Exudation Primes Human and Guinea Pig Neutrophils for Subsequent Responsiveness to the Chemotactic Peptide *N*-formylmethionylleucylphenylalanine and Increases Complement Component C3bi Receptor Expression

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Abstract

After circulating in the vascular system a short time, polymorphonuclear leukocytes (PMN) migrate to extravascular sites in response to chemotactic stimuli. Prestimulation of PMN in vitro by secretagogues has been shown to increase their number of *N*-formylmethionylleucylphenylalanine (fmet-leu-phe) and complement component C3bi (CR3) receptors. We investigated whether the same phenomenon occurred in vivo, comparing characteristics of human skin chamber and guinea pig peritoneal exudate and blood PMN. Exudate PMN of both species contained ~28% less of the specific granule marker vitamin B_{12}-binding protein ($P < 0.01$) but a similar amount of the azurophil granule marker β-glucuronidase. The total number of fmet-leu-phe receptors was 5.9 times higher in guinea pig exudate than in blood PMN ($P < 0.01$) and 2.9 times higher in human exudate than in blood PMN ($P < 0.02$). All exudate PMN and most blood PMN preparations showed a high affinity receptor ($K_d \sim 2.3 \times 10^{-8}$ M) and a low affinity receptor ($\sim 1.5 \times 10^{-7}$ M). The upregulation of fmet-leu-phe receptors in exudate PMN correlated with an improved responsiveness to fmet-leu-phe induced membrane depolarization, oxidative metabolism, and chemotaxis. In addition, the concentration of fmet-leu-phe that produced a half-maximal response of chemotaxis, superoxide production, and membrane potential depolarization was 10-fold lower in exudate PMN than in blood PMN. Human exudate PMN had a twofold increased C3bi receptor expression compared with blood PMN. Thus, a preferential loss of specific granules is associated with increased number of high and low affinity fmet-leu-phe receptors and increased C3bi receptor expression not only in vitro, but also in vivo. The data indicate that exudation primes PMN for their subsequent responsiveness to fmet-leu-phe, a modification that may be crucial for efficient antimicrobial host defense.

Introduction

Polymorphonuclear leukocytes (PMN) circulate in the bloodstream for only a few hours before they migrate to extravascular sites where they continue to perform functions as phagocytic cells (1, 2). The signals for exudation are chemotactic stimuli (3). Such stimuli not only attract cells, but also modify the characteristics of PMN (4–8). Ward and Becker (9) first introduced the term "chemotactic deactivation". They showed that PMN preincubated with activated complement have an irreversibly reduced chemotactic response toward the same stimulus. Later, chemotactic deactivation was shown to occur in vivo in different clinical situations (10–12). Deactivation is not limited to chemotaxis. In vitro stimulation of PMN can also lead to less superoxide (O_2^-)[1] production under certain experimental conditions (5, 13). We recently reported a similar deactivation of O_2^- production in PMN stimulated in vivo (i.e., peritoneal exudate PMN) (4).

Under certain conditions the decreased functional responsiveness of PMN after stimulation is graded and rapidly reversible, a phenomenon called adaptation (14, 15). Adaptation has been characterized by measuring the increase in *N*-formylmethionylleucylphenylalanine (fmet-leu-phe) concentration required to elicit a second depolarization of membrane potential after an initial exposure to low concentrations of fmet-leu-phe. Adapted cells are not deactivated, since they can respond maximally to a sufficiently high concentration of fmet-leu-phe, and since they return to their native state upon removal of the initial fmet-leu-phe dose.

Stimulation of PMN does not always result in deactivation or adaptation. Under certain conditions PMN can be primed by stimulation in vitro (7, 16, 17). For example, studies from our laboratory showed that limited degranulation increases the availability of fmet-leu-phe receptors and enhances the functional capacity of PMN to respond to fmet-leu-phe (17, 18).

Thus, data presently reported in the literature do not allow a general conclusion whether the subsequent functional responses of PMN stimulated in vivo by exudation will be deactivated, adapted, or primed. Cell exudation can be defined as migration of circulating PMN into intravascular sites, and interaction of extravascular PMN with the surrounding fluid. In this study we used guinea pig peritoneal PMN on the one hand, and human skin chamber PMN on the other hand, to compare PMN stimulated in vivo with unstimulated circulating PMN under controlled conditions in the same species. We anticipated that exudate PMN might be functionally primed, since Wright and

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1. *Abbreviations used in this paper:* di-O-C_5(3), 3,3′-dipentyloxacarbocyanine; DMSO, dimethyl sulfoxide; EAS, endotoxin-activated serum; ED_{50}, concentration of stimulus producing a half-maximal response; fmet-leu-phe, *N*-formylmethionylleucylphenylalanine; HBSS, phosphate-buffered Hanks' balanced salt solution; H_2O_2, hydrogen peroxide; mHBSS, calcium-free and magnesium-free Hanks' balanced salt solution; O_2^-, superoxide; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes.

Gallin (19) had shown that in vivo exudation leads to partial degranulation of specific granules.

This is the first report showing that C3bi receptor expression is increased in vivo by exudation in humans and that the number of fmet-leu-phe receptors is increased not only in guinea pig peritoneal, but also in human skin window exudate PMN. The upregulation of fmet-leu-phe receptors in different exudate PMN correlates with an increased responsiveness to fmet-leu-phe in chemotaxis and O_2^- and hydrogen peroxide (H_2O_2) production. In addition, the concentration of fmet-leu-phe producing a half-maximal response (ED_{50}) of chemotaxis, O_2^-, and H_2O_2 production, and stimulation of membrane potential depolarization was ~1 log lower, even though a comparable change in binding affinities was not measurable. These results suggest that guinea pig and human exudate PMN were primed during extravasation for their subsequent response to the chemotactic peptide fmet-leu-phe.

