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The Bacterial Peptide N-Formyl-Met-Leu-Phe Inhibits Killing of *Staphylococcus epidermidis* by Human Neutrophils in Fibrin Gels

Yongmei Li,* John D. Loike,* Julia A. Ember, † P. Patrick Cleary, ‡ Emily Lu,* Sadna Budhu,* Long Cao,§ and Samuel C. Silverstein*

To study human neutrophil (polymorphonuclear leukocyte (PMN)) migration and killing of bacteria in an environment similar to that found in inflamed tissues in vivo, we have used fibrin gels. Fibrin gels (1500 μm thick) containing *Staphylococcus epidermidis* were formed in Boyden-type chemotaxis chambers. PMN migrated <300 μm into these gels in 6 h and did not kill *S. epidermidis* when the gels contained heat-inactivated serum, C5-deficient serum, a streptococcal peptidase specific for a fragment of cleaved C5 (C5a), or anti-C5aR IgG. In contrast, in gels containing normal human serum, PMN migrated ~1000 μm into the gels in 4 h and into the full thickness of the gels in 6 h, and killed 90% of *S. epidermidis* in 6 h. FMLP reduced PMN migration into fibrin gels and allowed *S. epidermidis* to increase by ~300% in 4 h, whereas leukotriene B4 stimulated PMN to migrate the full thickness of the gels and to kill 80% of *S. epidermidis* in 4 h. We conclude that both complement opsonization and C5a-stimulated chemotaxis are required for PMN bacterial killing in fibrin gels, and that fMLP inhibits PMN bactericidal activity in fibrin gels. The latter finding is surprising and suggests that in the presence of fibrin fMLP promotes bacterial virulence. *The Journal of Immunology*, 2002, 168: 816–824.

The cellular mechanisms used by neutrophils (polymorpho-nuclear leukocytes (PMN)) to locate and kill bacteria in tissues have, until recently, been largely unexplored. Abundant evidence indicates that chemoattractants or chemokines are required to promote PMN emigration from the vasculature, but few investigators have reported the role(s) of these substances in enabling PMN to locate and kill bacteria that gain access to tissue spaces (1, 2).

Under physiological conditions, tissue spaces contain three-dimensional arrays of matrix proteins (e.g., collagens). Following wound or invasion of tissues by bacteria, fibrinogen-rich exudates lead to the formation of fibrin gels to which bacteria bind or in which they become embedded (3, 4). Fibrin also is deposited on prosthetic devices such as venous catheters, making them attractive substrates for adhesions expressed by *Staphylococci* and other bacteria (5). *Staphylococcus aureus* express a coagulase that promotes fibrin formation, while *Streptococci* express streptokinase, an enzyme that promotes fibrinolysis. Thus fibrin is involved in many aspects of bacterial infection.

We reported previously that PMN chemotaxis is under the dual regulation of chemotactic and extracellular matrix protein components (6, 7). For example, PMN stimulated with fMLP or TNF-α form zones of close apposition on fibrin and do not migrate into these gels. In contrast, PMN stimulated with leukotriene B4 (LTB4) or IL-8 form zones of loose apposition on fibrin and migrate effectively into these gels. In addition, fMLP or TNF-α inhibited PMN migration through fibrin gels in response to LTB4 or IL-8. These findings suggested that fMLP might exert an inhibitory effect on PMN killing of bacteria at sites of fibrin deposition by blocking PMN migration into and in these sites.

To explore mechanisms by which PMN locate and kill bacteria in tissues, and to examine the roles of various chemoattractants/chemokines in this process, we adapted a fibrin-gel system used by Hurst et al. (8) and by Rotstein et al. (9) to study phagocytosis and killing, respectively, of bacteria in a tissue matrix-like environment in vitro. In this report, we describe a method for quantitative assessment of PMN chemotaxis and killing of *Staphylococcus epidermidis* in fibrin gels, and of the effects of complement and of the chemoattractants fMLP, LTB4, and a fragment of cleaved C5 (C5a) on these processes. We report that C5a, generated by complement activation, is essential for PMN migration and killing of these bacteria in fibrin gels. LTB4 enhanced, and fMLP inhibited, PMN migration and bacterial killing. The latter finding demonstrates that in the presence of fibrin, fMLP can protect bacteria from attack by PMN.

**Materials and Methods**

**Materials**

Thrombin, fMLP, carboxypeptidase Y, cytochalasin D, and Histopaque 1077 and 1119 were from Sigma-Aldrich (St. Louis, MO). n-phenylanalyl-
-t-prolyl-l-arginine chloromethyl ketone and LTB4 were from Calbio-
chem-Novabiochem (San Diego, CA). Human fibrinogen was from Amer-
ican Diagnostica (Greenwich, CT). Cell culture inserts (0.4-μm pore size,
24-well plate format), tissue culture plates (24-well and 48-well format), agar, and tryptic soy broth (TSB) were from BD Biosciences (Franklin Lakes, NJ). Heparin was from Elkins-Sinn (Cherry Hill, NJ). Rabbit monoclonal anti-human C5aR (C85-4124) and an isotropic control anti-keyhole limpet hemocyanin rabbit mAb were a generous gift from Dr. J. A. Ember. Purified streptococcal C5a peptide (SCPA) was from Dr. P. P. Cleary.

