Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2213–2217, April 1987
Biochemistry

Platelet-derived growth factor stimulates phagocytosis and blocks agonist-induced activation of the neutrophil oxidative burst: A possible cellular mechanism to protect against oxygen radical damage

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Communicated by Richard M. Krause, December 22, 1986 (received for review October 31, 1986)

ABSTRACT **The effect of platelet-derived growth factor (PDGF) on agonist-induced activation of the superoxide-generating oxidative burst in human neutrophils was tested. PDGF had no effect on the resting level of superoxide generation but inhibited both the rate and the extent of fMet-Leu-Phe-stimulated superoxide production in a dose-dependent manner. The concentration required to inhibit the response by 50% was 95 ± 26 pM (n = 10). PDGF also blocked activation by other receptor-mediated agonists such as the complement protein C5a and opsonized zymosan, but not by phorbol myristate acetate or arachidonate, both of which may act at postreceptor sites. The growth factor, however, had no effect on the binding of fMet-Leu-Phe to its receptor. PDGF in concentrations that blocked the oxidative burst stimulated phagocytosis of opsonized latex particles. Thus, PDGF functions as a heterologous "down-regulator" of receptor-mediated activation of the neutrophil oxidative burst and an activator of phagocytosis. A model for a feedback regulatory loop between platelets and neutrophils is proposed.**

Neutrophils (polymorphonuclear leukocytes) are phagocytic leukocytes that function as the primary defense against bacterial infection. Their killing of bacteria involves a complex series of events (1, 2) in which neutrophils first migrate into the infected area in response to a variety of chemoattractants such as bacterial-derived formylated peptides [e.g., formylmethionylleucylphenylalanine (fMet-Leu-Phe) (3)], the complement protein C5a (4), and platelet-derived growth factor (PDGF) (5). The neutrophils then phagocytize the bacteria, a process that involves the infolding of the plasma membrane around the microorganism to form an enclosed vesicle, the phagosome. Bacterial killing then occurs via both oxygen-dependent (6) and oxygen-independent mechanisms.

Oxygen-dependent killing is characterized by an "oxidative burst," in which there is a marked increase in cyanide-insensitive oxygen consumption, which is mediated via an activated membrane-bound NADPH oxidase (7, 8). The process can be elicited in isolated cells by both particulate and soluble stimuli, such as opsonized particles (bacteria, zymosan, latex) or a variety of soluble stimuli including C5a, fMet-Leu-Phe [the concentration of fMet-Leu-Phe required to elicit the oxidative burst is ≈100-fold higher than that required for chemotaxis (9)], phorbol 12-myristate 13-acetate (PMA), or arachidonate. The burst generates several forms of reduced oxygen [e.g., superoxide, hydrogen peroxide, and products derived therefrom such as HOCl and perhaps hydroxyl radical (10, 11)], which are not only bactericidal, but when released also appear to be toxic to host cells and tissues (12). Rapidly proliferating cells (including those of the host) appear to be highly susceptible to DNA damage and mutation by these oxygen-derived radicals.

Neutrophils are particularly important in sterilizing wounds, or in areas where laceration of the skin and damage to the underlying tissue and blood vessels have occurred. In response to such an injury, there is an orchestrated sequence of events that involves several cell types and tissues and that leads to tissue repair. Early events, including blood coagulation and platelet deposition, promote hemostasis. Neutrophils and other leukocytes then migrate into the area and function in killing microbes, while fibroblasts and smooth muscle cells also migrate into and proliferate in the injured area (13) to participate in tissue repair.