Methods

Reagents. Phosphate-buffered Hanks' balanced salt solution with (HBSS) or modified without calcium and magnesium (mHBSS) (Whittaker; M. A. Bioproducts, Walkersville, MD) was used for all assays. The synthetic chemotactic peptide, fmet-leu-phe (Sigma Chemical Co., St. Louis, MO), was dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific Co., Pittsburgh, PA) to make appropriate stock solutions with the final DMSO concentration in the assay mixture below 0.1%, which does not interfere with cell function or viability (20). Fmet-leu-[^{3}H]phe (specific activity between 48.3 Ci/mmol and 60 Ci/mmol in different lots) and ^{57}Co-vitamin B_{12} were purchased from New England Nuclear, Boston, MA. Obtained as follows were: Cytochrome *c* (type VI from horse heart), glycogen (type II from oyster), phorbol myristate acetate (PMA), scopoletin, superoxide dismutase, Triton X-100, Hepes-buffer, and horseradish peroxidase (Sigma Chemical Co.); dextran T-500 and Percoll (Pharmacia Fine Chemicals, Piscataway, NJ); casein and lipopolysaccharide W *Escherichia coli* 0127:B8 (Difco Laboratories, Inc., Detroit, MI); fluoresceinated $F(ab')_2$ fragments of goat anti-mouse IgG (Tago, Inc., Burlingame, CA), OKM1 murine monoclonal antibodies anti-CR3 (C3bi receptors) (Ortho Pharmaceutical, Raritan, NJ), Na_2-EDTA (Fisher Scientific Co.); Versilube F50 (General Electric Co., Wilmington, MA); NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL); 3a20 counting solution (Research Products International Corp., Mt. Prospect, IL); glacial acetic acid (J. T. Baker Chemical Co., Phillipsburg, NJ); and activated charcoal (Matheson Coleman & Bell, Norwood, OH). Dr. Alan Waggoner (Carnegie-Mellon, Pittsburgh, PA) kindly provided 3,3'dipentyloxacarbocyanine [di-O-C_5(3)] (also available from Molecular Probes, Inc., Junction City, OR). Di-O-C_5(3) was dissolved in methanol to prepare a stock solution of 2.5×10^{-3} M dye from which a 1,000-fold dilution into HBSS was made giving the working stock solutions. The final assay concentration of di-O-C_5(3) was 2.5×10^{-8} M.

Animals. 600–800-g female albino outbred Hartley guinea pigs and in some control experiments tricolor inbred guinea pigs (strain, 131N) were used. The animals were housed in groups of 3–5 under optimal hygenic conditions with free access to food (guinea pig chow 5025, Ralston-Purina Co., St. Louis, MO) and water. The protocol for the animal experiments was approved by the National Institute of Allergy and Infectious Diseases animal user review committee.

Isolation of PMN. Guinea pig blood leukocytes were harvested by percutaneous heart puncture of CO_2-anaesthetized guinea pigs with a 21 gauge/1½ in. needle in a syringe containing Na_2-EDTA (5 mM final concentration). PMN were purified by dextran T-500 (4% in 0.85% saline) sedimentation and a discontinuous Percoll gradient by a method that was somewhat modified from the previously reported method (2). We found that the Percoll osmolarity measured by vapor pressure technique was variable and higher (over 116 mOsmol) than reported in the product description (20 mOsmol) (21). We measured the osmolarity of each new lot and calculated the amount of 10 times concentrated phosphate-buffered saline, which had to be added to render Percoll isotonic. This isotonic Percoll solution (usually 93% Percoll and 7% 10 times concentrated phosphate-buffered saline) was referred to as 100% Percoll. The separation was performed as previously described but on a 55/73% gradient prepared by diluting 100% Percoll with the appropriate volume of mHBSS (2). The volume of dextran sedimented EDTA-anticoagulated blood was reduced to 5 ml by centrifugation (350 *g* for 5 min/4°C) without washing and layered on top of this gradient and centrifuged 350 *g* for 25 min at 4°C. PMN accumulating at the interphase between the Percoll gradients were washed in mHBSS. Because of occasional contamination with erythrocytes, residual erythrocytes were lysed hypotonically (15-s distilled H_2O followed by correction of the osmolarity with 1.7% NaCl) after the first washing step. For experiments in which large PMN numbers were required, we pooled PMN of different animals after the hypotonic lysis. Control experiments showed that there was no difference in membrane potential response between PMN from an individual animal and pooled PMN from different outbred or inbred guinea pigs. Acute peritoneal exudates rich in PMN (~90% PMN, ~10% macrophages) were obtained by a single intraperitoneal injection of sterile 12% casein or 0.1% glycogen in 0.85% NaCl. 14 h after injection, guinea pigs were sacrificed with CO_2 overdose, the abdominal cavity was washed three times with mHBSS, and the leukocyte suspension was purified on a 50/70% Percoll gradient so that the final leukocyte preparation contained over 97% PMN, 1–2% eosinophils, and 1% esterase-positive cells as previously described (2). Control experiments (membrane depolarization and fmet-leu-[^{3}H]phe binding) were done with exudate cells that were sedimented on dextran before and hypotonically lysed after Percoll separation to be sure there were no functional differences between exudate and blood cells resulting from differences in cell isolation. These additional procedures, which were not necessary in exudate cells, did not alter cell characteristics, and therefore in most experiments were not applied.

Human blood PMN were purified from EDTA-anticoagulated (5 mM final concentration) blood by dextran T-500 (3% in 0.85% saline) sedimentation and subsequent centrifugation (350 *g* for 25 min at 4°C) on a 60/68% Percoll gradient. PMN accumulating at the interphase of the gradient were washed twice in mHBSS. Hypotonic lysis was not necessary because of the clean separation between the PMN and erythrocyte fractions. Human exudate PMN were prepared from 13 volunteers (five women and eight men, aged 18–37 yr) by the skin chamber technique in accordance with approved National Institute of Allergy and Infectious Diseases protocol No. 77-I-185. This technique was modified from previously reported methods (19, 22). In collaboration with Neuro Probe Inc., Bethesda, MD, we developed an eight-well polycarbonate skin suction unit and a congruous skin chamber unit, which will be commercially available (Neuro Probe Inc.). Briefly, we disinfected the volar surface of the forearm of the volunteers with ethanol, and produced eight blisters using a suction chamber with eight wells connected to a suction pump delivering a valve controlled suction of −360 mmHg (Spectrum Medical, Los Angeles, CA). After ~1½ h the blisters were complete and the tops were removed. The eight lesions with ~10 mm diam were covered with the skin chamber unit and filled with 0.8 ml 70% autologous serum in mHBSS. After ~14 h the chamber fluid was aspirated and the cell numbers were counted. The mean leukocyte number per milliliter chamber fluid was $2.4 \pm 0.4 \times 10^6$ (range, 5.3×10^5–5.6×10^6 in 41 chambers on nine different volunteers). Each chamber was rinsed twice with mHBSS to remove surface adherent cells. The chamber fluid, containing virtually no erythrocytes, >96% PMN, 1–3% eosinophils, and 1–2% monocytes, was either washed twice or centrifuged on a 50/70% Percoll gradient. In control experiments we found that the ED_{50} of the membrane depolarization was similar whether skin chamber leukocytes were only washed ($ED_{50} = 6 \times 10^{-8}$ M), resuspended in mHBSS and centrifuged on Percoll ($ED_{50} = 6 \times 10^{-8}$ M), or resuspended in 50% autologous serum/mHBSS before centrifugation on the Percoll gradient ($ED_{50} = 4 \times 10^{-8}$ M). Subsequently, all experiments were done without Percoll sedimentation, since unseparated skin chamber fluid contained >96% PMN.