Staphylococcus epidermidis

*S. epidermidis* H753, a clinical isolate from the cerebrospinal fluid (CSF) of a patient with an infected CSF shunt, was provided by the Diagnostic Microbiology Laboratory at Columbia-Presbyterian Hospital (New York, NY). For experiments, 3% TSB was inoculated with *S. epidermidis* from a single colony and incubated with shaking overnight at 37°C. The overnight culture was subcultured into fresh TSB, grown to late log phase, pelleted, washed three times in PBS, and resuspended in PBS. The OD of this suspension at 600 nm was monitored, and CFU of *S. epidermidis* were determined by reference to a standard curve relating OD at 600 nm to the CFU of *S. epidermidis*.

**Human sera**

C5-deficient human serum was from Sigma-Aldrich. Normal human serum (NHS) was prepared by incubating human AB plasma (New York Blood Center, New York, NY) with 1 U/ml thrombin at room temperature for 15 min and centrifuging the mixture at 8000 × g to remove fibrin. NHS was then filter sterilized using 0.22-µm filters (Pall Gelman Laboratory, Ann Arbor, MI). Heat-inactivated human serum (HIS) was prepared by heating NHS at 56°C for 30 min. Zymosan-activated serum (ZAS) was prepared as described (10). All sera were stored at −80°C until use.

**Human PMN**

PMN were prepared as described (6). Briefly, fresh heparinized blood was obtained from healthy adult volunteers after informed consent. PMN were isolated by centrifugation on Histopaque 1077 and 1119 gradients. Contaminating RBCs were removed by hypotonic lysis. The purity of PMN isolated by this method was >95%, as determined by Wright-Giemsa stain. Purified PMN were resuspended in PBG (PBS containing 0.5 mM MgCl₂, 1.1 mM CaCl₂, 5 mM glucose and 0.1% human serum albumin).

**Formation of fibrin gels containing *S. epidermidis***

Tissue culture inserts were filled sequentially with 5 µl of PBSG-BSA containing 0.1 U of thrombin and 100 µl of PBSG-BSA containing 1 mg/ml purified human fibrinogen. 1 × 10⁶ CFU of *S. epidermidis*, the indicated percentage (v/v) of serum (e.g., 1–40% NHS, 10 or 40% HIS, or 40% C5-deficient serum), with or without SCPA (0.01–10 µg/ml). The insert were incubated for 5 min at room temperature to allow the fibrin to gel. These gels were ~1500 µm in thickness. Once the fibrin gel formed, n-phenylalanyl-l-propionylarginine chloromethyl ketone (10⁻⁷ M, 10 µl) was added to the top of the gels to inhibit thrombin.

**PMN chemotaxis**

Inserts containing fibrin gels formed as described above were placed in 24-well tissue culture plates to form modified Boyden-type chemotaxis chambers. A total of 5 × 10⁶ PMN in 100 µl of PBSG-BSA were placed on top of each gel. Alternatively, before addition to the migration chamber, PMN (5 × 10⁶/ml) were pretreated at 4°C for 40 min in PBSG-BSA containing 1 µg/ml mAb against human C5aR, or 2 µg/ml control Ab. A total of 500 µl of PBSG-BSA alone or PBSG-BSA containing FMLP (10⁻⁷ M) or LTB₄ (10⁻⁷ M) was added to the bottom compartment. The chambers were incubated at 37°C in a humidified incubator containing 5% CO₂/95% air for 4–6 h, at which time the distance PMN penetrated into the fibrin gel was measured visually by focusing the distance between the gel surface and the leading front with a micrometer mounted on the focusing knob of a Nikon phase-contrast microscope (Nikon, Melville, NY).

**Enumeration of *S. epidermidis* in fibrin gels**

Fibrin gels (100 µl) containing 40% NHS, 1 × 10⁶ CFU of *S. epidermidis*, with or without 4 × 10⁻⁸–4 × 10⁻⁷ PMN were lysed as described above. A total of 200 µl of PBS (no Ca²⁺ and Mg²⁺) containing 5 mg/ml trypsin, with or without 20 µM EDTA and 20 µM cyclohexalin D (PH 10.4, 4°C), was added to each gel for 10 min to allow diffusion of phagocytosis inhibitors into the gel. The gels were then incubated at 37°C for 18 min. The liquefied gels were diluted with sterile distilled water and incubated for another 5 min at 37°C, as described (11), to completely lyse the PMN. Serially diluted samples were plated on TSB agar plates and incubated overnight at 37°C, and colonies were counted manually.

