Contact-Induced Neutrophil Activation by Platelets in Human Cell Suspensions and Whole Blood

By Andreas Ruf, Richard F. Schlenk, Antonios Maras, Eberhard Morgenstern, and Heinrich Patscheke

Platelet-dependent activation of polymorphonuclear neutrophils (PMNL) was investigated with a lumi-aggregometer in heparinized whole blood and platelet-PMNL suspensions. The lumi-aggregometer allowed us to simultaneously monitor increases in impedance or light transmission as consequences of platelet aggregation and luminol-enhanced chemiluminescence (CL) as a measure of the oxidative burst in PMNL. Aggregation and platelet-PMNL contacts were also checked by light and electron microscopy. In whole blood, adenosine diphosphate (ADP) and the thromboxane A2 mimetic U 46619 induced the aggregation (increase in impedance) and the CL, which were both suppressed by EDTA, arginyl-glycyl-aspartyl-serine (RGDS) peptide, and the absence of stirring. In contrast, FMLP caused only CL that was unaffected by EDTA, RGDS peptide, and nonstirring. Similar observations were obtained with mixed suspensions containing washed platelets and PMNL at their physiologic concentrations. ADP, U 46619, and thrombin induced both aggregation (increase in light transmission) and CL, whereas FMLP caused CL but only very weak aggregation. Exogenous fibrinogen strongly enhanced the effects of ADP and U 46619. Iloprost, EDTA, RGDS peptide, red blood cell (RBC) ghosts, and nonstirring inhibited the effects induced by the platelet

agonists, but were ineffective on the CL induced by FMLP. Treatment of platelets with aspirin did not affect the CL of PMNL induced by platelets. Microscopic examination, the requirements of stirring, Ca_2^+, and fibrinogen, and the inhibitory effects of RGDS peptide and RBC ghosts showed that stimulated platelets activate PMNL in a contact-dependent manner that depends on fibrinogen binding. This was confirmed by the immunochemical demonstration of fibrinogen (but not of fibronectin) in the contact spaces between activated platelets and PMNL. Because supernatants and lysates of resting or thrombin-stimulated platelets did not induce the CL of PMNL, soluble agonists did not appear to be involved. Nonstimulated washed platelets also caused CL of PMNL that required stirring and Ca_2^+ and was inhibited by RBC ghosts. No CL occurred in unstimulated stirred whole blood, suggesting that a preactivation of platelets during the preparation may be responsible for the effects of unstimulated washed platelets. The results show that platelets provide a strong stimulus for PMNL that requires intercellular contact. Fibrinogen exposure on the platelet surface seems to be necessary for the activation of PMNL by stimulated platelets.

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Platelets have been implicated as mediators in inflammatory responses, and platelet-neutrophil interactions may play a role in several diseases, such as the acute respiratory distress syndrome, myocardial ischemia, and atherosclerosis, in which the defense mechanisms of hemostasis and inflammation are concomitantly activated. Several mechanisms by which platelets affect polymorphonuclear neutrophils (PMNL) function have been suggested. Platelet factor 4 is released during platelet activation and acts chemotactically on PMNL. Platelet-derived growth factor, another component from the platelet α-granules, induces chemotaxis and phagocytosis, but blocks an agonist-induced oxidative burst in PMNL. Platelet-activated growth factor is also released from α-granules and can be cleaved by monocytotes to yield a neutrophil-activating peptide (NAP-2) that can activate PMNL dependent activation of neutrophils is far from certain because it has not been shown that they reach effective concentrations in coinoculation experiments at physiologic concentrations of both cell types. Unstimulated and stimulated platelets enhanced the lysozyme release induced by dimosan in PMNL, whereas supernatants derived from platelets at a physiologic concentration were ineffective. Platelets stimulated by thrombin also augmented the oxygen production of PMNL, an effect that required stirring of the platelet-neutrophil suspension. This effect was not reduced by nordihydroguaiadinic acid, eicosatetraynoic acid, aspirin, or the PAF receptor antagonist WEB 2086. These data suggest that neither eicosanoids, PAF, nor platelet release products were involved in the platelet-dependent enhancement of PMNL activity. As an alternative, intercellular contacts between platelets and neutrophils may have been necessary for the interactions observed.