Regulation of the cellular events of wound healing requires "signalling" among the various cell and tissue components (14). For example, it has long been known that collagen, which is exposed upon damage to the vascular endothelium, promotes blood clotting and platelet adherence (15). Platelets and monocytes in the damaged area provide peptide factors necessary for cell recruitment and for the initiation of cell division (14). One such compound, PDGF (16, 17), is released from the α granules of platelets in response to such stimuli as thrombin, collagen, and ADP. This heterodimer [30 kDa (18)] is also released from macrophages (19) and injured endothelial cells (20). PDGF is a potent mitogen for mesenchymal cells and is a primary initiator of cell growth during healing (17). It is also an important stimulus for cell recruitment [fibroblasts (21), smooth muscle cells (22), neutrophils and monocytes (5)].

A neutrophil response that generates cytotoxic and possibly mutagenic oxygen radicals would appear to be incompatible with the rapid cell proliferation that must accompany wound healing. In the present studies, we have investigated the effects of PDGF on the neutrophil oxidative burst. We find that this factor blocks the neutrophil oxidative burst, which is elicited by several activators including fMet-Leu-Phe, but stimulates phagocytosis. We suggest that this factor modulates activation to prevent oxidative damage in areas of active mitosis.

EXPERIMENTAL PROCEDURES

Materials

Hespan (6.0% Hetastarch/0.9% NaCl) was obtained from American Critical Care Division of American Hospital Supply Corporation (McGaw Park, IL). Lymphocyte separation medium (6.2% Ficoll/9.4% sodium diatrizoate) was obtained from Bionetics Laboratory Products (Kensington, MD).

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Abbreviations: PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate.

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fMet-Leu-Phe, PMA, zymosan, latex beads (0.8 μm), and cytochrome *c* (horse heart, type III) were from Sigma. [^{3}H]fMet-Leu-Phe (55.6 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. PDGF was obtained from Collaborative Research. PDGF used in these experiments was >90% pure as determined by $NaDodSO_4$/PAGE analysis; two bands of 31 and 28 kDa were present and correspond to the PDGF I and PDGF II fractions reported previously (18). The specific activity was >450,000 half-maximal units per mg of protein (a half-maximal unit is defined as the reciprocal of that dilution of PDGF that stimulates BALB/c-3T3 cells in 5% platelet-poor human plasma to incorporate half the amount of [^{3}H]thymidine that would maximally be incorporated under the influence of unlimited PDGF). C5a was generously provided by Thomas Van Dyke (Emory University School of Dentistry, Atlanta, GA).

Methods

Isolation of Human Neutrophils. Human neutrophils were obtained by continuous flow leukapheresis from normal adults. Residual erythrocytes were removed by hypotonic lysis with a resulting purity of >95% neutrophils (23). Alternatively, peripheral blood was obtained by phlebotomy and neutrophils were isolated by Hespan (6.2% Hetastarch/0.9% NaCl) sedimentation of erythrocytes, centrifugation of the resulting supernatant through lymphocyte separation medium (9.4% sodium diatrizoate/6.2% Ficoll), and hypotonic lysis of residual erythrocytes (24). Isolated cells are resuspended in phosphate-buffered saline (PBS)/glucose, containing 0.6 mM $CaCl_2$, 2.6 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 136 mM NaCl, 8 mM Na_2HPO_4, and 5.5 mM glucose.

Assay for Superoxide Production. Superoxide production was measured by using reduction of cytochrome *c* (25), monitored as an increase in absorbance at 550 nm and quantified using an extinction coefficient of 21,000 $M^{-1}\cdot cm^{-1}$ (26). Measurements were carried out at 37°C using $\approx 5 \times 10^5$ cells per ml, stirred continuously. The cells were allowed to equilibrate several minutes prior to the addition of effectors. Conditions for experiments are described in figure legends. Controls utilizing added superoxide dismutase verified that the cytochrome *c* reduction was mediated by superoxide.

Measurement of O_2 Consumption. Oxygen consumption was measured using a Clark-type electrode with a YSI model 53 oxygen monitor. Assays were conducted at 30°C with 1×10^7 neutrophils per ml and a total vol of 2.5 ml, stirred continuously. Zymosan was opsonized for these studies by incubating 40 mg of zymosan with 0.2 ml of human serum and 1 ml of Tris buffer (pH 8.5) for 1 hr at 37°C in a shaking water bath.

