophil leukocyte(s); PAGE, polyacrylamide gel electrophoresis; Fmoc, 9-fluorenylemethylchloroformate; PMA, phorbol 12-myristate 13-acetate.
The aim of this paper is to demonstrate that the receptor of type I collagen is the integrin $\alpha_2 \beta_1$, that this integrin mediates not only the binding but also transduction of the stimulation message, and that this phenomenon depends on the tyrosine-phosphorylation of both the $\alpha_1$ and $\beta_2$ subunits. We also point out some new details on the system of transduction operating intracellularly beyond the receptor.

**EXPERIMENTAL PROCEDURES**

**Materials—** Type I collagen was prepared from rat tail tendon by 0.1 M acetic acid extraction (6). Ferricytochrome c (type VI), superoxide dismutase from bovine erythrocytes, bovine serum albumin, fMet-Leu-Phe, pertussis toxin, chelera toxin, cytchalasin B, lactic dehydrogenase, H-7 (1-isoquinolinysulfonyl)-2-methylpiperazine), staurosporine, were all purchased from Sigma. Calphostin C was bought from Calbiochem (La Jolla, CA). The following monoclonal antibodies were used: P4C10, recognizing the $\beta_2$ integrin subunit (Life Technologies, Inc.); 4G7, raised toward the lymphocyte surface antigen Leu-12, n-12, recognizing CD11b, and 5H-13, recognizing CD11c, all from Becton-Dickenson (Mountain View, CA); MEM-25, Bear-1, FK-24, and MEM-48, recognizing CD11a, CD11b, CD11c, CD18, respectively, from Monosan (Tebu, Paris, France). Monoclonal antibodies to phosphotyrosine (clone 6G9, Life Technologies; and PY 20, Calbiochem) were used.

The synthetic peptides GRGD, DGGRRY, RFDS, CCGDSPC, and CGGRGESP are synthesized by Neosystem (Strasbourg, France). Fura-2 was bought from Molecular Probes Inc (Eugene, OR). Na$_3$H$_2$O and (H$^3$)Jodotilot were from DuPont NEN.

**Preparation of PMN—** Human blood was obtained on consent from healthy subjects. PMN were isolated according to a previously published method (4) involving a single centrifugation at 150 g through a Metrizoate-Polyprep gradient (Nycomed, Oslo, Norway) at 600 g for 35 min at room temperature. The PMN-rich layer was drawn, washed once in Dulbecco’s solution, pH 7.4, and centrifuged at 800 g for 10 min. The percentage of PMN in cell preparations exceeded 95%, and the cell viability, as determined by trypan blue exclusion, was over 98%.

**Experiments of Cell Adhesion—** Polystyrene 96-well plates (Nunc, Copenhagen, Denmark) were coated with type I collagen solubilized in saline or 0.3 M NaCl in the place of 150 mM. In some instances, the plates were washed three times with 0.1% (w/v) Nonidet P-40 and then incubated with 0.3 M collagen I. The production of superoxide was evaluated by spectrophotometry of the superoxide dismutase-inhibitable reduction of cytochrome c as indicated above. In another series of experiments, PMN were preincubated for 15 min

**Measurement of Granule Secretion—** A 0.1-ml aliquot containing 10$^6$ PMN was added to 0.8 ml of Dulbecco’s solution and 0.1 ml of collagen solution (final concentration 0.3 $\mu$M) already mixed in a glass test tube. The mixture was incubated for 30 min at 37°C. Cells were then removed by centrifugation at 800 $\times$ g for 5 min and the enzymatic activities measured in the supernatant.

The evaluations of elastase and of 92-kDa type IV collagenase were performed using N-methoxyoxycarbonyl-Ala-Ala-Pro-Val-p-nitroanilide and biotinylated type IV collagen as substrates, for incubation periods of 1 and 3 h, respectively, according to the methods of Nakajima et al. (9) and of Wilkinson et al. (10). The N-acetyl-$\beta$-glucosaminidase activity was measured according to the method of Troost et al. (11). Lactic dehydrogenase, whose enzyme permits to verify the absence of cell lysis, was evaluated as described by Buhl et al. (12). The enzymatic activities released in the medium were expressed as percentage of the corresponding total activity of the cell lysate.

**Effect of Monoclonal Antibodies on PMN—** Treh polypropylene microtubes (PolyLabo, Strasbourg, France) were coated with 1.5 ml of fetal calf serum 37°C for 1 h and then extensively washed with a 0.15 M NaCl solution. A 0.1-ml volume of antibody solution, at the appropriate titer, was added to each tube, followed by the PMN suspension in Dulbecco’s solution, in order to achieve a final concentration of 6 $\times$ 10$^6$ cells in 0.75 ml. Test tubes were shaken horizontally in a mechanical shaker for 90 min at 150 cycles/min (13).