To investigate whether PMNL respond to platelets in a contact-dependent manner, we applied a method that rendered it possible to monitor the activity of both cell types simultaneously in the same sample. Aggregation as a predominant phenomenon of platelet activation and the luminol-amplified chemiluminescence (CL) as a measure of the oxidative burst in PMNL were recorded with a
lumi-aggregometer. This methodologic approach supplemented with light and electron microscopy allowed us to show that contacts between platelets and PMNL induce a strong neutrophil activation in platelet-neutrophil suspensions and whole blood.

MATERIALS AND METHODS

Biochemicals. Biochemicals were purchased as follows: hydroxyethylstarch (4%, molecular weight [MW] 450,000) from Fresenius AG (Bad Homburg, Germany); human serum albumin (20%) from Immuno GmbH (Heidelberg, Germany); Percoll (1.13 g/mL), paraformaldehyde, 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol), N-formyl-methionyl-leucyl-phenylalanine (FMLP), N-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid (TES), arginyln-glycyl-aspartyl-serine (RGDS peptide), human fibronectin, and anticoagulated with acid citrate dextrose (ACD; National Institute of Health [NIH] formula A). For experiments with aspirin-stimulated platelets, PRP was treated with 5 mmol/L acetylsalicylic acid for 30 minutes at room temperature, and the platelets were washed according to our procedure previously described. The washed platelets were resuspended in a suspending fluid containing NaCl 90 mmol/L, KCl 5 mmol/L, CaCl2 2 mmol/L, MgCl2 1 mmol/L, glucose 5 mmol/L, human albumin 2 mg/mL, apyrase 200 μg/mL, and phosphate buffer 30 mmol/L, pH 6.5. They were suspended with a Sysmex PL-100 cell counter (Digitana AG, Hamburg, Germany) and were adjusted to a concentration of 1 × 10^9 platelets/mL and kept at 20°C until use.

Preparation of lysates and supernatants of platelets. Washed platelets (6 × 10^9/mL) were incubated with saline or thrombin 0.06 U/mL for 5 minutes at 37°C to obtain either unstimulated or stimulated platelets, respectively. Supernatants were separated by centrifugation at 8,000 g for 1 minute. For the preparation of lysates, the platelets were treated on ice for 1 minute with a sonifier 12-B (Branson Sonic Power Company, Danbury, MA) before centrifugation at 8,000 g for 1 minute. Supernatants and lysates were added 1.9 (vol/vol) to the warmed PMNL suspension immediately after their preparation.

Preparation of RBC ghosts. Hemoglobin exerts a strong quench effect on the luminol-enhanced CL. Therefore, RBC ghosts were used instead of RBCs to examine a sterical hindrance elicited by RBCs on platelet-PMNL contacts. The low layer of the RBC sediment was used from the blood vials after separation of the PRP from ACD-anticoagulated blood. Ghosts were prepared according to Marchesi and Palade. The milky white ghosts were washed three times with TES-buffered saline containing NaCl 139 mmol/L and TES buffer 33 mmol/L, pH 7.4. Ghosts were counted in a Neubauer chamber by using interference contrast microscopy and resuspended at a concentration of 1 × 10^9/L in test medium (see below).