**PMN killing of *S. epidermidis* embedded in fibrin gels**

Two killing assays were used. In the first, fibrin gels (100 µl in volume) containing 1–2 × 10⁶ CFU of *S. epidermidis* and 40% NHS were formed in culture inserts as described above. The inserts were then placed in a 24-well plate to form Boyden-type chemotaxis chambers. A total of 5 × 10⁶ PMN in 100 µl of PBSG-BSA were over laid on top of the gels, and the indicated chemoattractants in PBSG-BSA were placed in the bottom compartment. The chambers were incubated at 37°C for 4–6 h in a humidified incubator containing 5% CO₂/95% air. The gels then were lysed and their content of viable *S. epidermidis* was assayed as described above. Thus, this assay measures the rate of PMN migration into the fibrin gels and killing of the embedded bacteria.

In the second assay, fibrin gels (100 µl in volume) containing 1 × 10⁶ CFU of *S. epidermidis*, 5 × 10⁵ PMN, 10% NHS, FMLP (0, 0.01 nM to 1 µM), and, where indicated, 1 U/ml carboxypeptidase Y were formed in 48-well tissue culture plates, incubated at 37°C for 90 min, and lysed, and the number of viable bacteria remaining was assayed, all as described above. Thus, the second assay measures PMN killing of bacteria within the fibrin gel.

**PMN killing of *S. epidermidis* in suspension**

Killing of *S. epidermidis* in suspension was assayed as described (12). Briefly, 500 µl of PBSG-BSA containing 10% NHS or 10% C5-deficient serum, 2.5 × 10⁶ PMN, and 0.5 × 10⁶ CFU of *S. epidermidis* with or without FMLP (10⁻⁶ M) or LTB₄ (10⁻⁷ M) was placed in a sterile 1.5-ml Eppendorf tube, and the tube was incubated at 37°C on an Orbit Environ shaker (Lab-Line Instruments, Melrose Park, IL) rotating at 200 rpm. After 90 min, a 100-µl sample was diluted in sterile distilled water to lyse PMN. Serial dilutions of the sample were plated on TSB agar plates and incubated at 37°C overnight, and the CFU of *S. epidermidis* was counted manually as described above.

**Statistics**

Experiments were performed at least three times in duplicate and are reported as the means ± SEM for the number of experiments indicated. Significance was obtained using a two-sample paired Student’s t test.

**Results**

**Opsonization with IgG and the third component of complement are required for PMN to kill *S. epidermidis* H753**

Preliminary experiments showed that >90% of *S. epidermidis* H753 was killed when incubated in suspension or in fibrin gels with 4 × 10⁶/ml PMN in the presence of NHS, but not in the presence of NHS that had been preadsorbed with protein A to remove IgG class Abs (data not shown), or heated to inactivate complement (data not shown). Immunofluorescence studies using anti-human IgG and anti-human C3 confirmed that *S. epidermidis* incubated in NHS or in C5-deficient serum was coated with both IgG and C3 (data not shown) and that IgG directed against *S. epidermidis* found in NHS was needed to promote C3 fixation on these bacteria (data not shown). Thus, opsonization of *S. epidermidis* H753 with both IgG and complement was required for PMN to kill these bacteria under the conditions of these experiments.

**Quantitative recovery of *S. epidermidis* from fibrin gels**

Rotstein et al. (9) reported that PMN killed 90% of *Escherichia coli* embedded in gels formed with 1 mg/ml fibrinogen. In their study, bacteria were recovered from fibrin gels after trypsin digestion. However, they did not report the efficiency of recovery of bacteria from these gels, and they did not report the effects of digestion of the gels on recovery of viable bacteria. Therefore, we examined the recovery of bacteria in our system of fibrin gels that contained *S. epidermidis*, NHS, and the indicated number of PMN (Table I). The gels were digested as described in Materials and Methods, and we compared recovery of *S. epidermidis* in the presence or absence of cytochalasin D and EDTA.
Table I. Effect of EDTA and cytochalasin D on recovery of S. epidermidis from fibrin gels

<table>
<thead>
<tr>
<th>PMN</th>
<th>S. epidermidis Recovered (% inoculum)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cyto D + EDTA</td>
</tr>
<tr>
<td>0</td>
<td>98 ± 1</td>
</tr>
<tr>
<td>4 × 10^5</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>4 × 10^6</td>
<td>100 ± 3</td>
</tr>
</tbody>
</table>

*a Fibrin gels (1500 μm thick, 100 μl in volume) containing 1 × 10^6 CFU S. epidermidis, 40% NHS, and the indicated number of PMN were incubated with 200 μl of PBS containing trypsin (5 mg/ml) alone (no inhibitors) or with PBS containing trypsin (5 mg/ml), EDTA (20 mM), and cytochalasin D (20 μM) (Cyto D + EDTA). Shown is the percentage of the inoculum (1 × 10^6 CFU) recovered from digested gels. Data represent the means ± SEM of three experiments, each performed in duplicate.

b p < 0.01 compared to control of zero PMN.