**Affinity Chromatography—** Ten mg of purified type I collagen were covalently coupled to 1 ml of packed beads of CNBr-activated Sepharose 4B (Pharmacia Biotech Inc.) according to the manufacturer’s instructions. In parallel, a suspension of 20 $\times$ 10$^6$ cells in 1.5 ml of phosphate-buffered saline was iodinated using the lactoperoxidase method of Leiden et al. (14). Iodination was carried out on ice by addition of 200 $\mu$g of lactoperoxidase followed by the addition of 100 $\mu$g of 125I and of Na$_2$H$_2$O$_2$ with a $\mu$M hydrogen peroxide at a 5-min interval. Reaction was terminated by adding 2 ml of ice-cold phosphate-buffered saline, and the cells were washed three times with this solution. The cells were then extracted in 1 ml of a 100 mM Tris- HCl buffer pH 7.4, containing 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 0.1% (w/v) sodium deoxycholate (SDS), 1% protein (w/v) sodium methylsulfonyl fluoride, 10 $\mu$g/ml leupeptin. Following centrifugation at 12,000 x $g$ for 15 min at 4°C, the lysate was diluted 1/5 with a 10 mM Tris- HCl buffer pH 7.4 containing 1.3 mM CaCl$_2$, 1 mM MgCl$_2$, 2 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml leupeptin. This dilution was necessary to reduce the concentration of SDS. The extract was transferred to the collagen-Sepharose 4B beads in batch and incubated overnight in the same conditions. The suspension was then packed into a 1.0-cm diameter glass column and washed at 4°C by buffer A at a flow of 5 ml/h.

The material was eluted first with a 10 mM Tris- HCl buffer, pH 7.4, containing 5 mM EDTA, 0.1% (w/v) n-actyl $\beta$-glucopyranoside, 150 mM NaCl, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml leupeptin. This dilution was necessary to reduce the concentration of SDS. The proteins were sequentially eluted by the specific peptides, first by 4 ml of a 3 mM solution of synthetic DGGRRY, then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS. Both peptides were dissolved in a 10 mM Tris- HCl buffer, pH 7.4, containing 0.1% (w/v) n-actyl $\beta$-glucopyranoside, 150 mM NaCl, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml leupeptin.

The flow rate was adjusted to 0.1 ml/h, then with the same buffer containing 1.0 M NaCl in the place of 150 mM. In some instances, the proteins were sequentially eluted by the specific peptides, first by 4 ml of a 3 mM solution of synthetic DGGRRY, then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS, and then by 4 ml of a 3 mM solution of CRGDS. Both peptides were dissolved in a 10 mM Tris- HCl buffer, pH 7.4, containing 0.1% (w/v) n-actyl $\beta$-glucopyranoside, 150 mM NaCl, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml leupeptin.

Fractions of 0.1 ml were collected and their radioactivity counted with an automatic $\gamma$ counter (Kontron MR-480) in order to monitor the elution. For characterization of the eluted substances, the proteins contained in 200-$\mu$l aliquots corresponding to the peaks of radioactivity were precipitated by addition of ethanol to 80% (v/v). The precipitates were re-dissolved in Laemmli sample buffer (15) and submitted to electrophoresis in a 7.5% polyacrylamide gel under reducing conditions. Radiolabeled bands were revealed by exposure to a Hyperfilm MP (Amersharn Corp.) for convenient periods of time.

**Immunoprecipitations—** Aliquots of 200 $\mu$l of the eluted fractions were incubated with antibodies and triton X-100 by incubation at 4°C for 2 h. The Sepharose-adsorbed material was washed three times with 3 ml of Tris-buffered saline. The eluted proteins were analyzed by SDS-PAGE under reducing conditions and detected by autoradiography as described above (16).

**Preincubation of PMN with Specific or Competitive Inhibitors—** PMN were preincubated for 10 min at 37°C either with pertussis toxin (final concentration 100–500 ng/ml), with cholera toxin (2.5 $\mu$g/ml), with protein kinase C inhibitors staurosporine (0–500 $\mu$m), H-7 (0–1000 $\mu$m), calphostin C (0–500 $\mu$m), or with the inhibitor of tyrosine kinase genistein (0–2.0 $\mu$g/ml). After this preincubation, they were stimulated by addition of 0.1 $\mu$M fMet-Leu-Phe or of 0.3 $\mu$M collagen I. The production of superoxide was evaluated by spectrophotometry of the superoxide dismutase-inhibitable reduction of cytochrome c as indicated above.

In a series of experiments, PMN were preincubated for 15 min
at 37°C with the synthetic peptides RFDS, GRGD, CGRGDSPC, CGRGESP, or DGGRYY at various concentrations. At the end of this incubation, they were stimulated by addition of 0.3 μM collagen I. The O₂ production and enzyme-rich granule release were evaluated as described above.