Lumi-aggregometry. The lumi-aggregometer model 560 (ChronoLog Corp, Havertown, PA) allowed us to measure aggregation and CL in two samples simultaneously. Aggregation was recorded with a 6-channel strip chart recorder (Kontron Electronic GmbH, Munich, Germany) either optically in platelet and PMNL suspensions or with the impedance module in whole blood. The optical range was calibrated according to the manufacturer's instruction with the cell suspensions in the test cuvette and the clear test medium in the reference cuvette. Then, 80 mV were measured with the unstimulated cell suspension and 0 mV with the suspending medium at the analog exit of the instrument. Percent aggregation is the maximal change within 3 minutes upon stimulation, given in percent of the 80 mV range. In the experiments with RBC ghosts, percent aggregation was calculated. In these experiments, washed PMNL were used instead of RBCs to examine a sterical hindrance elicited by RBCs on platelet-PMNL contacts. After the experiments, the cell suspensions containing platelets, PMNL, and ghosts was calibrated against the pure ghost suspension to compensate for the turbidity of the nonaggregating ghosts. With the impedance module used for whole blood experiments, aggregation started at 0% and reached approximately 15% with a strong stimulus such as U 46619 2(5Z)16R3 (U 46619 2(5Z)16R3) and reached approximately 15% with a strong stimulus such as U 46619 2(5Z)16R3 (U 46619 2(5Z)16R3). All experiments were performed with a final volume of 1 mL in disposable plastic cuvettes. One hundred microliters of the concentrated PMNL suspension was added to 800 μL of test medium containing NaCl 125 mmol/L, KCl 5.14 mmol/L, glucose 4.9 mmol/L, CaCl2 1.12 mmol/L, MgCl2 0.75 mmol/L, albumin 300 μg/mL, apyrase 37 μg/mL, TES buffer 33 mmol/L, pH 7.4, and warmed to 37°C within 10 minutes in the lumi-aggregometer. In a separate vial, 80 μL of the concentrated platelet suspension was diluted with 160 μL of a solution containing NaCl 127 mmol/L, KCl 5 mmol/L, glucose 4.9 mmol/L, CaCl2 0.3 mmol/L, albumin 2 mg/mL, and apyrase 200 μg/mL and simultaneously warmed to 37°C. One hundred microliters of that platelet suspension was added to the PMNL suspension in the aggregometer cuvette.
After the addition of the washed platelets, the CL increased within approximately 6 minutes, and remained at a plateau for at least 2 minutes before it slowly decreased. Stimulations were induced after CL had reached the plateau. CL induced by unstimulated platelets was reported as the increment upon addition of 5 μL of thrombin, before the platelet suspensions or the stimulation of the mixed cell suspension. Corresponding volumes of test medium were omitted. After the addition of the washed platelets, the CL increased within approximately 6 minutes, and remained at a plateau for at least 2 minutes before it slowly decreased. Stimulations were induced after CL had reached the plateau. CL induced by unstimulated platelets was reported as the plateau value, whereas stimulated CL was reported as the increment upon addition of 5 μL of thrombin, ADP (aqueous buffered solutions), or 2 μL of U 46619 or FMLP (both dissolved in DMSO). All additions occurred under stirring with 1,000 rpm. If "unstirred" tests were performed, the stirrer was switched off 2 seconds after addition of the platelets or the stimulant.

Whole blood experiments were performed with heparinized blood diluted 1:4 with TES-buffered saline (see above). The tables show peak values of the CL curves. Luminol 2 μL, drugs, and stimulants were added at the expense of TES-buffered saline to show peak values of the CL curves. Both phenomena led to aggregation, as measured by the increase in impedance, and to a CL signal (Fig 1). Both phenomena were significantly decreased if the stirrer was switched off immediately after the addition of the agonists ("unstirred" on Table 1). The presence of EDTA or the RGDS peptide suppressed both CL and aggregation response (Table 1). In contrast, FMLP did not induce a change in impedance but triggered a CL signal that was neither influenced by EDTA, the RGDS peptide, nor stirring (Table 1).

### RESULTS

**CL and aggregation in anticoagulated whole blood.** CL was not observed in stirred unstimulated whole blood anticoagulated with heparin and diluted (1:4) with TES-buffered saline. The addition of ADP or U 46619 to whole blood led to aggregation, as measured by the increase in impedance, and to a CL signal (Fig 1). Both phenomena were significantly decreased if the stirrer was switched off immediately after the addition of the agonists ("unstirred" on Table 1). The presence of EDTA or the RGDS peptide suppressed both CL and aggregation response (Table 1). In contrast, FMLP did not induce a change in impedance but triggered a CL signal that was neither influenced by EDTA, the RGDS peptide, nor stirring (Table 1).