Measurement of [^{3}H]fMet-Leu-Phe Binding to Neutrophils. Cells (1×10^6) in 100 μl of PBS plus glucose were incubated with [^{3}H]fMet-Leu-Phe for 1 hr on a shaking water bath at 25°C. The incubation was terminated by addition of 5 ml of ice-cold PBS, followed immediately by filtration on an Amicon VFM-III filtration apparatus, using 0.45 μm nitrocellulose filters, as described (27). Each sample was washed five times with 5 ml each of PBS, and the filters were counted. Nonspecific binding was measured in the same manner, except that 100 μM unlabeled fMet-Leu-Phe was included in the incubation. Specific binding was calculated for each concentration of labeled fMet-Leu-Phe as total filter-associated counts minus nonspecific counts.

Quantitation of Phagocytosis. Latex particles were opsonized by incubating at 37°C 1 ml of particles with 0.2 ml of human serum plus 1 ml of Tris buffer, pH 8.5 (28). Neutrophils (2×10^6 cells) in 0.1 ml of PBS/glucose (either containing 300 pM PDGF or with no addition) were incubated with 10^8 particles at 37°C for 5 min. The reaction was terminated by the addition of 0.4 ml of ice-cold buffer containing 1 mM EDTA. The cells were then diluted by 80% with 0.15 M NaCl and layered onto a Ficoll/sodium diatrozoate solution (d = 1.077) and centrifuged 20 min at 400 × g at 4°C. Cells are sedimented while beads remain at the Ficoll/saline interface. The number of latex beads internalized per cell and the number of cells containing at least one bead were determined by microscopic examination (29).

RESULTS

Inhibition of the fMet-Leu-Phe-Induced Oxidative Burst by PDGF. The effect of PDGF on human neutrophils was tested by monitoring superoxide-mediated cytochrome *c* reduction. Fig. 1 shows spectrophotometric tracings of fMet-Leu-Phe-activated reduction. Tracing a shows the effect of fMet-Leu-Phe (1 μM); activation is characterized by a rapid increase in superoxide production with spontaneous cessation after ≈2 min. Superoxide production can be reactivated at this point by addition of phorbol ester (PMA). Tracings b and c show the effects of treating neutrophils with 50 and 100 pM PDGF ≈80 sec prior to addition of fMet-Leu-Phe. There is a dose-dependent decrease in both the initial rate of superoxide production and the size of the oxidative burst. The primary effect was on the size of the burst. Changes in the initial rate were observed at higher concentrations. Tracing d shows that 200 pM PDGF abolishes fMet-Leu-Phe response but does not affect the PMA-induced activation. The apparent decrease in initial rate of PMA activation of superoxide production seen in tracing d was not consistently seen in replicate experiments. In tracing e, 600 pM PDGF alone had no effect on the oxidative burst. In a large number of similar experiments using a variety of PDGF concentrations and various cell preparations, we did not observe activation and were thus