Intracellular Calcium Ion Measurement—The intracellular Ca²⁺
concentration was measured according to Grzymkiewicz et al. (17). A suspension of PMN (10⁷/ml) in a Dulbecco's solution containing 1.3 mM CaCl₂, 0.5 mM MgCl₂, and 10 mM glucose, was loaded with 0.5 μM Fura-2-AM for 30 min at 37°C, rinsed twice with Dulbecco's solution, and sedimented. The cells were resuspended in Dulbecco's solution in a solution at an amount of 10⁷ cells/ml. Two ml of this suspension were transferred into a cuvette of a Shimadzu RF 5000 spectrofluorometer. The apparatus was set up at two excitation wavelengths of 340 and 380 nm, and the emitted fluorescence monitored at 510 nm for 3 min in order to determine the basal level, the stimulating agent (final concentration 0.1 μM fMet-Leu-Phe or 0.3 μM type I collagen) was added and fluorescence recorded for 10 min. At the end of each experiment, cells were lysed by adding a 0.1% (w/v) Triton X-100 solution and the maximal fluorescence measured. Minimal fluorescence was also determined after addition of 2.0 mM MnCl₂ solution. Results were expressed as nanomoles of Ca²⁺/10⁷ cells.

Intracellular Inositol Trisphosphate Measurement—PMN were suspended at a concentration of 10⁷ cells/ml in Dulbecco's solution containing 0.0025% (w/v) bovine serum albumin and were incubated with 1.1 M LiCl₂ for 120 min at 37°C. Ten minutes before the end of this incubation, 1 ml of a solution of 10 mM LiCl₂ was added in order to inhibit the hydrolysis of inositol phosphate. Finally, the cells loaded with [³H]inositol were rinsed twice with Dulbecco's solution containing 10 μM LiCl₂ and resuspended at 2 × 10⁶ cells/ml in fresh Dulbecco's solution.

The experiment was performed after several types of stimuli had been applied to the cells, either 0.1 μM fMet-Leu-Phe or 0.3 μM type I collagen. The incubation was stopped at various times (0, 0.5, 1.0, 2.0, or 5.0 min) by addition of 0.15 ml of a 35% (w/v) sodium perchlorate solution. The reaction mixture was centrifuged at 5,000 g for 15 min at 4°C. The supernatants were neutralized with a 9.0 M KOH solution and equilibrated under the formate form. The column was eluted sequentially with 3 ml of distilled water (for [³H]inositol), 10 ml of 5 M sodium tetraborate/60 mM sodium formate (for glycerophospho-[³H]inositol), 14 ml of 0.1 M formic acid/0.2 M ammonium formate (for [³H]inositol phosphate), 18 ml of 0.1 M formic acid/0.5 M ammonium formate (for [³H]inositol bisphosphate), 18 ml of 0.1 M formic acid/1.0 M ammonium formate (for [³H]inositol trisphosphate), according to the method of Berriidge (18). The radioactivity of the eluted fractions was measured in a Packard 1900 TR liquid scintillation counter. Results were expressed as percentage of total inositol phosphate.

Measurement of Protein Kinase C Activity—Protein kinase C activity was evaluated in the particular or in the cytosolic fractions of PMN stimulated with fMet-Leu-Phe, PMA, or type I collagen, according to the method described by Kikkawa et al. (19).

Tyrosine Phosphorylation of the αβ₃ Integrin—The suspension of PMN (10⁷ cells/ml) in Dulbecco's solution containing 1.3 mM CaCl₂, 0.5 mM MgCl₂, was first preincubated for 30 min at 20°C in the presence of 2.5 mM phenylmethylsulfonyl fluoride. Then temperature was raised to 37°C for 5 min prior to the addition of the stimulating agent (Dulbecco's solution as a control, 0.1 μM fMet-Leu-Phe peptide in Dulbecco's solution, or 0.3 μM type I collagen in Dulbecco's solution). The incubation was stopped after 2 min by centrifugation at 1000 × g for 4 s at 4°C. The supernatants were discarded and the cells lysed by addition of 150 μl of a 0.05 M Hepes buffer, pH 7.5, containing 150 mM NaCl, 5 mM EGTA, 10 mM sodium pyrophosphate, 15 mM sodium fluoride, 5 mM orthovanadate, 1% (w/v) SDS, 1% (w/v) Nonidet P-40, 10% (w/v) glycerol, and a mixture of proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A) (20). Cell lysis took place within 10 min at 4°C. A 100-μl aliquot of cell extract was immunoprecipitated by addition of a 200-μl aliquot of anti-CD11a or anti-CD11b monoclonal antibodies (DE21) followed by adsorption on protein A-Sepharose for 2 h at 4°C. The immunoprecipitated material was fractionated by SDS-PAGE in 10% polyacrylamide gel under reducing conditions and blotted on a transfer Immobilon membrane (Millipore, Bedford, MA). The membrane was saturated by incubation for 1 h with a 10 mM Tris buffer, pH 7.4, containing 150 mM NaCl and 5% (w/v) bovine serum albumin, then incubated for 2 h in the presence of monoclonal antibodies to phosphotyrosine (clone 659 or clone PY-20), Alkaline phosphatase-conjugated anti-mouse IgG antibody (Organon Teknika, Durham, NC) was used as a secondary antibody, for detection of the positive protein bands visualized by the reaction with 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitro blue tetrazolium.