More than 99% of S. epidermidis was recovered from gels containing S. epidermidis alone or S. epidermidis and 4 × 10^5 PMN, whether or not EDTA, an inhibitor of integrins (13), and cytochalasin D, an inhibitor of chemotaxis and phagocytosis (14), were included in the lysis buffer (Table I). However, when using a 10-fold greater number of PMN (4 × 10^6), only 75% of S. epidermidis was recovered from gels lyed in the absence of EDTA and cytochalasin D, a significant decrease in recovery (p < 0.01) as compared with >99% recovery from gels lyed in the presence of these inhibitors (Table I). Further studies showed >99% recovery of S. epidermidis when cytochalasin D was the sole inhibitor in the lysis buffer (data not shown). Thus, inclusion of cytochalasin D in the lysis buffer ensures full recovery of S. epidermidis from fibrin gels, even when the gels contain PMN at a concentration 8-fold in excess of that used in subsequent experiments.

**PMN migrate into fibrin gels in a complement-dependent manner**

To examine the factors that influence PMN chemotaxis into fibrin gels, gels containing NHS or HIS without or with S. epidermidis were formed in Boyden-type migration chambers as described in Materials and Methods. PMN were placed into the upper compartment of these chambers, and migration was measured by the distance PMN penetrated into the gels after a 6-h incubation at 37°C. PMN migration into these gels depended on the presence and concentration of NHS in the gels. As shown in Fig. 1, PMN migrated through the full thickness of the gels (1500 μm) in 6 h when the gels contained 40% NHS and S. epidermidis, but only ~80% of the thickness of the gels (~1200 μm) when S. epidermidis was not added to the gels. In contrast, PMN penetrated only 20% of the thickness of the gels (~300 μm) into fibrin gels containing 40% HIS or low concentrations (1–5%) of NHS, regardless of whether they contained S. epidermidis.

These results indicate that components released following complement activation are required to stimulate PMN migration into fibrin gels under these conditions. These results are consistent with Hurst and Wilton’s observation (8) that heating plasma clots that contained Capnocytophaga ochracea abolished PMN migration into the clots. These experiments indicate that if S. epidermidis produce chemoattractant substances (15, 16), they do not release them in quantities sufficient to promote PMN migration under these conditions.

To determine whether activated complement components stimulate PMN migration into fibrin gels, we placed 10% ZAS (a source of C3a and C5a) in the bottom compartment of chemotaxis chambers and examined PMN migration into gels containing HIS alone or HIS and S. epidermidis. PMN migrated 90% of the full thickness of these gels in the presence of ZAS regardless of the presence or absence of bacteria (data not shown).

To identify the specific complement components involved, we performed two complementary experiments. First, we examined PMN migration into fibrin gels containing 40% C5-deficient serum and S. epidermidis. Under these conditions, PMN migrated only ~50 μm into these gels (Fig. 2). Second, we tested the effect of inhibiting C5aR function with a blocking mAb. PMN treated with anti-C5aR Ab appeared round and penetrated only ~100 μm after 6 h into gels containing 40% NHS and S. epidermidis (Fig. 2). PMN not treated with Ab or treated with an isotype control Ab appeared polarized and penetrated the full thickness (1500 μm) of the gels after 6 h. These experiments indicate that C5a is the primary chemoattractant mediating PMN migration into these gels.

**SCPA inhibits PMN migration and clearance of S. epidermidis in fibrin gels**

SCPA is a serine protease that specifically cleaves and inactivates C5a (17). To determine whether degradation of C5a affects PMN bacterial killing in fibrin gels, we measured the effect of SCPA on PMN migration and bacterial killing in fibrin gels containing 40% NHS and S. epidermidis. In the absence of SCPA, PMN penetrated the full thickness of these gels (1500 μm) after 6 h (Fig. 1). SCPA reduced PMN migration in a dose-dependent manner (Fig. 3) with an IC50 of ~0.7 μg/ml. At 1 μg/ml SCPA maximally reduced PMN migration to ~400 μm (p < 0.01), a distance similar to that migrated by PMN into fibrin gels containing HIS (Fig. 1). Inhibition of PMN migration was associated with bacteria growth of 3-fold increase over the initial inoculum (Table II). In contrast, at lower SCPA concentrations (0.01 and 0.1 μg/ml), PMN penetrated the full thickness of the gels (Fig. 3) and killed ~90% of the
S. epidermidis bacteria. Thus, reduces PMN migration into the gels and subsequent killing of unable to grow.


Those experiments were performed with albumin as the only isotype control Ab, were placed on top of the gels. The bottom compartments were filled with PBSG-HSA, and the migration chambers were incubated at 37°C. Shown is PMN migration into fibrin gels after 6 h, measured as described in Fig. 1. Data represent the means ± SEM of three experiments, each done in duplicate. **, p < 0.01 compared with control where PMN migrated into NHS-containing fibrin gels in the absence of Ab.