Fluorescence assay of membrane potential. The essay for bulk PMN suspension has been previously described (23). In our experiments we

used a fluorescence spectrophotometer (Perkin-Elmer LS5; Perkin-Elmer Corp., Oak Brook, IL) interfaced with a Perkin-Elmer 3600 Data Station. PMN (2.5×10^5/ml) were equilibrated in a stirred and thermostated (37°C) five-cell cuvette holder with di-0-C_5(3) (2.5×10^{-8} M) at 37°C for 15 min in HBSS to allow PMN to reach thermal, ionic, and dye equilibrium. In the fluorometer PMN suspensions were maintained with a magnetic stir bar, and fluorescence was monitored continuously (excitation wavelength, 460 nm and fluorescence wavelength, 510 nm) before and after addition of the stimulus.

Superoxide production. O_2^- production was determined spectrophotometrically (549 nm, mM extinction coefficient of 21,100 M^{-1} cm^{-1}) by monitoring at 1-min intervals the superoxide dismutase (300 U/ml) inhibitable reduction of cytochrome *c* (120 μg/ml) by a stirred suspension of PMN (1.25×10^6 PMN/ml) in a five-cell cuvette holder of a Lambda 3 spectrophotometer (Perkin-Elmer Corp.) interfaced with a 3600 Data Station. The stimulus was added after a 10-min incubation in the thermostated (37°C) cuvette holder. The change in optical density was followed for 20 min after stimulation. This modification of techniques described previously (2, 24) allows the simultaneous recording of five different PMN suspensions. Resting exudate and blood PMN produced both <0.03 nmol superoxide/10 min per 10^6 PMN.

Hydrogen peroxide production. H_2O_2 production was measured with the scopoletin assay as described (25). Briefly, fluorescence of scopoletin (final concentration, 4 μM; excitation wavelength, 350 nm; and fluorescence wavelength, 460 nm) was monitored continuously in a stirred, thermostated cuvette containing PMN (2.5×10^5 PMN, final concentration) and horseradish peroxidase (final concentration, 0.24 μM). The loss of fluorescence before and after adding a stimulus allowed us to calculate the rate of H_2O_2 production in nmol/min (standardization with ethylperoxide, Polysciences, Inc., Warrington, PA). Resting exudate and blood PMN produced <0.06 nmol H_2O_2/5 min per 10^6 PMN.

Chemotaxis assay. PMN migration was quantitated using a micropore filter technique in a 48-well microchamber (Neuro Probe, Inc.). The distribution of PMN in a 3.0-μm cellulose nitrate filter (Sartorius, Testing Machines, Inc., Amityville, NY) was determined after 45 min incubation (37°C) according to the method of Zigmond and Hirsch (26). The filters were counted with a photomicroscope (2; Carl Zeiss, Inc., Thornwood, NY) connected to an image analyzer (Optomax CPU-2; Micromeasurements, Cambridge, England) interfaced with a Hewlett-Packard 9815 calculator and 7225A plotter (Hewlett-Packard Co., Palo Alto, CA) as previously described (27). Results were calculated by analyzing two fields in each of four different filters in vertical intervals of 10 μm.

Fmet-leu-[^{3}H]phe binding assay. Peptide binding to PMN was carried out at 4°C using a silicone oil technique as described previously (18). The incubation time was 20 min. Binding equilibrium was reached between 2 and 10 min. For each experimental condition the total binding (fmet-leu-[^{3}H]phe in mHBSS only) and the nonspecific binding (fmet-leu-[^{3}H]phe plus 1,000-fold excess of nonradioactive fmet-leu-phe) was determined. For Scatchard analyses final concentrations of fmet-leu-[^{3}H]phe were chosen between 3 and 400 nM (in some experiments 600 nM), as previously described (17, 18). Scatchard plots of the experimental data in this range were fitted by linear regression and the dissociation constants and receptor number were estimated from the fitted slopes and *x*-intercepts. Corresponding estimates were also made by a computer modeling method based on mass action principles (28) using a nonlinear least squares algorithm (29).

Staining with OKM1 antibody to C3bi(CR3) receptor and flow cytometry. Human exudate and blood PMN (10^6 in 75 μl mHBSS) were incubated in Eppendorf microfuge tubes with 25 μl OKM1 (200 μg/ml) for 30 min on ice. The tubes were then spun in a tabletop microfuge (Eppendorf Model 5412) for 5 s, the supernatant was aspirated, and the cells were resuspended in 75 μl mHBSS and 25-μl fluoresceinated $F(ab')_2$ fragments of goat anti-mouse IgG were added. After a second incubation for 30 min on ice, the cells were spun again (5 s in microfuge) and resuspended in 0.5 ml paraformaldehyde (2%). Parallel preparations were done with both PMN types without adding any antibodies in order to control for autofluorescence, which was low in each experiment. After 1 h cells were centrifuged (5 s in microfuge) and resuspended in 0.4 ml mHBSS. Flow cytometry was performed on a FACS II cell sorter (Becton-Dickinson & Co., Oxnard, CA) as described (30). After computer transformation to a linear scale, the mean fluorescence of each sample was calculated.

Enzyme assays. Vitamin B_{12}-binding protein and β-glucuronidase were determined as described previously (31).