**CL and aggregation in suspensions of pure PMNL or platelets.** The PMNL suspensions used did not show CL. Addition of the chemotactic peptide FMLP 5 μmol/L induced a strong CL, but only very weak aggregation, as measured by the increase in light transmission. These effects were not affected by the presence of RBC ghosts at a concentration of 1 x 10^9/mL (Fig 2B). FMLP did not show any effects on pure platelet suspensions. Platelet stimuli such as thrombin, U 46619, and ADP induced neither CL nor aggregation in pure PMNL suspensions, but strong aggregation without CL in a pure platelet suspension (data not shown). Supernatants or lysates from unstimulated platelets or platelets stimulated with thrombin 0.06 U/mL neither induced CL nor aggregation when added to a PMNL suspension (data not shown).

**CL and aggregation in mixed suspensions of PMNL and platelets.** If human washed platelets were added to a...
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The CL reached a plateau within 6.3 minutes before the agonists. In contrast to platelet-PMNL suspensions, thrombin led to a further intense CL signal that was associated with strong aggregation (Fig 2A). The aggregometry trace (Fig 2A) and confirmed by microscopic examination. If RBC ghosts were present at a concentration of $1 \times 10^9$/mL, the CL responses induced by these agonists were completely inhibited when the aggregation was substantially reduced (Fig 2A). RBC ghosts also inhibited the aggregation in thrombin-stimulated mixed cell suspensions.

The CL of PMNL induced by resting thrombin-stimulated platelets was approximately proportional to the platelet concentration between 0.05 and $1 \times 10^9$ platelets/mL (Fig 3) and was unaffected by aspirin (Table 2). Therefore, aspirinated washed platelets at a physiologic concentration and cell ratio of 1 PMNL to 100 platelets were used, if not otherwise stated.

The CL decreased if the stirrer was switched off immediately after the addition of the platelets and no CL was observed in the presence of EDTA. The addition of EDTA or switching off the stirrer was ineffective after the CL induced by unstimulated platelets reached its plateau (data not shown). Thrombin-induced CL was not only inhibited by EDTA and nonstirring, but also by iloprost and the RGDS peptide (Table 3). In contrast, the RFDS peptide (1 mmol/L) had no inhibitory effect (data not shown). The chemotactic peptide FMLP did not induce substantial aggregation, but did induce a strong CL signal that was unaffected by iloprost, EDTA, fibrinogen, the RGDS peptide, and nonstirring (Table 3). In contrast to thrombin, both aggregation and CL induced by the platelet agonists U 46619 and ADP depended on added fibrinogen. Nonstirring reduced and EDTA or RGDS peptide completely inhibited the responses induced by these agonists, even in the presence of exogenous fibrinogen (Table 4). However, mixed cell suspensions stimulated with U 46619 in the presence of added fibrinogen nearly reached the CL signals induced by thrombin, whereas those stimulated with ADP remained at low levels despite the presence of exogenous fibrinogen (Table 4).

Morphologic investigations. The platelets and PMNL used in this study appeared as resting cells. The platelets

| Table 1. Luminol-Enhanced CL and Aggregation (increase in impedance) Induced by U 46619, ADP, and FMLP in Heparinized and Diluted (1/4) Whole Blood. Differential Effects of Nonstirring, EDTA, and RGDS Peptide |
|-----------------|-----------------|
| U 46619 2 μmol/L | CL (μL)          | Aggregation (μL) |
| Control         | 740 ± 120       | 14.5 ± 0.5 (10)  |
| Unstirred       | 40 ± 20*        | 0.9 ± 0.2* (5)   |
| EDTA 2.5 mmol/L | 0*              | 0* (5)           |
| RGDS peptide 1 mg/mL | 0*          | 0 (3)            |
| ADP 10 μmol/L   |                 |                  |
| Control         | 440 ± 30        | 10.3 ± 0.6 (10)  |
| Unstirred       | 30 ± 10*        | 0.7 ± 0.1* (5)   |
| EDTA 2.5 mmol/L | 0*              | 0* (5)           |
| RGDS peptide 1 mg/mL | 0*          | 0 (3)            |
| FMLP 1 μM       |                 |                  |
| Control         | 760 ± 120       | 0 (10)           |
| Unstirred       | 850 ± 90 NS     | 0 (5)            |
| EDTA 2.5 mmol/L | 830 ± 110 NS    | 0 (5)            |
| RGDS peptide 1 mg/mL | 820 ± 100 NS  | 0 (3)            |