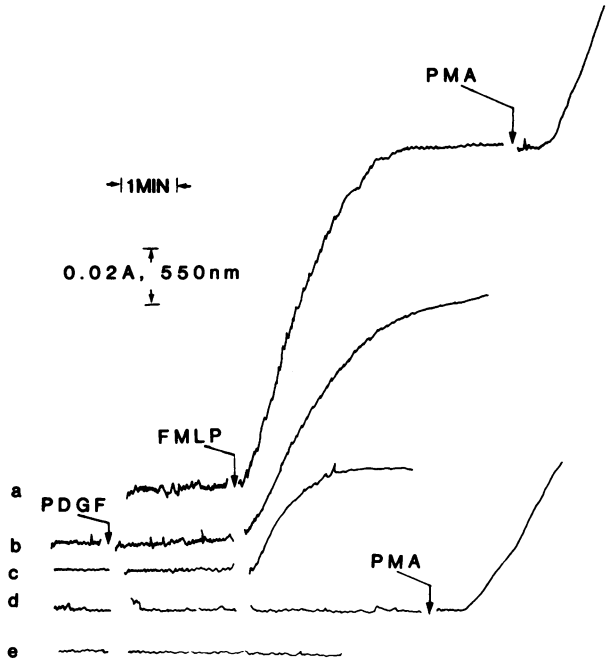

FIG. 1. Effects of PDGF on fMet-Leu-Phe-induced superoxide production. Neutrophils (5×10^5) were assayed for fMet-Leu-Phe-stimulated superoxide-mediated cytochrome *c* reduction as described. Tracing a, control levels of activation with 1 μM fMet-Leu-Phe (FMLP), with subsequent reactivation by PMA. Tracings b, c, and d, effects of preincubating with increasing concentrations of PDGF (50, 100, and 200 pM, respectively) prior to addition of fMet-Leu-Phe. Tracing e, incubation with 600 pM PDGF alone.

unable to confirm the earlier study of Tzeng *et al.* (30) in which activation of the oxidative burst by PDGF was reported. The initial rate of superoxide production calculated from their data was only 3–4% of that seen when either PMA or fMet-Leu-Phe is used to activate the oxidative burst, a value that would be difficult to detect above baseline noise. Thus, PDGF does not appear to affect basal superoxide generation, but blocks activation of the oxidative burst by fMet-Leu-Phe.

Concentration Dependence for Inhibition of the fMet-Leu-Phe-Induced Oxidative Burst by PDGF. The concentration dependence for the inhibition of the extent of the oxidative burst is shown in Fig. 2; 50% inhibition occurred in this experiment at 75 pM. In repeated determinations using different cell preparations, this value varied from 60 to 150 pM with an average of 95 ± 26 pM (SEM; n = 10). Thus, neutrophils are exquisitely sensitive to modulation by PDGF, suggesting a physiological role for this factor.

The Effect of PDGF on the Activation of the Oxidative Burst by Other Agonists. Table 1 shows the effect of PDGF pretreatment on the activation of the oxidative burst by a variety of agonists. Activation of superoxide generation by either arachidonate or PMA was not affected by 200 pM PDGF, a dose that was completely inhibitory for fMet-Leu-Phe activation. In contrast, activation by the complement protein C5a was completely blocked and zymosan-induced activation was 30–50% inhibited. Activation by opsonized zymosan was assayed by oxygen uptake because the turbidity of the reaction mixture precluded the use of the spectrophotometric assay for superoxide. Thus, PDGF blocks activation of the oxidative burst by some but not all of the agonists tested.

PDGF Does Not Affect Binding of fMet-Leu-Phe to Its Cellular Receptor. The binding of radiolabeled fMet-Leu-Phe to neutrophils was determined as a function of fMet-Leu-Phe concentration, using a filtration binding assay, both in the presence and absence of pretreatment for 5 min with PDGF. Binding data are shown in Fig. 3. In both cases, a single dissociation constant for fMet-Leu-Phe binding of 25 nM was calculated, in good agreement with a value of 22.3 nM reported by Koo *et al.* (27). The number of receptors per cell was calculated to be 25,300 and 27,000 in the absence and presence of PDGF, respectively. Thus, PDGF did not appear to affect significantly the number or affinity of neutrophil fMet-Leu-Phe receptors.

Effect of PDGF on Phagocytosis. Table 2 shows the effect of PDGF on phagocytosis of opsonized latex beads, determined by light microscopy as described in *Experimental Procedures*. Pretreatment of cells with PDGF for 5 min prior to addition of beads increased significantly both the number of cells that contained one or more beads and the average number of beads per cell. Calculations revealed that the increased number of beads per cell was not accounted for completely by an increase in the percentage of phagocytosing cells. Rather, each active cell ingested more particles. Thus, although the oxidative burst is blocked, phagocytosis is unimpaired and in fact is stimulated by PDGF.