To test whether C5a per se is required for PMN to kill S. epidermidis, we mixed PMN and S. epidermidis in suspension in C5-deficient serum for 90 min at 37°C, as described in Materials and Methods. When PMN and bacteria were mixed together mechanically, PMN bactericidal activity occurred independent of chemotaxis because inhibitors of chemotaxis such as pertussis toxin did not affect bactericidal activity in suspension (data not shown). Under these conditions, PMN killed S. epidermidis in C5-deficient serum as effectively as in NHS (Table III). This indicates that C5a-mediated PMN activation is not needed for these cells to kill S. epidermidis. It strongly suggests that C5a serves an important role as a chemoattractant in PMN killing of S. epidermidis in fibrin gels.

fMLP inhibits, and LTB4 facilitates, C5a-stimulated PMN migration into S. epidermidis-containing fibrin gels

We previously reported (6) that PMN migration through fibrin gels is differentially regulated by chemoattractants. LTB4 and IL-8 stimulate PMN chemotaxis through fibrin gels. In contrast, fMLP and TNF-α inhibit PMN chemotaxis by activating β1 integrins and causing the cells to adhere tightly to the underlying fibrin matrix (7). Those experiments were performed with albumin as the only serum protein in the gels and without bacteria.

To determine whether fMLP exerts similar effects in the presence of serum and/or C5a, we formed fibrin gels containing S. epidermidis and NHS in Boyden-type chemotaxis chambers. With no additional chemoattractant added in the bottom compartment (control), PMN migrated 1035 ± 66 μm (n = 6) or two-thirds the thickness of these gels after 4 h, and the full thickness of the gels (1465 ± 63, n = 5) after 6 h (Fig. 4). Placement of 10⁻⁶ M fMLP in the bottom compartment significantly (p < 0.001) reduced the migration distance to 536 ± 37 μm at 4 h (n = 8) and 843 ± 55 μm at 6 h (n = 5). At a 100-fold lower concentration (10⁻⁸ M), fMLP had no significant inhibitory effect (data not shown). In contrast, placement of LTB4 (10⁻⁷ M) in the bottom compartment enhanced the rate of PMN migration (Fig. 4). At 4 h, PMN penetrated the full thickness of fibrin gels incubated with LTB4. Thus, fMLP inhibits and LTB4 promotes C5a-mediated chemotaxis into S. epidermidis-containing fibrin gels.

Extent of PMN migration into S. epidermidis-containing fibrin gels is a critical determinant of the ability of these cells to control S. epidermidis growth in these gels

We anticipated that enhanced PMN migration into S. epidermidis-containing fibrin gels would facilitate contact between the bacteria

### Table II. SCPA inhibits PMN bacterial killing in fibrin gels

<table>
<thead>
<tr>
<th>SCPA (µg/ml)</th>
<th>S. epidermidis Recovered (% inoculum)</th>
</tr>
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<tbody>
<tr>
<td>0.01</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>0.1</td>
<td>8 ± 5</td>
</tr>
<tr>
<td>1</td>
<td>283 ± 10b</td>
</tr>
<tr>
<td>10</td>
<td>293 ± 11b</td>
</tr>
</tbody>
</table>

a Fibrin gels (1500 μm thick, 100 μl in volume) containing 40% NHS and 1 × 10⁶ CFU of S. epidermidis with or without SCPA at the indicated concentrations were formed in Boyden-type migration chambers. PMN (5 × 10⁵) were placed on top of each gel. PBSG-HSA with or without SCPA at the same concentrations used in the gels was placed in the bottom compartment, and the migration chambers were incubated at 37°C. Shown is the percentage of bacterial inoculum (1 × 10⁶ CFU) remaining in fibrin gels after a 6-h incubation. Data represent means and SEM from three experiments, each done in duplicate.

b Values of p < 0.05 compared to control where no SCPA was added.
and the PMN, thereby promoting phagocytosis and killing. Conversely, we anticipated that inhibition of PMN migration into these gels would reduce contact between the PMN and the bacteria and thereby inhibit killing. We used LTB₄ and fMLP to test these ideas.