EDTA and RGDS peptide were added 1 minute after luminol and 2 minutes before the agonists. In contrast to platelet-PMNL suspensions, no CL occurred in whole blood without added stimuli. Values in parentheses indicate number of different experiments.

Abbreviation: NS, not significant.

**P ≤ .05.

stirred suspension of PMNL, a CL response occurred but no aggregation. The CL reached a plateau within 6.3 ± 0.9 (n = 10) minutes (Fig 2A). Subsequent stimulation with thrombin led to a further intense CL signal that was associated with strong aggregation (Fig 2A). The aggregation comprised platelets and PMNL, as suggested by the aggregometer trace (Fig 2A) and confirmed by microscopic examination. If RBC ghosts were present at a concentration of $1 \times 10^9$/mL, the CL upon addition of resting platelets and subsequent thrombin was substantially reduced (Fig 2A). RBC ghosts also inhibited the aggregation in thrombin-stimulated mixed cell suspensions.

The CL of PMNL induced by resting and thrombin-stimulated platelets was approximately proportional to the platelet concentration between 0.05 and $1 \times 10^9$ platelets/mL (Fig 3) and was unaffected by aspirin (Table 2). Therefore, aspirinated washed platelets at a physiologic concentration and cell ratio of 1 PMNL to 100 platelets were used, if not otherwise stated.

The CL decreased if the stirrer was switched off immediately after the addition of the platelets and no CL was observed in the presence of EDTA. The addition of EDTA or switching off the stirrer was ineffective after the CL induced by unstimulated platelets reached its plateau (data not shown). Thrombin-induced CL was not only inhibited by EDTA and nonstirring, but also by iloprost and the RGDS peptide (Table 3). In contrast, the RFDS peptide (1 mmol/L) had no inhibitory effect (data not shown). The chemotactic peptide FMLP did not induce substantial aggregation, but did induce a strong CL signal that was unaffected by iloprost, EDTA, fibrinogen, the RGDS peptide, and nonstirring (Table 3). In contrast to thrombin, both aggregation and CL induced by the platelet agonists U 46619 and ADP depended on added fibrinogen. Nonstirring reduced and EDTA or RGDS peptide completely inhibited the responses induced by these agonists, even in the presence of exogenous fibrinogen (Table 4). However, mixed cell suspensions stimulated with U 46619 in the presence of added fibrinogen nearly reached the CL signals induced by thrombin, whereas those stimulated with ADP remained at low levels despite the presence of exogenous fibrinogen (Table 4).

Morphologic investigations. The platelets and PMNL used in this study appeared as resting cells. The platelets

Fig 2. Simultaneous monitoring of aggregation and luminol-enhanced CL in platelet-PMNL suspensions (A) and in pure PMNL suspensions (B). Platelets (PL), thrombin 0.06 U/mL, or FMLP 5 μmol/L was added in the presence (+) or absence (-) of RBC ghosts ($1 \times 10^9$/mL). Curves are representative examples of four series of experiments.