Table 1. Effect of PDGF on the rate of the oxidative burst using a variety of agonists

Agonist	Rate, nmol per min per 10^6 cells	
	No PDGF	PDGF (200 pM)
fMet-Leu-Phe (1 μM)*	6.1 ± 0.5	0
C5a (150 ng/ml)*	4.9 ± 0.8	0
Opsonized zymosan† (30 μg/ml)	5.3 ± 0.5	2.7 ± 0.4
PMA (100 nM)*	7.2 ± 0.6	7.4 ± 0.3
Arachidonate* (83 μM)	6.2 ± 0.5	6.1 ± 0.5

*Superoxide production.
†Oxygen consumption.

DISCUSSION

Is the Effect of PDGF Physiological? The concentration of PDGF required for 50% inhibition of the fMet-Leu-Phe-induced oxidative burst is very low (95 pM). For comparison, Bowen-Pope and coworkers (31) have shown that the concentration of PDGF in serum is ≈570 pM (17.5 ng/ml), due to the presence of released platelet products. (In serum, the clotting products have apparently activated platelets to release their granule contents including PDGF, while in plasma, wherein coagulation has been inhibited, platelet contents have not been released.) Levels in plasma, however, are not detectable, so that circulating levels are probably very low in the absence of platelet activation. Although PDGF levels have not to our knowledge been measured in sites of vascular injury, Bowen-Pope and coworkers concluded (31) that because of the high content of activated platelets at sites of vascular injury, localized PDGF concentrations would be expected to be high. Levels in the range used in the present studies would not be unreasonable considering the quantity in serum. We therefore suggest that PDGF released into an

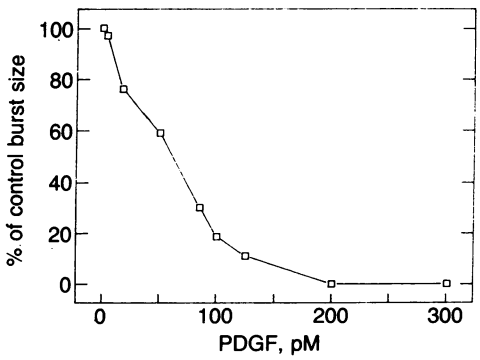

FIG. 2. Concentration dependence for PDGF inhibition of fMet-Leu-Phe-induced superoxide production. Cells (5 × 10^5) were preincubated with the indicated concentration of PDGF for ≈80 sec prior to activation with 1 μM fMet-Leu-Phe. Superoxide production was monitored as described.

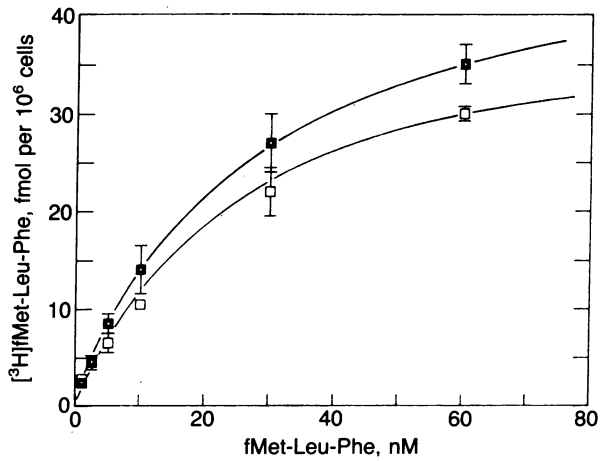

FIG. 3. Binding of fMet-Leu-Phe to PDGF-pretreated neutrophils. Neutrophils (1 × 10^6 cells) were incubated with various concentrations of [^{3}H]fMet-Leu-Phe and specific binding was measured as described. Nonspecific binding has been subtracted for each concentration and was typically ≈10% of specific binding. Measurements were carried out either with (■) or without (□) preincubation (5 min) with PDGF.