Fibrin gels (1500 μm thick and 100 μl in volume) containing 1.9 × 10⁶ S. epidermidis and 40% NHS were formed in Boyden-type chemotaxis chambers. Buffer alone or buffer containing LTB₄ (10⁻⁶ M) or fMLP (10⁻⁸ or 10⁻⁸ M) were placed in the bottom compartment and the chambers were incubated for 4–6 h at 37°C. In the absence of added PMN, S. epidermidis grew to ~10 times the initial inoculum after 4 h of incubation, and to ~100 times the initial inoculum after 6 h (Fig. 5). The presence of 5 × 10⁵ PMN initially added to the top of the gels markedly suppressed bacterial growth (Fig. 5). Under these conditions, the number of S. epidermidis grew to only two times the initial inoculum (from 1.9 × 10⁴ to 4 × 10⁴ CFU) after 4 h of incubation, and by 6 h had decreased by a log to 2 × 10³ CFU (Fig. 5). LTB₄ added to the bottom chamber significantly enhanced the rate at which PMN killed S. epidermidis in fibrin gels (Fig. 5). By 4 h, the number of bacteria in gels incubated with LTB₄ had decreased to 20% of the initial inoculum and was 10 times lower than the number of bacteria in gels not incubated with LTB₄ (3.7 × 10³ CFU with LTB₄ vs 4 × 10⁴ CFU without LTB₄). However, by 6 h there was no significant difference in the number of bacteria in gels incubated with or without LTB₄. In contrast, 10⁻⁶ M fMLP added to the bottom compartment consistently inhibited PMN killing of S. epidermidis. By 4 h, significantly more S. epidermidis were recovered from gels incubated with fMLP than from gels incubated without fMLP (p < 0.05). By 6 h, the number of S. epidermidis in gels incubated with fMLP was about twice the initial inoculum, whereas only 10% of the initial inoculum remained viable in gels incubated without fMLP (p < 0.01) (Fig. 5).

To confirm that the inhibitory effect of fMLP reflected its capacity to inhibit PMN migration within these gels, we formed fibrin gels with PMN, S. epidermidis, and fMLP embedded in them, as described in Materials and Methods, and measured their content of viable S. epidermidis after a 90-min incubation at 37°C. fMLP dose-dependently inhibited PMN killing of S. epidermidis with IC₅₀ ~ 10 nM. Maximal inhibition occurred at a concentration of 1 μM (Fig. 6). In the absence of fMLP, PMN embedded together with S. epidermidis efficiently killed these bacteria (Table IV). When carboxypeptidase Y was added to the gels to degrade fMLP (18), the inhibitory effect of fMLP on PMN killing of S. epidermidis was abolished (Table IV). Control experiments reveal that

**Table III.** Killing of S. epidermidis in suspension is unaffected by the absence of C5 or the presence of fMLP or LTB₄ *a*.  

<table>
<thead>
<tr>
<th>Serum</th>
<th>Chemoattractant</th>
<th>S. epidermidis Killed</th>
<th>(10₃ inoculum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHS</td>
<td>None</td>
<td>96 ± 1</td>
<td></td>
</tr>
<tr>
<td>C5-deficient</td>
<td>None</td>
<td>97 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>NHS</td>
<td>fMLP</td>
<td>97 ± 1</td>
<td></td>
</tr>
<tr>
<td>NHS</td>
<td>LTB₄</td>
<td>97 ± 1</td>
<td></td>
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</tbody>
</table>

*a* Killing was carried out in suspension in 500 μl of PBSG-HSA containing 0.5 × 10⁷ CFU of S. epidermidis; 2 × 10⁵ PMN, 10% NHS, or 10% C5-deficient serum in the absence or presence of fMLP (10⁻⁷ M) or LTB₄ (10⁻⁷ M). Shown is the percentage of the bacterial inoculum killed after incubation at 37°C for 90 min. Data are means ± SEM of three to five experiments, each done in duplicate.
carboxypeptidase Y alone did not affect killing of *S. epidermidis* in fibrin gels (Table IV). To be certain that neither fMLP nor LTB4 affected PMN killing of *S. epidermidis* per se, we measured PMN killing of these bacteria in suspension in the presence of fMLP (10^-6 M) or LTB4 (10^-7 M), as described in Materials and Methods. Neither fMLP nor LTB4 had any effect on the efficiency with which PMN killed *S. epidermidis* under these conditions (Table III). Taken together, the findings reported in Figs. 4--6 and Tables III-IV show that fMLP inhibits and LTB4 enhances PMN bacterial killing in fibrin gels, and that these effects were due to the inhibitory or stimulatory effects of fMLP and LTB4, respectively, on PMN chemotaxis through fibrin matrices.

**Discussion**

Since Wright and Douglas (19, 20) first demonstrated that Abs and complement cooperate in opsonizing *Staphylococci* for phagocytosis by leukocytes, in vitro systems that mimic in vivo environments have profoundly influenced our understanding of the interactions of phagocytic leukocytes with microbial pathogens. While many investigators (12, 21--23) have used a “tumble” system to study the effects of opsonins and their cognate receptors on uptake and killing of bacteria by PMN in suspension, comparatively little attention has been given to in vitro systems that mimic tissue environments. The fibrin gel system described in this work has many of the properties of inflamed or injured tissue in vivo. It appears to be useful for examining the roles of chemoattractants and of PMN membrane proteins in regulating PMN migration in three-dimensional matrices and in binding and phagocytosis of bacteria embedded in these matrices.