Statistics. Standard error was used as an estimate of variance, and means were compared by Student's *t* test (two-tailed). For some comparative data in which the distribution was uneven, the geometric mean $\times/\div$ relative standard error was calculated, and the corresponding mean logs were compared using *t* test on summary data.

Results

Degranulation of PMN in vivo. We first characterized the granule content of each PMN type used in our experiments by measuring vitamin B_{12}-binding protein as a marker of specific granules and β-glucuronidase as a marker of azurophil granules. Guinea pig ~14-h peritoneal exudate PMN contained 28% less vitamin B_{12}-binding protein than blood PMN (289±8 pg/10^6 PMN vs. 401±6 pg/10^6 PMN, three experiments, $P < 0.001$). The in vivo degranulation of human PMN was very similar with 14-h exudate cells having 27% less vitamin B_{12}-binding protein than blood cells (72±7 pg/10^6 exudate PMN vs. 98±9 pg/10^6 blood PMN, six experiments, $P < 0.01$). In contrast, the total content of β-glucuronidase was not significantly different in exudate and blood PMN. Guinea pig PMN contained 191±14 vs. 186±2 μg phenolphthalein released/6 h per 10^6 exudate and blood PMN, respectively ($P > 0.05$). Human PMN contained 144±8 vs. 164±12 μg phenolphthalein released/4 per 10^6 exudate and blood PMN, respectively ($P > 0.05$). We therefore concluded that guinea pig and human exudate PMN had both preferentially lost their specific granules.

Membrane potential. Since limited degranulation may modify cell characteristics, we measured the fmet-leu-phe induced depolarization in exudate and blood PMN of guinea pigs and men. In guinea pig exudate PMN the loss of di-0-C_5(3) fluorescence in response to 10^{-5} M fmet-leu-phe (i.e., depolarization) was between 5 and 50% greater than in blood PMN in different experiments. In human exudate PMN 10^{-5} M fmet-leu-phe induced also a variably greater depolarization response (0–60% in three experiments) than in blood PMN. Furthermore, the ED_{50} for fmet-leu-phe induced depolarization was ~1 log lower in guinea pig exudate than blood PMN in each experiment (Fig. 1, left, <0.0001). Control experiments showed that the lower ED_{50} in exudate PMN was not due to particle (casein) interaction, because glycogen elicited peritoneal PMN had an ED_{50} (7.5×10^{-10} M) that was even more than 1 log lower than the ED_{50} of blood PMN. Furthermore, the low ED_{50} was not due to endotoxin contamination of the casein, because the ED_{50} of blood PMN was not significantly different whether PMN were preincubated in endotoxin (30 μg/ml, 30 min at 37°C) or buffer (1.8×10^{-8} M vs. 2.4×10^{-8} M). Fig. 1, right shows the ED_{50} of experiments using human exudate and blood PMN. The geometric mean of the fmet-leu-phe concentration leading to a half-maximal depolarization was 0.75 log lower in the exudate cells in four experiments ($P < 0.02$).

These results indicate that purified exudate PMN, whether casein or glycogen elicited from guinea pig peritonea or migrating toward autologous serum in human skin chambers, were not deactivated with respect to a depolarization response but were rather primed for fmet-leu-phe stimulation.

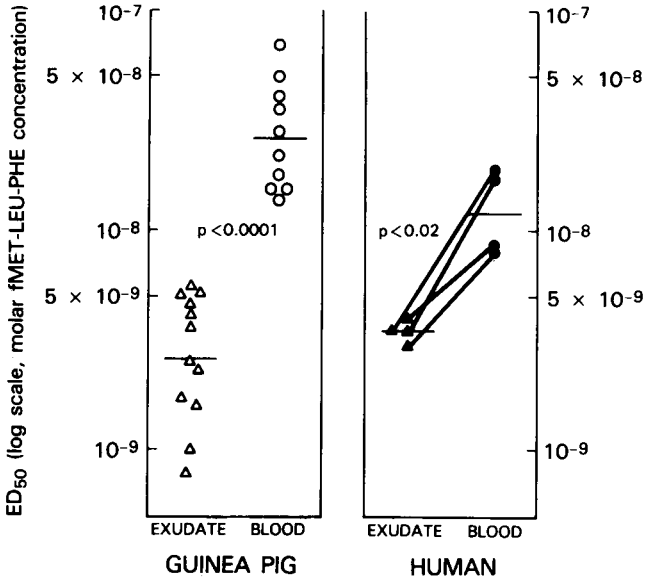

Figure 1. Fmet-leu-phe dose leading to half-maximal depolarization in guinea pig (*left*) exudate (△), blood (○) PMN, human (*right*) exudate (▲), and blood (●) PMN. Each symbol is calculated from a complete dose-response curve performed in triplicate. The significance of the difference between the geometric means of the values of the two cell types is determined by the two-tailed Student's *t* test.

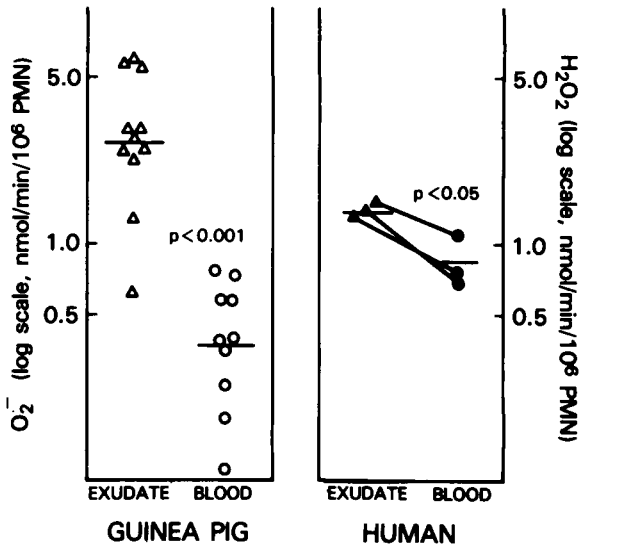

Figure 2. Maximal linear rate of superoxide (O_2^-) production in guinea pig (*left*) exudate (△), blood (○) PMN, and of hydrogen peroxide (H_2O_2) production in human (*right*) exudate (▲) and blood (●) PMN in response to fmet-leu-phe (10^{-5} M). In human data, lines connect exudate and blood PMN from the same individual, tested the same day. The significance of the difference between the geometric means is determined by the two-tailed Student's *t* test.