Table 2. Effect of PDGF pretreatment on phagocytosis of opsonized latex beads

Pretreatment	% of cells containing beads	Beads per cell, mean ± SEM
None	65	6.0 ± 2.5 (n = 100)*
PDGF (300 pM)	90	12.7 ± 3.0 (n = 100)*

*Number of cells examined.

area of platelet aggregation (e.g., a wound) will physiologically modulate the responses of neutrophils in the area.

Generality and Mechanism of the PDGF Effect on the Oxidative Burst. PDGF blocked the response to receptor-mediated activation but had no effect when PMA and arachidonate were used as agonists. PMA is a direct activator of protein kinase C (32, 33). The mechanism of activation by arachidonate is poorly understood, but there is general agreement that it acts at a postreceptor site, perhaps protein kinase C (34) or the oxidase itself (35, 36). Thus, these postreceptor mechanisms do not appear to be affected by PDGF.

Activation by fMet-Leu-Phe and C5a, however, was completely inhibited and that by opsonized zymosan was partially inhibited by PDGF. Like fMet-Leu-Phe (37, 38), C5a acts via a plasma membrane receptor (39). Similarly, opsonized zymosan (40) action is receptor mediated (e.g., Fc, C3b). We have provided evidence that protein kinase C participates in the activation of the neutrophil oxidative burst not only by PMA, but also by fMet-Leu-Phe, opsonized zymosan, and arachidonate (41). fMet-Leu-Phe has been shown to act through receptor-mediated hydrolysis of phosphatidylinositol phosphates to generate the protein kinase C activator diacylglycerol (42), and the other receptor-mediated activators may act similarly. Since PMA and arachidonate both appear to activate at postreceptor steps, but C5a, fMet-Leu-Phe, and opsonized zymosan all act via plasma membrane receptors, we suggest that PDGF exerts its effects on the activation pathway prior to protein kinase C, probably at the level of plasma membrane receptors or receptor-coupled mechanisms.

The finding that PDGF does not affect the binding of fMet-Leu-Phe suggests that signal transduction mechanisms rather than receptor binding is modulated. Thus, it is possible that inhibition may be occurring at the level of either a guanine nucleotide regulatory protein (43) or phospholipase C (42), both of which have been implicated in the fMet-Leu-Phe-signal transduction mechanism.

PDGF has been previously shown to down-regulate the response of the receptor for epidermal growth factor (EGF) (44). PDGF appears to function at least in part by decreasing the affinity of the EGF receptor for its ligand (44, 45). The mechanism in this case appears to involve activation by PDGF of protein kinase C (46, 47), with phosphorylation of the EGF receptor (48); phosphorylation results in decreased binding affinity. Mechanisms that do not involve protein kinase C may also function (49). The mechanism by which PDGF down-regulates the fMet-Leu-Phe response appears to be distinct from that for PDGF modulation of the EGF receptor, since fMet-Leu-Phe binding is unaffected. We speculate that in the neutrophil, protein kinase C activation by PDGF does not participate in the down-regulation, since activation of this enzyme would be expected to initiate the oxidative burst as is seen with phorbol esters or diacylglycerol.

A Model for Feedback Interactions Between Neutrophils and Platelets. Two of the cell types that participate prominently in the initial events of wound healing are platelets (which function in hemostasis and provide growth factors and chemoattractants) and neutrophils (which serve as the primary defense against microbial infection). Because the bactericidal oxygen radicals generated during bacterial killing are also toxic and mutagenic in proliferating cells, temporal and/or spatial regulation of the oxidative burst in a wound site may be important.