Identification of the Sequences of Type I Collagen Responsible for Adhesion and Stimulation—Preparations of PMN were submitted to the effect of solutions of synthetic peptides containing sequences RGD and DGGGRYY. After a 15-min treatment, an aliquot of cells was added onto type 1 collagen molecules layered to the bottom of the wells of culture polystyrene plates. Adhesion to collagen and production of O₂ by these cells was measured (Table I). The linear peptide GRGD and the control peptide RFDS did not exert any significant action. On the other hand, the peptide CGRGDSPC, which contains RGD in a loop formed under the influence of a disulfide bridge, exerted an inhibiting effect of about 50% on both adhesion and superoxide formation, whereas peptide CGRGESP did not. This effect was slightly dose-dependent. Peptide DGGGRYY did not inhibit the binding of PMN to type 1 collagen but inhibited the formation of O₂ in a dose-dependent manner. The secretion by PMN of lytic enzymes such as elastase or gelatinase was found to be inhibited by the peptides CGRGDSPC or DGGGRYY in parallel to superoxide liberation (Table II).

Identification of LFA 1 as the Receptor for Type I Collagen on PMN Membrane—In experiments similar to the previous one, monoclonal antibodies to the various subunits of the β₃ inte-

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**Table I**

| Adhesion          | Production of O₂ | PMN × 10⁷/mg well | nmol/10⁷ cells |
|-------------------|------------------|--------------------|---------------|
| Control           | 91 ± 13          | 15.3 ± 1.0         |
| GRGD μM           |                  |                    |               |
| 500               | 86 ± 10 (5.5 NS) | 15.2 ± 1.3 (0.7 NS)|               |
| 625               | 87 ± 12 (4.4 NS) | 15.5 ± 1.4 (1.3 NS)|               |
| 1250              | 86 ± 10 (5.5 NS) | 15.4 ± 1.3 (0.7 NS)|               |
| CGRGDSPC          |                  |                    |               |
| 250               | 52 ± 5 (42.9 p 0.001 | 8.4 ± 1.0 (45.1 p 0.001)|               |
| 625               | 42 ± 4 (53.8 p 0.001 | 7.3 ± 1.1 (52.3 p 0.001)|               |
| 1250              | 32 ± 4 (64.8 p 0.001 | 6.5 ± 0.9 (57.5 p 0.001)|               |
| CGRGESP          |                  |                    |               |
| 250               | 89 ± 10 (2.2 NS) | 15.2 ± 1.3 (0.7 NS)|               |
| 625               | 88 ± 10 (3.3 NS) | 15.1 ± 1.2 (1.3 NS)|               |
| 1250              | 87 ± 10 (4.4 NS) | 14.9 ± 1.3 (2.6 NS)|               |
| DGGGRYY          |                  |                    |               |
| 250               | 82 ± 10 (9.9 NS) | 11.8 ± 1.2 (22.9 p 0.05)|               |
| 625               | 85 ± 9 (6.6 NS)  | 7.7 ± 1.1 (49.7 p 0.001)|               |
| 1250              | 85 ± 9 (6.6 NS)  | 2.3 ± 0.8 (85.0 p 0.001)|               |
| RFDS             |                  |                    |               |
| 250               | 87 ± 13 (4.4 NS) | 15.8 ± 1.2 (3.3 NS)|               |
| 625               | 86 ± 12 (5.5 NS) | 15.7 ± 1.2 (2.6 NS)|               |
| 1250              | 86 ± 10 (5.5 NS) | 15.3 ± 1.3 (0.0 NS)|               |
PMN (5 × 10⁶) were preincubated for 15 min with type I collagen in a test tube in order to measure the production of superoxide induced by this collagen by the spectrophotometric technique of ferricytochrome c granule exocytosis (Table IV). Two monoclonal antibodies recognizing the β₂ chain of integrins, MHH 23 and MEM 48, were found to inhibit both adhesion of PMN to collagen and secretion of superoxide and lytic enzymes. On the other hand, one

Table II

| Preincubation with | Elastase | Collagenase IV | N-Acetyl-β-D-glucosaminidase | Lactic dehydrogenase |
|-------------------|----------|----------------|-----------------------------|---------------------|
| (a) Control (Dulbeco’s solution for both preincubation and incubation) | 1.3 ± 0.4 | 1.3 ± 0.7 | 1.0 ± 0.8 | 1.2 ± 0.4 |
| (b) Dulbeco’s solution | 9.6 ± 1.2 | 9.2 ± 0.7 | 10.1 ± 1.3 | 1.3 ± 0.4 |
| RFSD | 9.4 ± 1.1 NS | 9.1 ± 0.7 NS | 9.8 ± 1.1 NS | 1.1 ± 0.5 NS |
| GRGD | 9.3 ± 1.0 NS | 9.0 ± 0.9 NS | 9.7 ± 1.2 NS | 1.0 ± 0.5 NS |
| GRGDSPC | 4.1 ± 1.0 p < 0.01 | 3.9 ± 0.8 p < 0.001 | 4.7 ± 1.0 p < 0.01 | 1.2 ± 0.5 NS |
| DGGRRY | 3.9 ± 1.0 p < 0.01 | 3.7 ± 0.8 p < 0.001 | 4.2 ± 1.0 p < 0.01 | 1.2 ± 0.6 NS |