Superoxide and hydrogen peroxide production. To confirm the priming of exudate PMN to fmet-leu-phe, we tested the O_2^- and H_2O_2 production of guinea pig and human PMN, respectively, in response to fmet-leu-phe and PMA. The maximal linear rate of O_2^- production in response to 100 ng/ml PMA was similar in guinea pig blood and exudate PMN (4.9±0.7 nM O_2^-/min per 10^6 blood PMN, $n = 7$ vs. 5±0.5 nM O_2^-/min per 10^6 exudate PMN, $n = 10$, $P > 0.05$). However, similar to the membrane potential results, guinea pig blood PMN produced O_2^- at a slower rate in response to 10^{-5} M fmet-leu-phe than exudate PMN (0.37 nM O_2^-/min per 10^6 PMN, geometric mean, $n = 10$ vs. 2.7 nM O_2^-/min per 10^6 PMN, geometric mean, $n = 11$, $P < 0.001$) (Fig. 2, left). The difference between the average rate of O_2^- produced during 20 min was even more impressive, because the O_2^- production decreased in blood PMN earlier than in exudate PMN (2.0 nM $O_2^-/10^6$ PMN, $n = 10$ vs. 20.0 nM $O_2^-/10^6$ PMN, geometric means, $n = 11$, $P < 0.001$). The ED_{50} for O_2^- production was significantly lower in exudate PMN (5.8×10^{-9} M geometric mean, $n = 6$) than in blood PMN (3.2×10^{-7} M geometric mean, $n = 3$, $P < 0.025$).

In studies with human cells, H_2O_2 production was used as an indicator of oxidative metabolism, since too few exudate cells were obtained to assay O_2^-; the data were similar to guinea pigs. The maximal linear rate of H_2O_2 production in response to 100 ng/ml PMA was identical in blood and exudate PMN (0.98±0.16 vs. 0.98±0.09 nM H_2O_2/min per 10^6 PMN). However, blood PMN produced less H_2O_2 in response to 10^{-5} M fmet-leu-phe than exudate PMN (0.84 vs. 1.37 nM H_2O_2/min per 10^6 PMN, geometric means; Fig. 2, right). Due to the shorter duration of the oxidative burst in blood than in exudate PMN (<70 s vs. >70 s), the total H_2O_2-production during 2 min was striking, with 0.94 nM $H_2O_2/10^6$ blood PMN ($n = 3$) vs. 1.93 nM $H_2O_2/10^6$ exudate PMN ($n = 3$) geometric means, $P < 0.01$). The geometric means of the ED_{50} for H_2O_2-production were 0.75 log lower in human exudate (1.4×10^{-8} M ×/÷ 1.9, $n = 3$) than in blood PMN (9.1×10^{-8} M ×/÷ 1.4, $n = 3$, $P < 0.01$).

Chemotaxis. In guinea pig cells, spontaneous nondirected migration (to buffer) was lower in exudate than in blood PMN in each of five comparative assays ($P < 0.01$; Fig. 3). Directed migration toward endotoxin-activated guinea pig serum (EAS) was also significantly lower in exudate PMN compared with blood PMN (five experiments, $P < 0.005$). However, when casein was used, the migration was similar in both cell types (four experiments, $P > 0.05$). In contrast, the stimulated migration toward fmet-leu-phe was significantly greater in exudate than in blood PMN (10^{-9} M fmet-leu-phe, $P < 0.01$, or 10^{-8} M, $P < 0.001$, three experiments). Additionally, the optimal migration for exudate PMN was seen at lower concentrations of fmet-leu-phe (10^{-8} M vs. 10^{-7} M).

Experiments with human skin chamber exudate PMN showed a nonsignificantly increased spontaneous nondirected migration (174% of blood PMN migration in four experiments,

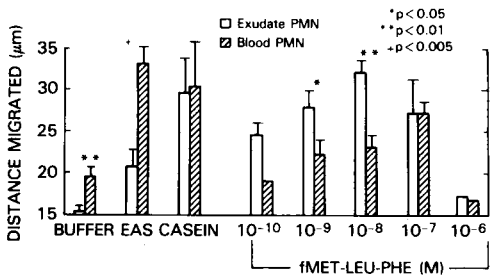

Figure 3. Locomotion of exudate (□) and blood (■) PMN. The ordinate gives the average distance migrated (micrometers) by PMN into nitrocellulose filters between microwell chambers. Results are means±SE of 3–5 experiments. *P* values are the levels of significance between exudate and blood PMN (two-tailed Student's *t* test).

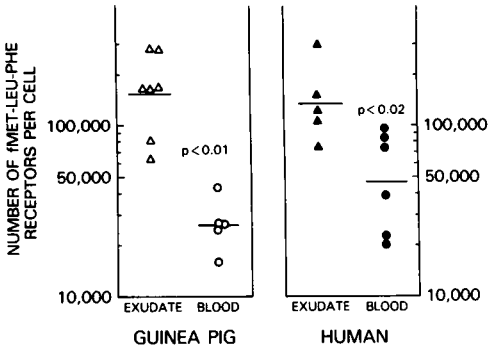

Figure 4. Total number of fmet-leu-phe receptors per cell. Results of guinea pig exudate (△) and blood (○) PMN are given (*left*) and those of human exudate (▲) and blood (●) PMN (*right*). The data are plotted on a logarithmic scale. The significance of the difference between the geometric means is determined by the two-tailed Student's *t* test.

$P > 0.05$), a result that contrasted with the animal experiments. However, similar to guinea pig peritoneal exudate PMN, human exudate PMN had a decreased directed migration toward EAS (13% of blood PMN migration in three experiments, $P < 0.05$). The migration toward casein was similar in both PMN types (exudate PMN 120% migration of blood PMN in three experiments, $P > 0.05$). The better migration of guinea pig exudate PMN toward fmet-leu-phe was also confirmed in human PMN. The migration toward each fmet-leu-phe concentration (10^{-6}–10^{-9} M) was better in exudate PMN (average of 236% of blood PMN migration in three experiments, $P < 0.01$).