Fig 3. Influence of the platelet concentration on the CL of PMNL ($2 \times 10^9$/mL). The CL is reported as plateau value of the CL curve reached after addition of unstimulated platelets (C) and as the increment in CL induced by thrombin 0.06 U/mL in the mixed platelet PMNL suspensions (B). Data represent the results from three experimental series.
were discoid in shape in interference contrast microscopy. Electron micrographs showed the typical marginal bundle of microtubules, the granules were evenly distributed, and no remnant membranes of discharged granules could be seen. The PMNL exhibited a smooth surface without ruffling, a large number of granules, and no vacuolization. In stirred suspensions of unstimulated platelets and PMNL, no aggregates consisting of a single cell type but platelets surrounded by PMNL. Nonstirring and EDTA contacts was strongly reduced in the absence of stirring and EDTA, RGDS peptide, and omission of fibrinogen.

but no fibronectin could be identified in the contacts between platelets as well as between PMNL and platelets stimulated with thrombin, U 46619, or ADP (Fig 4). The same electron micrographs showed labeled fibronectin and fibrinogen in the platelet a-granules, and fibronectin in the granular compartments of PMNL (Fig 4). In crossblocking experiments and in controls using normal rabbit serum instead of the specific antibodies, no label was detected on such thin sections.

**DISCUSSION**

To further define the mechanism of platelet-dependent PMNL activation, we used a lumi-aggregometer to monitor the CL of PMNL and the platelet aggregation in mixed cell suspensions and whole blood. The control experiments with pure platelet or PMNL suspensions indicated that the CL specifically reflected PMNL activation, whereas the increase in light transmission resulted predominantly from platelet aggregation. PMNL aggregation in response to FMLP contributed to the changes in light transmission only to a very small extent. Consistent with reports of others, thrombin, U 46619, and ADP have no direct stimulatory effects on PMNL, although U 46619 and ADP may enhance agonist-induced PMNL responses.15,40,41

By using this technique we found that unstimulated platelets, but more so stimulated platelets, cause a strong neutrophil activation dependent on stirring and the presence of bivalent cations. Supported by the morphologic data, these observations indicated that platelet-PMNL contacts were necessary for the CL response. Nonstirring and RBC ghosts diminished the rate of cell collisions, whereas bivalent cations appeared to be essential cofactors for the platelet-PMNL contacts. It has recently been reported that PMNL stimulated by FMLP are able to induce activation of coincubated platelets. This effect depends on cytochalasin B, which enhances PMNL secretion. The major platelet activator appeared to be cathepsin G.42 In contrast, a soluble platelet-derived mediator does not seem to be involved in platelet-induced activation of PMNL. Neither supernatants nor lysates from thrombin-

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**Table 2. Luminol-Enhanced CL Induced by Washed Platelets Added to a PMNL Suspension and by Thrombin, ADP, U46619, and FMLP in Platelet-PMNL Suspensions. Comparison of Aspirin-Treated With Untreated Control Platelets**

<table>
<thead>
<tr>
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<th>PMNL</th>
<th>Platelets</th>
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</thead>
<tbody>
<tr>
<td><strong>CL (U)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>70.1 (4)</td>
<td>69.5 ± 6.2 (5) NS</td>
</tr>
<tr>
<td>Stimulated</td>
<td>153.2 (6)</td>
<td>160.7 ± 32.2 (5) NS</td>
</tr>
<tr>
<td>Thrombin 0.06 U/mL</td>
<td>129.6 (4)</td>
<td>133.9 ± 10.6 (8) NS</td>
</tr>
<tr>
<td>ADP 10 μmol/L</td>
<td>19.9 ± 3.7 (4)</td>
<td>17.0 ± 3.9 (8) NS</td>
</tr>
<tr>
<td>FMLP 5 μmol/L</td>
<td>117.4 ± 17.7 (4)</td>
<td>111.8 ± 15.1 (8) NS</td>
</tr>
</tbody>
</table>

All experiments were performed in the presence of exogenous fibrinogen 300 μg/mL. Values in parentheses indicate number of different experiments.