Effect of several monoclonal antibodies to the integrin subunits on PMN adhesion and superoxide production elicited by type I collagen

A first incubation of 6 × 10⁶ PMN was performed for 90 min with monoclonal antibodies (6 µg of IgG/ml), and then an aliquot of these PMN (1.5 × 10⁶) was layered on a type I collagen-coated plate and their adhesion measured by staining nuclei with crystal violet (7). Another aliquot (1 × 10⁶) was incubated for 30 min with 0.3 µM type I collagen and centrifuged at 800 × g for 5 min. Enzymatic activities were measured in the supernatant. Control a, preincubation with Dulbeco’s solution alone, then incubation with Dulbecco’s solution again. Control b, control stimulated by type I collagen. Values of p were calculated by reference to control b. Results were expressed as percent of total activity present in every cell lysate. Each value represents mean of quadruplicate determinations.

Table III

| Preincubation with | Adhesion | O₂ production |
|-------------------|----------|---------------|
| (a) Control without preincubation | PMN × 10⁻³ | nmol/10⁶ cells |
| 105 ± 10 | 21 ± 1.9 |
| (b) Preincubation with Dulbecco’s solution | 103 ± 10 | 20.7 ± 1.8 |
| CD19 clone 4-G7 | 101 ± 9 (1.9) NS | 21.0 ± 2.3 (−1.5) NS |
| CD11a clone MHM 24 | 99 ± 9 (3.9) NS | 20.3 ± 2.0 (1.9) NS |
| Clone MEM 25 | 8 ± 4 (92.2) p < 0.001 | 3.3 ± 1.2 (84.1) p < 0.001 |
| CD11b clone D12 | 101 ± 10 (1.9) NS | 20.2 ± 2.5 (2.4) NS |
| Clone Bear 1 | 98 ± 9 (4.9) NS | 16.0 ± 21 (22.7) NS |
| CD11c clone S-HCl-3 | 95 ± 9 (7.8) NS | 20.2 ± 2.3 (2.4) NS |
| Clone FK-24 | 95 ± 9 (7.8) NS | 19.2 ± 2.1 (7.2) NS |
| CD18 clone MHM 23 | 33 ± 7 (68.0) p < 0.001 | 3.3 ± 1.0 (84.1) p < 0.001 |
| Clone CEM 48 | 12 ± 5 (88.3) p < 0.001 | 3.0 ± 0.9 (85.5) p < 0.001 |
| β₂ integrin clone P4C 10 | 100 ± 10 (2.9) NS | 20.5 ± 2.4 (1.0) NS |

Table IV

| Preincubation with | Elastase | Collagenase IV | N-Acetyl-β-D-glucosaminidase | Lactic dehydrogenase |
|-------------------|----------|----------------|-----------------------------|---------------------|
| (a) Control (Dulbeco’s solution for both preincubation and incubation) | 1.3 ± 0.4 | 1.3 ± 0.7 | 1.0 ± 0.8 | 1.3 ± 0.4 |
| (b) Dulbecco’s solution | 9.6 ± 1.2 | 9.2 ± 0.7 | 10.1 ± 1.3 | 1.3 ± 0.5 |
| CD11a clone MEM 25 | 3.7 ± 1.0 p < 0.01 | 3.9 ± 0.7 p < 0.01 | 4.2 ± 1.2 p < 0.01 | 1.2 ± 0.5 NS |
| CD11b clone Bear 1 | 9.7 ± 1.1 NS | 9.3 ± 0.9 NS | 9.9 ± 1.2 NS | 1.1 ± 0.4 NS |
| CD11c clone FK 24 | 9.6 ± 1.2 NS | 9.2 ± 0.8 NS | 10.0 ± 1.3 NS | 1.3 ± 0.5 NS |
| CD18 clone MEM 48 | 4.8 ± 1.0 p < 0.01 | 4.6 ± 0.8 p < 0.01 | 5.1 ± 1.2 p < 0.01 | 1.1 ± 0.4 NS |

An inhibitory effect of synthetic peptides reproducing characteristic sequences of α(I) collagen chain on granule exocytosis by PMN stimulated by type I collagen

PMN (5 × 10⁶) were preincubated for 15 min with the synthetic peptides (625 µM at final concentration). Aliquots containing 10⁶ PMN were incubated for 30 min with 0.3 µM type I collagen and centrifuged at 800 × g for 5 min. Enzymatic activities were measured in the supernatant. Control a, preincubation with Dulbecco’s solution alone, then incubation with Dulbecco’s solution again. Control b, control stimulated by type I collagen. Values of p were calculated by reference to control b. Results were expressed as percent of total activity present in every cell lysate. Each value represents mean of quadruplicate determinations.