Fmet-leu-[^3H]phe binding. Since guinea pig and human exudate PMN showed an increased response to fmet-leu-phe in respect to membrane depolarization, oxidative metabolism, and chemotaxis, we tested whether this improved responsiveness was correlated with a higher fmet-leu-[^{3}H]phe binding in exudate than in blood PMN. Fig. 4 shows the total number of fmet-leu-[^{3}H]phe binding sites in guinea pig (left) and human (right) PMN. The geometric mean of the total number of receptors was 5.9 times higher in guinea pig exudate than in blood PMN ($P < 0.001$). Human exudate PMN bound 2.9 times more fmet-leu-[^{3}H]phe than blood PMN ($P < 0.02$). In individual experiments done simultaneously with exudate and blood PMN from the same donor this difference was between 1.4-fold and 3.4-fold. The number of fmet-leu-phe receptors per cell and the receptor affinities were estimated from the fmet-leu-[^{3}H]phe saturation curves (Figs. 5 *A* and 6 *A*), and from Scatchard plots (Figs. 5 *B* and 6 *B*), and confirmed by computer modeling (Tables I and II). For both exudate and blood cells from guinea pigs and humans, fmet-leu-[^{3}H]phe binding approached saturation at 400 nM (Figs. 5 *A* and 6 *A*). In each experiment guinea pig and human exudate PMN had a curvilinear Scatchard plot of fmet-leu-[^{3}H]phe binding (Figs. 5 *B* and 6 *B*), indicating the presence of two receptors with different affinities or negative cooperativity (15, 32). Computer modeling indicated that three quarters of the guinea pig and human exudate PMN fmet-leu-phe receptors were in the low affinity state (Tables I and II). Furthermore, it revealed two classes of receptors in five of seven experiments using guinea pig blood PMN (Table I) and in four out of six experiments using human cells (Table II). In two guinea pig experiments and two human experiments the Scatchard plot as well as the computerized data suggested the presence of only one type of fmet-leu-phe receptor on blood PMN. As summarized in Table I (guinea pig PMN) and Table II (human PMN), in cells with two classes of receptors (high and low affinity) the same class had similar values in all cell types. In guinea pig and human PMN the fraction of high affinity receptors was similar in exudate and blood PMN.

C3bi receptor expression. Since fmet-leu-phe receptors were increased on the surface of exudate PMN, we tested whether the expression of C3bi receptors, measured as OKM1 antibody fluorescence with flow cytometry, showed a similar upregulation. Fig. 7 *A* shows the result of an individual experiment done on blood and exudate PMN of the same volunteer. Unstained PMN showed identical low autofluorescence for blood and exudate

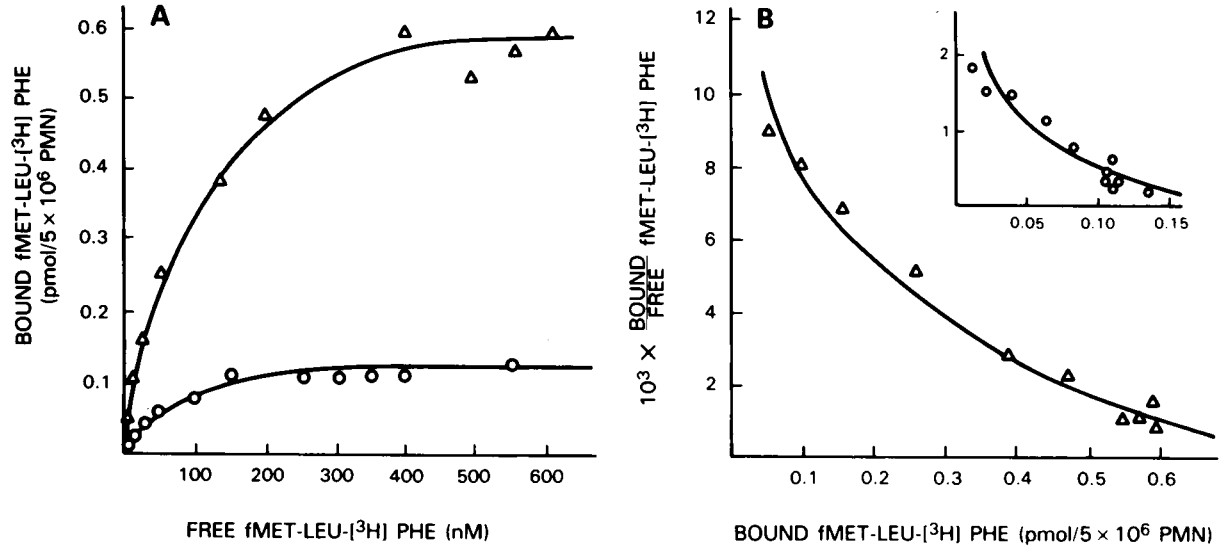

Figure 5. Representative experiment of specific fmet-leu-[^{3}H]phe binding to guinea pig exudate (△) and blood (○) PMN. *A* shows binding isotherms to both cell types. PMN were incubated with varying concentrations of fmet-leu-[^{3}H]phe at 4°C for 20 min. Each symbol represents the specific binding (total binding minus nonspecific binding in presence of 1,000-fold excess of cold fmet-leu-phe). *B* shows a transformation of the same data in a Scatchard plot. The inset shows the results of blood (○) PMN in a twofold expanded scale.