Fig. 4 illustrates a hypothetical feedback loop in the interaction between platelets and neutrophils. Platelets are the first cells to arrive at a wound or intravascular thrombus and are activated by thrombin and collagen to secrete their granular contents, including PDGF (14). The latter is also secreted by damaged endothelial cells. In relation to the model of neutrophil–platelet interaction, PDGF serves as a chemoattractant to promote recruitment of neutrophils to the wound (5). Bacterial peptides (formylated) and complement proteins serve similar chemoattractant roles. The present studies have shown that PDGF also functions as a negative modulator of the oxidative burst and as activator of phagocytosis. This phenomenon may be important in separating temporally or spatially the generation of cytotoxic oxygen compounds from induced mitotic activity. It is tempting to speculate that phagocytosis and perhaps some nonoxidative killing mechanisms may occur immediately, but that oxidative events may be delayed until the neutrophil moves away from the site of healing.

The other arm of a proposed feedback loop (Fig. 4) may be provided by neutrophil products that affect platelet function. One such compound is platelet-activating factor (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF), which has been shown to have multiple effects on various cellular components (reviewed in ref. 50). This factor is released, predominantly by granulocytes, in response to stimuli such as opsonized particles and formylated peptides (51–53). The platelet appears to be particularly sensitive to the effects of PAF, which causes platelet activation at concentrations as low as 1 pM (50), a range several orders of magnitude lower than that which causes effects in other cell types, including the neutrophil (54). In addition, another neutrophil-derived platelet-regulating factor, the protein "neutrophilin," which stimulates platelet calcium mobilization, secretion, and aggregation has recently been identified (55). The activated platelets would in turn deactivate or block the neutrophil oxidative mechanisms. (By deactivation, we are referring to effects on a population of neutrophils rather than on a single cell. Thus, if platelets are triggered by activation of a small subset of neutrophils, the platelet-released products should prevent activation of the larger population. It has not been

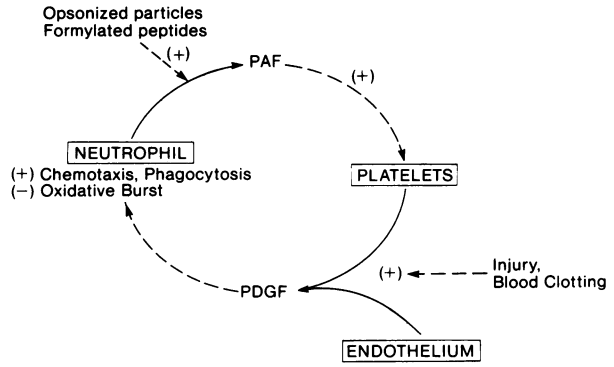

FIG. 4. A model for feedback interaction of platelets and neutrophils. Platelet-activating factor (PAF), released by activated neutrophils, promotes degranulation of platelets with release of PDGF. The latter can also be released in response to endothelial damage or clotting products. The released PDGF then down-regulates the oxidative response of the neutrophil to activating stimuli.

possible yet to test whether the oxidative burst in an individual neutrophil can be reversed by these mechanisms.) Thus, if a neutrophil becomes activated in an area where platelets are present, deactivation should occur via this cell–cell feedback loop. The model thus predicts that communication between these cells regulates the temporal and spatial events during wound repair, thus allowing for healing and avoiding deleterious effects due to the oxidative inflammatory response.

We thank Dr. W. R. Vogler, Dr. E. F. Winton, and the staff of the Cell Separator Department of Emory University Hospital for help in obtaining neutrophils and for performing phlebotomies. We thank Bill Shaffer for help with the phagocytosis experiments. We are grateful to Mark Fuller for carrying out the C5a purification and to Jack Kinkade, Jr., and Julie Taylor for helpful discussions. This work was supported by National Institutes of Health Grants AI22809 (J.D.L.) and CA40266 (L.R.G.). J.D.L. is recipient of National Institutes of Health Research Career Development Award 01085.

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