Grains were incubated with PMN, then the adhesion of the cells was measured as well as superoxide secretion (Table III) and granule exocytosis (Table IV). Two monoclonal antibodies recognizing the β₂ chain of integrins, MHH 23 and MEM 48, were found to inhibit both adhesion of PMN to collagen and secretion of superoxide and lytic enzymes. On the other hand, one
of the monoclonal antibodies recognizing the \(\alpha_\ell\) chain, MEM 25, inhibited both adhesion and stimulation of the cells, whereas the second monoclonal antibody to the \(\alpha_\ell\) chain did not induce any inhibition. The secretions of lytic enzymes by PMN, such as elastase or gelatinase induced by type I collagen, were found to be inhibited by the same monoclonal antibodies as superoxide liberation. The monoclonal antibodies inhibited the secretions by PMN in a dose-dependent manner (Fig. 1). None of the monoclonal antibodies recognizing \(\alpha_M\) or \(\alpha_X\) chains inhibited adhesion or activation of PMN.

Isolation of the Receptor—The isolation of the receptor membrane protein was undertaken by the method of affinity chromatography on a column of type I collagen-Sepharose. The elution diagram is shown on Fig. 2. Two peaks were found, one eluted with buffer A containing 5 mM EDTA and 150 mM NaCl and the second with the same buffer A containing 1 mM NaCl.

SDS-PAGE was performed on the proteins contained in both peaks (Fig. 2, inset). The first peak of elution contained two major bands with respective apparent molecular masses of 95 and 185 kDa as estimated by comparison to control globular proteins of known molecular mass. In addition, this peak contained two minor bands, of 31 and 35 kDa, respectively. Peak two contained these 31- and 35-kDa proteins as major bands.

The identity of the proteins contained in these peaks was verified by immunoprecipitation with the monoclonal antibodies anti-CD18 and anti-CD11a. The precipitated molecules were analyzed by SDS-PAGE. The antibody anti-CD18 precipitated the two proteins of molecular masses 95 and 185 kDa from peak 1 (Fig. 3A). No material from peak 2 was precipitated by this antibody. Similar results were obtained with the antibody anti-CD11a (Fig. 3B). Monoclonal antibodies to CD11b and CD11c did not precipitate any material.

The synthetic peptides CGRGDSPC and DGGRYY used either separately or together were not able to elute significant amounts of proteins from the column by themselves (data not shown).

Further Steps of Transduction of the Message beyond the Receptor: G Proteins—The necessity of a \(G_\alpha\) protein for transduction of the message was checked by addition of cholera toxin to the preparation of PMN and measurement of the production of superoxide in the presence of type I collagen. No influence of this toxin was noticed either on collagen-stimulated PMN or on control fMet-Leu-Phe-stimulated PMN (data not shown).

In the same type of experiments, the effect of pertussis toxin (at concentrations ranging from 0 to 500 ng/ml) on the collagen-dependent formation of superoxide was compared to the effect of this toxin on the fMet-Leu-Phe-dependent stimulation. Pertussis toxin was found to be inactive on the transduction of the message from type I collagen, whereas it exerted a dose-dependent inhibiting effect on the stimulation by the peptide fMet-Leu-Phe used as a control.

Effect of Cytochalasin B and Colchicine on the Production of Superoxide—These inhibitors of cytoskeleton assembly were added to PMN at concentrations 2.5 \(\mu\)g/ml and 10 \(\mu\)M, respectively, 10 min prior to stimulation by addition of type I collagen or control fMet-Leu-Phe. The result of this addition on the production of superoxide by the cells was measured (Table V). While cytochalasin B or colchicine increased the production of superoxide in the presence of fMet-Leu-Phe, they suppress this effect in the presence of type I collagen. Similar results were obtained when measuring granule exocytosis (data not shown).

Inositol Trisphosphate—The results of the evaluation of the inositol trisphosphate in the cytoplasm of PMN after various stimulations are shown on Fig. 4. There was a fast rise in the concentration of inositol trisphosphate, with a peak about 0.5 min after the onset of stimulation in the case of the use of type I collagen or peptide fMet-Leu-Phe, whereas in the case of treatment with pepsinized type I collagen the amount of inositol trisphosphate remained comparable to the baseline levels obtained with Dulbecco’s solution as a control. Preincubation of PMN with monoclonal antibodies anti-CD11a or anti-CD18 inhibited the rise in the concentration of inositol trisphosphate triggered by collagen I, without affecting that induced by fMet-Leu-Phe.

Calcium Ion—The results of the evaluation of calcium ion concentrations in the cytoplasm of PMN are shown on Fig. 5. Once again, type I collagen elicited a sudden rise of the calcium concentration, comparable to that elicited by the control peptide fMet-Leu-Phe, followed by a decrease within 2 min after the onset of stimulation. Pepsinized collagen was inactive. On the other hand, preincubation of PMN with monoclonal antibodies anti-CD11a or anti-CD18 inhibited the calcium mobilization triggered by collagen I without affecting that induced by fMet-Leu-Phe (data not shown).