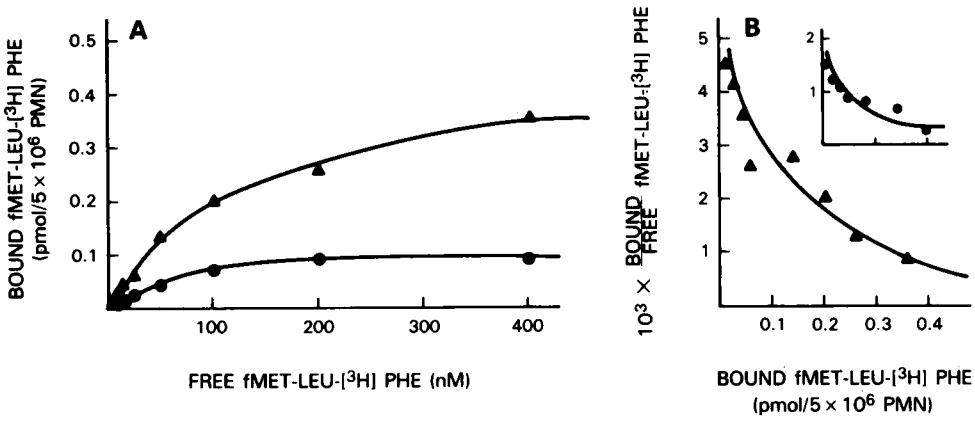

Figure 6. Representative experiment of the specific fmet-leu-[^{3}H]phe binding to human exudate (▲) and blood (●) PMN from the same individual. *A* shows binding isotherms to both cell types. PMN were incubated with varying concentrations of fmet-leu-[^{3}H]phe at 4°C for 20 min. Each symbol represents the specific binding (total binding minus nonspecific binding in presence of 1,000-fold excess of cold fmet-leu-phe). *B* shows a transformation of the same data in a Scatchard plot. The *inset* shows the results of blood (●) PMN in a two-fold expanded scale.

PMN. The results of the mean OKM1 fluorescence in five volunteers in whom blood and exudate PMN were tested simultaneously are presented in Fig. 7 *B*. The mean OKM1 fluorescence, i.e., the C3bi expression, was two times higher in exudate than blood PMN ($P < 0.001$).

Discussion

PMN interacting with chemotactic factors change their characteristics with respect to surface charge, chemotaxis, oxygen metabolism, and bactericidal activity (4–9). During exudation, PMN are exposed to chemotactic stimuli for a prolonged time. We therefore asked the question of whether casein-induced exudation in guinea pigs and skin window exudation in humans, as paradigms of in vivo stimulation, led to modification of cell functions. In previous studies, human exudate PMN from synovial or crevicular fluid were compared with circulating blood PMN (11, 12, 33). The main conclusion of these studies was that exudation leads to deactivation of chemotaxis and phagocytosis. However, these exudate PMN were from sites harboring bacteria (11) and immune complexes (12), respectively. The deactivation of these PMN is therefore not necessarily due to exudation, but possibly to previous interaction with immune complexes or bacteria. It has recently been shown that bacteria, such as *E. coli*, produce fmet-leu-phe in culture (34). One can therefore argue that fmet-leu-phe receptors on crevicular PMN may be occupied by bacterial formylpeptides or down-regulated by previous exposure to high doses of fmet-leu-phe (35).

In our experiments we used exudate PMN from a sterile peritonitis in guinea pigs on the one hand and human skin chamber PMN on the other hand and compared them with circulating PMN of the same species. By this approach we could study the effect of exudation into sterile sites. For the purposes of our study exudation refers to the migration of cells from blood to the extravascular space and all the events that occur to the cells after migration and before sampling. In previous studies exudate and blood cells were prepared by dissimilar methods, resulting in cell suspensions that may have been differently activated by the purification procedures (4, 7, 36). In the present study we exposed all cell types to a similar Percoll purification procedure, which resulted in cell preparations of similar high purity. To exclude a specific casein-effect in guinea pig exudate PMN, we measured similar fmet-leu-phe-induced membrane depolarization with glycogen-elicited exudate PMN. Since casein may be contaminated with endotoxin, we also performed studies to exclude nonspecific effects of endotoxin as an explanation for the difference between exudate and blood cell. It is, therefore, unlikely that the described differences are artifact of the purification procedures, especially since human exudate PMN elicited by autologous serum showed similar differences from blood cells.

*Table I. F-met-leu-[^{3}H]phe Binding to Guinea Pig Blood and Exudate PMN**

Cell type	Number of experiments	Dissociation constants (M)		Receptors/cell	
		Low affinity	High affinity	Low affinity	High affinity
Blood PMN (One receptor)	2	3.8×10^{-8}		20,000	
Blood PMN (Two receptors)	5	1.4×10^{-7} ×/÷ 1.7‡	2.8×10^{-8} ×/÷ 2.3‡	18,800 ×/÷ 1.4§	6,600 ×/÷ 2.1‖
Exudate PMN (Two receptors)	7	9.6×10^{-8} ×/÷ 2.3‡	2.2×10^{-8} ×/÷ 1.6‡	106,500 ×/÷ 1.8§	39,000 ×/÷ 1.6‖

* 5×10^6 PMN/ml were incubated during 20 min with different concentrations of fmet-leu[^{3}H]phe in the presence or absence of a 1,000-fold excess of cold fmet-leu-phe as described in Methods. The dissociation constants and receptor numbers were calculated as described in Methods. Data were calculated separately for experiments in which the computed results suggested the presence of only one affinity receptor. All results are reported as geometric means ×/÷ relative SE. ‡ $P > 0.05$; § $P < 0.01$; and ‖ $P < 0.001$; all are Student's t test on means, comparing exudate and blood PMN for experiments with two affinity receptors.

*Table II. F-met-leu-[^{3}H]phe Binding to Human Blood and Exudate PMN**

Cell type	Number of experiments	Dissociation constants (M)		Receptors/cell	
		Low affinity	High affinity	Low affinity	High affinity
Blood PMN (One receptor)	2	9.3×10^{-8}		29,800	
Blood PMN (Two receptors)	4	$2.1 \times 10^{-7} \times/\div 1.7$‡	$2.4 \times 10^{-8} \times/\div 2.3$‡	47,900 ×/÷ 2.3‡	12,000 ×/÷ 2.1§
Exudate PMN (Two receptors)	5	$1.2 \times 10^{-7} \times/\div 1.3$‡	$1.8 \times 10^{-8} \times/\div 2.4$‡	96,500 ×/÷ 1.8‡	35,400 ×/÷ 1.7§

* 5×10^6 PMN/ml were incubated during 20 min with different concentrations of fmet-leu[^{3}H]phe in the presence or absence of a 1,000-fold excess of cold fmet-leu-phe as described in Methods. The dissociation constants and receptor numbers were calculated as described in Methods. Data were calculated separately for experiments in which the computed results suggested the presence of only one affinity receptor. All results are reported as geometric means ×/÷ relative SE. ‡ $P > 0.05$; and § $P < 0.05$; all are Student's t test on geometric means, comparing exudate and blood PMN for experiments with two affinity receptors.