Three technical details about this fibrin gel system merit consideration here. The first is the importance of adding phagocytosis inhibitors during lysis of the fibrin gel. In the few studies in which PMN killing of bacteria in fibrin gels has been examined (8, 9), phagocytosis inhibitors were not used. As shown in Table I, in the absence of such inhibitors, recovery of viable bacteria from gels containing 4 X 10^7 PMN/ml was incomplete. In these studies, we used both cytochalasin D and EDTA as phagocytosis inhibitors. In subsequent studies (data not shown) we have found that cytochalasin D in the absence of EDTA blocks PMN bactericidal activity during gel lysis. Because EDTA has a deleterious effect on the viability of Gram-negative bacteria, this finding indicates that this system also can be used to analyze PMN killing of Gram-negative bacteria. Indeed, we have used it to study PMN killing of *Pseudomonas aeruginosa* (Y. Li and S. C. Silverstein, unpublished observations). The second is the use of chemotaxis chambers formed with filters that do not permit bacteria to diffuse into the lower compartment. By this means we achieved virtually complete recovery of bacteria (Table I). The third is the apparently spontaneous formation of C5a when NHS was placed in chambers containing fibrin gels. Formation of C5a is evidenced by the large number of PMN that migrated into fibrin gels containing NHS but lacking *S. epidermidis* (Fig. 1) and the dearth of PMN that migrated into fibrin gels containing *S. epidermidis* and C5-deficient serum (Fig. 2). We have not investigated the mechanism(s) responsible for generation of chemotactants by NHS in fibrin gels lacking *S. epidermidis*. It seems likely that contact of serum with the plastic and/or filter that form the chemotaxis chambers, and/or the matrix proteins they contain, promotes complement activation. Clearly, future studies would be facilitated by use of chambers formed of materials and/or containing matrix proteins that do not cause such activation.

In vivo, PMN also encounter bacteria in tissues such as bone and cartilage, which are collagen-rich and are preferential sites of Staphylococcal infections (24, 25). In principle, it should be relatively easy to form three-dimensional matrices with proteins other than fibrin (e.g., collagens) and to examine the effects of these proteins on a variety of leukocyte functions (7, 26--28).

### Roles of complement

Complement played at least two roles in PMN killing of *S. epidermidis* in fibrin gels. First, C3 opsonization of *S. epidermidis* was required for PMN to kill them. Immunofluorescence studies confirmed that *S. epidermidis* incubated in HIS were coated with IgG but not C3, while *S. epidermidis* incubated in NHS serum were coated with both IgG and C3 (data not shown). PMN were incapable of killing *S. epidermidis* in suspensions or gels containing HIS, but killed these bacteria efficiently when incubated with them in suspension or in fibrin gels containing NHS (Fig. 5). C3b and C3bi bound to the surfaces of *Staphylococci* have been identified as key ligands for PMN phagocytosis of these bacteria (29, 30).

**Table IV.** Carboxypeptidase Y blocks fMLP’s inhibitory effect on killing of *S. epidermidis* in fibrin gels

<table>
<thead>
<tr>
<th>fMLP</th>
<th>Carboxypeptidase Y</th>
<th><em>S. epidermidis</em> Killed (% inoculum)</th>
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</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>83 ± 8</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>−9 ± 10b</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>92 ± 2</td>
</tr>
</tbody>
</table>

Fibrin gels (1500 μm thick, 100 μl in volume) containing 4 X 10^7 PMN, 1 X 10^4 CFU of *S. epidermidis*, with or without fMLP (10^-6 M) and carboxypeptidase Y (1 U/ml), were incubated at 37°C for 90 min. The gels were then digested and the number of viable bacteria in the gels was assayed as described in Materials and Methods. Shown is the percentage of the bacterial inoculum killed after a 90-min incubation. Data are the means ± SEM from three to five experiments, each done in duplicate.

p < 0.001 compared to control where no fMLP or carboxypeptidase Y was added.
Second, C5 was required to promote PMN migration into fibrin gels. Addition of SCPA, a C5a-specific peptidase, or of anti-C5aR IgG, blocked PMN migration into fibrin gels containing *S. epidermidis* and NHS (Figs. 2 and 3). These findings strongly suggest that C5 was required to generate C5a, and that C5a was the principal chemoattractant that stimulated PMN to migrate into fibrin gels. These results also indicate that substances such as fibrin peptides generated during clotting (31, 32), C3a generated during C3b fixation (33), or products released from *S. epidermidis* (15, 16) play an insignificant role, compared with C5a, in stimulating PMN migration under the condition of these experiments. They lend support to the concept that SCPA is a virulence factor (34) and suggest that *Streptococci* expressing SCPA may facilitate infections mediated by bacteria that do not express this C5a-specific peptidase by degrading C5a.

C5a could enhance PMN phagocytosis by activating complement and FcR (13, 35). *S. epidermidis* incubated in C5-deficient serum were coated with C3 (data not shown). However, the finding that PMN kill *S. epidermidis* in suspension in the absence of C5, fMLP, or LTB₄ as efficiently as in their presence (Table III) indicates that these chemoattractants are not required to activate PMN for phagocytosis or bacterial killing under the conditions of these experiments. We conclude that in contrast to bacterial killing in suspension, the generation of C5a by C5 convertases on the surface of bacteria in fibrin gels is required to guide PMN to contact and ingest them.