**Abbreviation:** NS, not significant.

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**Table 3. Luminol-Enhanced CL and Aggregation Induced by Thrombin or FMLP in Mixed Suspensions of PMNL and Aspirin-Treated Platelets. Differential Effects of Nonstirring, EDTA, RGDS Peptide, and Omission of Fibrinogen**

<table>
<thead>
<tr>
<th></th>
<th>CL (U)</th>
<th>Aggregation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Control Platelets</td>
<td>20.2 ± 3.9 (8)</td>
<td>11.2 ± 3.1 (3)</td>
</tr>
<tr>
<td>Unstirred</td>
<td>4.3 ± 0.6* (5)</td>
<td>1.5 ± 0.5* (5)</td>
</tr>
<tr>
<td>EDTA 2.5 mmol/L</td>
<td>0*</td>
<td>0* (5)</td>
</tr>
<tr>
<td>RGDS peptide 1 mg/mL</td>
<td>0*</td>
<td>0* (3)</td>
</tr>
<tr>
<td>Without fibrinogen</td>
<td>5.2 ± 1* (5)</td>
<td>4.5 ± 0.8* (5)</td>
</tr>
</tbody>
</table>

**Values in parentheses indicate number of different experiments.** *P ≤ .05.

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**Table 4. Luminol-Enhanced CL and Aggregation Induced by U46619 or ADP in Mixed Fibrinogen (300 μg/mL) Containing Suspensions of PMNL and Aspirin-Treated Platelets. Inhibitory Effects of Nonstirring, EDTA, RGDS Peptide, and Omission of Fibrinogen**

<table>
<thead>
<tr>
<th></th>
<th>CL (U)</th>
<th>Aggregation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U 46619 0.1 μmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>133.9 ± 10.6</td>
<td>52.6 ± 4.6 (8)</td>
</tr>
<tr>
<td>Unstirred</td>
<td>5.2 ± 0.5*</td>
<td>3.6 ± 0.5* (5)</td>
</tr>
<tr>
<td>EDTA 2.5 mmol/L</td>
<td>0*</td>
<td>3.4 ± 0.6* (5)</td>
</tr>
<tr>
<td>RGDS peptide 1 mg/mL</td>
<td>0*</td>
<td>3.3 ± 0.4* (3)</td>
</tr>
<tr>
<td>Without fibrinogen</td>
<td>13.4 ± 1.7*</td>
<td>11.2 ± 3.1* (3)</td>
</tr>
</tbody>
</table>

**Values in parentheses indicate number of different experiments.** *P ≤ .05.
stimulated or unstimulated platelets could evoke CL of PMNL. This is in particular true for cyclooxygenase products such as thromboxane A₂ because aspirin did not interfere with the observed effects. In contrast, an added soluble agonist such as FMLP induced the CL irrespective of stirring, EDTA, RGDS peptide, or the presence of RBC ghosts. Recent studies have already suggested that the O₂⁻ production of PMNL caused by stimulated platelets may not depend on platelet-derived soluble mediators. Supernatants from stimulated platelets augmented the zymosan-induced lysosomal release of PMNL only if they were prepared from suspensions with threefold higher than physiologic platelet concentrations. On the other hand, inhibitory effects of platelet products on FMLP-induced O₂⁻ generation have also been described. McGarrity et al claimed adenine nucleotides as inhibitors, which Ward et al identified as adenosine and AMP. In contrast to those observations, we found in coincubation experiments that platelets are potent stimuli for PMNL in a contact-dependent manner.
It has recently been suggested that glycoprotein 140 (GMP 140) mediates the Ca<sup>2+</sup>-dependent binding of PMNL to thrombin-activated fixed platelets. Thus, it is likely that GMP 140 is involved in platelet-PMNL adhesion in platelet-PMNL suspensions or whole blood. However, our results suggest that GMP 140-mediated contacts are not sufficient for the activation of PMNL. Exogenous fibrinogen substantially enhanced the PMNL response to stimulated platelets, whereas it did not affect an FMLP-induced CL of PMNL. This effect of fibrinogen was most clearly expressed with ADP and U 46619, which caused almost no or only limited release of fibrinogen in aspirinated platelets. Because thrombin is a strong secretagogue in platelets and releases high amounts of fibrinogen from the platelet α-granules, added fibrinogen was neither necessary nor could it enhance thrombin-induced PMNL activation as well as the accompanying platelet aggregation. The role of fibrinogen for PMNL activation is also substantiated by the inhibitory effect that RGDS peptide has on the platelet-dependent CL upon stimulation with ADP, U 46619, and thrombin. Peptides containing the RGD sequence inhibit fibrinogen binding to the Gp IIb/IIIa complex at the platelet surface. RGDS peptide did not affect the FMLP-induced CL of PMNL and RFDs peptide did not inhibit the platelet triggered PMNL response, confirming the specificity of the inhibitory effect that RGDS peptide has on the platelet-dependent activation of PMNL. Exogenous fibrinogen substituted for the activation of PMNL. Because RGDS peptide contains the RGD sequence, it inhibits platelet activation and the binding of fibrinogen<sup>58,49</sup> by increasing cyclic AMP formation in platelets<sup>50</sup> and may thereby block the CL of PMNL in response to stimulated platelets. In support of the concept that contact-induced neutrophil activation by platelets requires fibrinogen binding, we found fibrinogen but not fibronectin in the contact area. Fibrinogen but not fibronectin in the contact areas was found in a certain degree and may mediate platelet-PMNL interaction upon stirring. On the other hand, fresh whole blood may not contain preactivated platelets but also between platelets and neutrophils. Unstimulated washed platelets also produce a contact-dependent activation of PMNL. Although they showed morphologically no sign of activation, the washed platelets could have been preactivated by the preparation procedure. As a consequence, GMP 140 and cytoadhesins like fibrinogen may already be expressed to a certain degree and mediate platelet-PMNL interaction upon stirring. On the other hand, fresh whole blood may not contain preactivated platelets and, therefore, did not produce luminol-enhanced CL. However, if stimulated with ADP or U 46619, whole blood aggregated and emitted CL, which was again inhibited by nonstirring, EDTA, and RGDS peptide. Thus, it is reasonable to conclude that the activation of PMNL by stimulated platelets in whole blood also depends on intercellular contacts and fibrinogen exposure.