Our results show that limited degranulation increases the number of fmet-leu-phe receptors, the C3bi receptor expression, and the functional properties not only in vitro but also in vivo. The increased number of fmet-leu-phe and C3bi receptors in exudate PMN, together with their lower content of specific granules, are compatible with the concept that the upregulation results from translocation of a putative pool of fmet-leu-phe and C3bi receptors associated with specific granules or a closely related intracellular compartment (18, 37–39).

Our fmet-leu-phe receptor results confirm and extend data from Tsung et al. (40), who showed a sevenfold increased fmet-leu-phe receptor number and an improved chemotactic responsiveness of rabbit peritoneal PMN compared with blood PMN of the same species. In contrast to their data (40), we found curvilinear Scatchard plots in all studies using human and guinea pig exudate PMN and in most experiments using blood PMN. Since Tsung et al. (40) used a filtration technique for binding assays in contrast to our method of centrifuging cells through silicone oil, the ligand on low affinity receptors may have been removed by the washing procedure necessary in Tsung's study (40). Interestingly, using the oil centrifugation technique, the same group later described two binding sites with different affinities on rabbit peritoneal PMN, as we describe here on human and guinea pig PMN (41).

Human and guinea pig exudate PMN increased the number of high and low affinity fmet-leu-phe receptors at the same degree. It has been suggested that whereas a high affinity state of the receptors is associated with chemotaxis, a low affinity state may be associated with degranulation and the respiratory burst (15, 18). The increase of both high and low affinity fmet-leu-phe receptors in exudate PMN may explain the improvement of fmet-leu-phe-induced chemotaxis on the one hand and generation of products of the oxygen metabolism on the other hand.

In our study exudate PMN showed a half-maximal membrane depolarization and H_2O_2 or O_2^- production at lower fmet-leu-phe concentrations than blood PMN. Since the affinities of fmet-leu-phe receptors were similar in exudate and blood PMN, it was not obvious why exudate PMN responded to lower fmet-leu-phe concentrations. One possible explanation could be that the absolute number of high affinity receptors on exudate PMN, being higher than on blood PMN, is sufficient to trigger the respiratory burst, a function known to require a high receptor occupancy (50%) and to be induced only by relatively high fmet-leu-phe concentrations (42). If a critical absolute number of occupied receptors is crucial to trigger cell functions, the higher sensitivity of exudate PMN to fmet-leu-phe would be explained. This could be the physiological rational of the upregulation of fmet-leu-phe receptors by exudation, since superoxide produc-

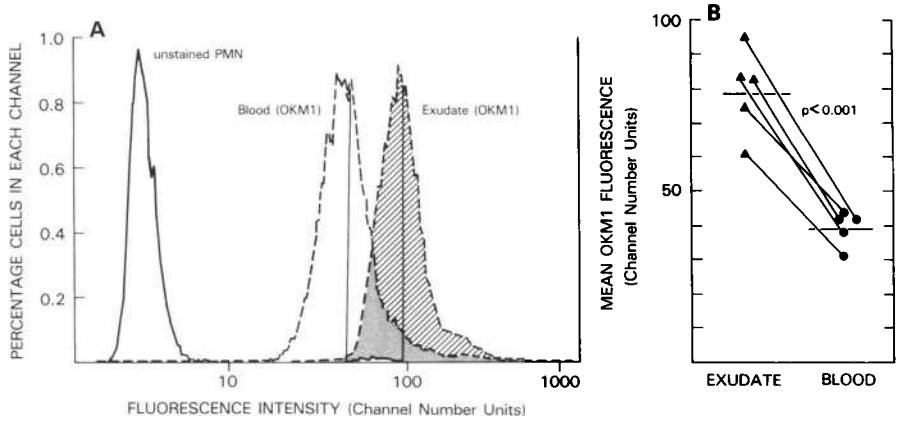

Figure 7. C3bi receptor expression of human exudate (▲) and blood (●) PMN. PMN (10^6) were incubated with 5 μg OKM1 for 30 min on ice and subsequently with 25 μl fluoresceinated F(ab')$_2$ fragments of goat anti-mouse IgG for an additional 30 min. Green fluorescence is measured on paraformaldehyde-fixed cells on a cell sorter and reported as mean fluorescence of the >95% cells that were OKM1 positive. *A* shows an individual experiment. Autofluorescence of exudate PMN, OKM1 fluorescence of blood, and exudate PMN are shown. The bars give the mean fluorescence. *B* shows the mean OKM1 fluorescence on paired exudate and blood samples. The significance of the difference between the arithmetic means is determined by the two-tailed Student's t test.

tion of toxic oxygen products during circulation or migration would be detrimental to the cell, due to autooxidation and premature exhaustion (2, 43).

All our functional studies showed an improvement of fmet-leu-phe-induced responsiveness in exudate PMN. Variable effects of exudation on subsequent chemotaxis have been reported in the literature. Whereas crevicular PMN show a markedly decreased migration toward fmet-leu-phe and C5a, synovial PMN and monocytes from patients with rheumatoid arthritis have a decreased chemotaxis only toward C5a, but a normal responsiveness toward fmet-leu-phe (12, 44). In the latter study synovial monocytes had increased numbers of fmet-leu-phe receptors and a decreased number of C5a receptors compared with circulating monocytes (44). Peritoneal exudate PMN from rabbits were shown to have an impaired chemotaxis toward immune complexes activated plasma (36). A similar chemotactic deactivation for activated serum was shown in burn patients (10). The reason for this was shown to be a down-regulation of C5a receptors (45) and may be a consequence of previous exposure of cells to C5adesArg (46). Interestingly, the turnover of C5a receptors is much slower (several hours) than the turnover of fmet-leu-phe receptors (minutes) (45). Since, on the one hand, infected or necrotized sites may contain high concentrations of *N*-formyl peptides coming from host cells' mitochondria (47) or microbial products (34), and on the other hand, circulating blood may contain considerable levels of C5adesArg, the down-regulation of C5a receptors (44, 45) and up-regulation of fmet-leu-phe receptors may help keep cells in inflammatory sites.

Thus, the data show that human exudate PMN have an increase in C3bi receptor expression. Furthermore, human and guinea pig exudate PMN have a greater functional responsiveness to fmet-leu-phe compared with blood PMN. This priming may be explained by the observed increased number of both high and low affinity fmet-leu-phe receptors during exudation.

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