Protein Kinase C—The participation of protein kinase C in the process was tested with three inhibitors: H-7, staurosporine, and calphostin C. The first inhibitor had no effect on the production of superoxide by PMN stimulated by collagen.
(whereas under the same conditions it decreased this formation when induced by contact with peptide fMet-Leu-Phe) (Fig. 6). Staurosporine and calphostin C decreased the stimulation exerted by type I collagen in a concentration-dependent manner parallel to that induced by the control peptide fMet-Leu-Phe but in greater proportion (Fig. 6). The stimulation of PMN with collagen I triggered the translocation of protein kinase C from cytosolic to particular fraction as fMet-Leu-Phe or PMA did (Fig. 7).

Effect of Genistein—The stimulation of the production of superoxide by type I collagen and by the peptide fMet-Leu-Phe was found to be decreased by this inhibitor of protein tyrosine kinase (Fig. 8).

Detection of the Tyrosine Phosphorylation of the Subunits of Integrin $\alpha_\text{L}$/$\beta_2$—After stimulation of PMN by type I collagen, the proteins extracted from plasma membrane have been precipitated by anti-CD18 or anti-CD11a monoclonal antibodies. Fig. 9 shows that the precipitated proteins, analyzed by SDS-PAGE, migrate with electrophoretic mobilities identical to that of the $\alpha_\text{L}$ and $\beta_2$ subunits. Immunoblotting by an antibody to phosphotyrosine was inhibited by an excess of free phosphotyrosine. The electrophoregram showed that a tyrosine-phosphorylation of the $\beta_2$ subunit of the integrin was taking place in case of adhesion of PMN to pepsinized type I collagen, whereas stimulation by complete collagen induced the tyrosine-phosphorylation of both $\alpha_\text{L}$ and $\beta_2$ subunits. Phosphoamino acid analysis confirmed the presence of $^{32}$P-labeled phosphotyrosine in the $\beta_2$-integrin subunit in PMN incubated with pepsinized type I collagen, and in both $\alpha_\text{L}$ and $\beta_2$ subunits in PMN stimulated with complete type I collagen.

**DISCUSSION**

The $\alpha_1$ chain of type I collagen stimulates PMN functions such as respiratory burst and degranulation through two distinct sequences, an RGD sequence corresponding to residues 915–917 and a DGGRYY sequence corresponding to the C-terminal extremity of this polypeptide chain, residues 1034–1039 (4).

The synthetic CGRGDSPC peptide, which exposes the RGD sequence on a loop, inhibits the adhesion of PMN to type I collagen, whereas linear GRGD and CGRGESPC do not. The conformation of RGD sequences is critical for fulfilling its function of fixation or message transmission (22). The peptide DGGRYY does not inhibit the binding of type I collagen to PMN, whereas it inhibits superoxide and lytic enzyme secretions. The functions of binding and stimulating are distributed between the two peptide sequences involved in the ligand. Apparently, RGD, when correctly folded, is in charge of binding, whereas DGGRYY is responsible for the transmission of stimulation. Pepsinized $\alpha_1$(I) chain of collagen I, which does not contain the sequence DGGRYY, still binds PMN, but this binding is not followed by stimulation. It must be noted that the binding of PMN onto type I collagen seems necessary for the
stimulation to occur but that a specific inhibition of the stimulation may occur independently of the binding. Finally, this binding also necessitates the presence of Mg$^{2+}$ and Ca$^{2+}$ ions, as we have already demonstrated (5).

Previously we found that a $\beta_2$ integrin is involved in the stimulation of PMN by type I collagen (5), but we had not yet identified the $\alpha$ chain. In this paper, we demonstrate that the membrane receptor of type I collagen on PMN is constituted by $\alpha$ and $\beta_2$ chains of the $\alpha\beta_2$ integrin, which is a receptor for collagen in various cells (25). This domain may represent a common marker for the adhesion of type I collagen, despite the restricted level of identity (36%) between the $\alpha$ chains of $\beta_2$ integrins (26). Other authors suggested the adhesion of PMN to collagen I through the $\alpha_{II}\beta_2$ integrin (27).

Characterization of the receptor protein by radioactive iodine labeling of whole cells, followed by preparation of plasma membranes and affinity chromatography of proteins on Sepharose-type I collagen columns, permitted isolation of two major peaks. In SDS-PAGE, peak 1 was found to contain two protein bands of 95 and 185 kDa, respectively, apparent molecular masses corresponding to those proposed in literature for $\beta_2$ and $\alpha_2$ chains (16). The identity of the two chains was assessed by immunoprecipitation of the fractions with anti-$\beta_2$ and anti-$\alpha_2$ monoclonal antibodies. Anti-$\alpha_2M$ and anti-$\alpha_2X$ monoclonal antibodies were devoid of effect. In addition, peak 1 contained traces of two other proteins, whose apparent molecular masses, as estimated by SDS-PAGE, corresponded to 31 and 35 kDa. We have no information on their nature and relationship with the $\alpha_2\beta_2$ integrin.