Complement receptor type three of PMNL (CD11b/CD18) binds fibrinogen in an RGDS peptide-insensitive manner. Another member of the leukocyte integrin family, CD11c/CD18, has recently been reported to mediate the adherence of tumor necrosis factor-α (TNF-α)-stimulated PMNL to fibrinogen-coated surfaces. CD11b/CD18 also serves on monocytes as a fibrinogen receptor<sup>53</sup> and exposure of monocytes to fibrinogen-coated surfaces induces the oxidative burst. Thus, a possible explanation for our results may be that stimulation of the PMNL fibrinogen receptor by platelet-expressed fibrinogen also elicits an oxidative burst in neutrophils. The question of whether CD11c/CD18 or CD11b/CD18 triggers the signal for the oxidative burst is currently being investigated. CD11/CD18 integrins also seem to be involved in the adherence of PMNL to substrates like fibronectin, vitronectin, and laminin. The adherence of PMNL to these subendothelial matrix proteins is dependent on PMNL activation.<sup>55,56</sup> On the other hand, the initial interaction with endothelial cells is thought to be independent of PMNL activation. The transient adhesion, as it occurs during the so-called ‘‘rolling’’ of PMNL on endothelial cells, seems to be mediated by selectins such as GMP140 or lectin adhesion molecule 1 (LECAM-1).<sup>57,58</sup> For a stable and shear stress-resistant adhesion, the interaction of CD11/CD18 integrins on PMNL with counterreceptors of the IgG family such as intercellular adhesion molecule 1 on the endothelium appears to be necessary. At sites of endothelial damage, platelets rapidly adhere to the subendothelial matrix and form aggregates. GMP140 expressed on these platelets may substitute for an endothelial selectin and promote transient PMNL adhesion. A shear stress-resistant adhesion may also depend on CD11/CD18 integrins that bind to platelet-associated glycoproteins such as fibrinogen. Our results suggest that such an interaction strongly activates the PMNL. However, the methods used are not suited to study the sequence of events involved in PMNL-platelet adhesion at the sites of thrombus formation. For this purpose, studies based on a perfusion chamber may permit better insight into these processes because they render it possible to investigate cell-surface and cell-cell interactions sequentially.

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