As regards the transduction pathways linking the membrane receptor of type I collagen to the effector systems of superoxide formation and enzyme granule secretion, neither G$_{\alpha}$ nor G$_{\beta\gamma}$ proteins were involved (absence of effect of cholera and pertussis toxins). The final result of PMN stimulations by the bacterial peptide fMet-Leu-Phe and by type I collagen is the same, but the former depends on a G$_{\alpha}$ protein associated to a seven-transmembrane domain receptor, whereas the second depends on the $\alpha_2\beta_2$ integrin; one can question at which point the transduction pathways for both systems become identical.

Cytocchalasin B, an inhibitor of F actin assembly, and colchicine, a disrupting agent for tubulin, exert severe inhibitory effects on type I collagen induced superoxide formation. In contrast, the same cytochalasin B enhances the stimulation by the peptide fMet-Leu-Phe. The latter effect points out to the intervention of actin and tubulin in the reaction to type I collagen.

Inositol trisphosphate increases sharply after collagen stimulation, and calcium ion also increases for a short period of about 30 s. From these results, it is inferred that the activation of the type I collagen receptor is transmitted to a phospholipase C, which liberates inositol trisphosphate and diacylglycerol. Few studies have pointed to cascades of stimulations involving $\beta_2$ integrins (28). Inositol trisphosphate stimulates the output of calcium ions from the endoplasmic reticulum. Calcium signaling through MAC 1 has been described (29, 30), in the case of cross-linking of this integrin by monoclonal antibodies. The
stimulation depends on the presence of Ca\(^{2+}\) in the medium surrounding cells. This requirement is at present unexplained. It may be related to the binding of collagen to the receptor. The effect of Ca\(^{2+}\) on enzyme-containing granules probably depends on the actomyosin system responsible for the exocytosis of these granules. The effect of calcium on the activation of the NADPH-oxidase system of PMN is difficult to explain and may be due to a calcium-dependent isoform of protein kinase C able to phosphorylate the cytoplasmic protein p47, a preliminary step for the assembly of the NADPH-oxidase at the membrane and to its activation.

A role for diacylglycerol in the process of PMN stimulation with type I collagen by activating a protein kinase C was confirmed by the inhibiting effect exerted by staurosporine and calphostin C. Nevertheless, another inhibitor of protein kinase C, H-7, did not inhibit the process, whereas it inhibited the stimulation exerted by the peptide fMet-Leu-Phe. Collagen stimulates the translocation of protein kinase C to the plasma membrane, as demonstrated by measurements of changes in enzyme activity.

The \(\alpha_\text{L}\beta_2\) integrin, when liganded by type I collagen, is phosphorylated on tyrosyl residues as demonstrated by the use of monoclonal antibodies to phosphotyrosine reacting with the subunits of the integrin separated by PAGE. PMA or fMet-Leu-Phe treatments of monocytes induce the phosphorylation of CD18, mainly on seryl residues, and to a lesser extent on threonyl and tyrosyl residues (16, 31). Phosphoserine was also detected on CD11a (16). On which residues of the \(\alpha_\text{L}\) and \(\beta_2\) peptide chains does this reaction take place? The \(\beta_2\) chain contains 1 tyrosyl residue in its intracellular domain (32). Four seryl residues but no tyrosyl are present in the intracellular domain of \(\alpha_\text{L}\). However, in the transmembrane domain, the 6th residue closer to the cytoplasmic domain is a tyrosine (23). Our results suggest that it might be accessible to an intracellular tyrosine kinase. Tyrosine phosphorylation of several intracel-
Bars represent 1 S.D. from the mean.

Polymerons under multiple stimulations in order to cope with all the situations of defense of the organism against the many invader cells or toxic substances. Specific membrane receptors exist for every stimuli. At the present time, only a few steps of the transduction pathways for these messages are known and most of them concern the effect of the peptide fMet-Leu-Phe. In this paper, we describe a somewhat different system of signaling, depending on the type I collagen or, more probably, on the large fragments of collagen that are liberated during the initial degradative steps of tissue inflammation or wound healing. The transduction system is characterized by several distinct features; the receptor is an integrin (namely αLβ2), the integrin is phosphorylated on tyrosine residues belonging to both subunits, there is no G protein involved, and cytoskeleton participates in the transduction pathway, which involves inositol trisphosphate, calcium ion, and protein kinase C.

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The Binding of Type I Collagen to Lymphocyte Function-associated Antigen (LFA) 1 Integrin Triggers the Respiratory Burst of Human Polymorphonuclear Neutrophils: ROLE OF CALCIUM SIGNALING AND TYROSINE PHOSPHORYLATION OF LFA 1
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