**Role of C5a in promoting PMN functions in vivo**

Our findings suggest that C5a plays an essential role in PMN killing of *S. epidermidis* in fibrin-containing sites in vivo. They imply that in vivo C5a is important for guiding PMN to microbes enmeshed in tissues. This interpretation is consistent with the findings of Hopken et al. (1), Larsen et al. (36), and Ashman et al. (37), who showed that C5- or C5aR-deficient mice were unable to control the number of *P. aeruginosa* in the trachea or *Candida albicans* in CSF as compared with wild-type mice, despite a more intense infiltrate of PMN into the lungs or brains of C5- or C5aR-deficient mice. They also showed that PMN isolated from C5- or C5aR-deficient mice were as effective as PMN from wild-type mice in phagocytosis/killing of bacteria in vitro. Their studies showed that neither C5 nor C5aR is required for PMN to migrate out of the vasculature into mouse lung or brain (1, 36, 37). However, in C5- or C5aR-deficient mice, these extravasated PMN are ineffective in phagocytosis and killing of bacteria/yeast at these sites. This is consistent with what we have observed in fibrin gels (Table II). These results suggest that in vivo the most important role of C5a is the guidance of extravasated PMN to microbes enmeshed in tissues, matrices, and/or mucus.

Our studies also indicate that C5a plays an essential role in PMN killing of bacteria in fibrin gels (Table II and Fig. 3) but not in suspension (Table III). This suggests that bacterial killing by PMN in the bloodstream or in anatomical sites where bacteria are not entrapped in fibrin-containing matrices may occur independently of the presence of C5a. Indeed, C5 or C5aR deficiency in mice is not associated with increased susceptibility to bacterial infections. C5-deficient humans are generally healthy but do exhibit increased susceptibility to bacterial infection by neisserial organisms (38). C5aR knockout mice clear peritoneal infections with *P. aeruginosa* as effectively as wild-type mice (1). Therefore, PMN extravasation and migration at this and other sites must involve chemoattractants/chemokines other than C5a. Indeed, there is substantial evidence for this conclusion. For example, IL-8, not C5a, appears to play a dominant role in promoting PMN emigration from the vasculature into the CSF of rabbits infected intracranially with *S. pneumoniae* (39–41).

Taken together, these studies lead us to make three additional observations about the roles of chemoattractants/chemokines in PMN-mediated host defense. First, they support our suggestion that the receptor for each chemoattractant/chemokine initiates a unique set of signals and that differences in PMN functions initiated by these signals become apparent only in the context of specific matrix proteins (6). Second, these findings support the hypothesis of Foxman et al. (42) that PMN migration and orientation in tissues is a multistep process in which the effects of different chemoattractants/chemokines predominate at each step along the way. Third, they call attention to the role of chemoattractants/chemokines other than C5a in body compartments and tissues that contain low concentrations of C5 (e.g., cerebrospinal space, alveoli, bronchi, and pleural and peritoneal cavities). Normal human plasma contains ~75 µg/ml C5 (43). As shown in Fig. 1, a serum concentration of at least 10% (~7.5 µg/ml C5) is required to induce a large number of PMN to migrate into *S. epidermidis*-containing fibrin gels. In body compartments containing low concentrations of C5, production of chemoattractants/chemokines by resident tissue macrophages may play an important role in recruiting and stimulating PMN migrate to sites of bacterial invasion.

**LTB₄ facilitates PMN chemotaxis and bacterial killing**

In the presence of 40% NHS and of *S. epidermidis*, PMN crawled through fibrin gels in response to C5a at a rate of ~4 µm/min (Fig. 4). Addition of LTB₄ to the compartment underlying these gels increased the rate of PMN chemotaxis to ~6 µm/min (Fig. 4). These results suggest that in tissues containing suboptimal C5 concentrations, such as those noted above, LTB₄ generated by pioneer PMN, or by macrophages (44), speeds PMN migration to sites of bacterial invasion.

fMLP may be a bacterial virulence factor in the presence of fibrin

fMLP reduced PMN chemotaxis to ~2 µm/min, thereby slowing C5a-stimulated PMN chemotaxis in fibrin gels (Fig. 4) and PMN killing of *S. epidermidis* embedded in them (Figs. 5 and 6). These results lend physiological relevance to our previous reports that fMLP inhibits LTB₄-stimulated PMN chemotaxis through fibrin gels and plasma clots (6).

PMN express on their plasma membranes a neutral endopeptidase, CD10, for which fMLP is an excellent substrate (45). Our finding that carboxypeptidase Y blocks the inhibitory effects of fMLP on PMN bactericidal activity suggests that CD10 may facilitate PMN migration into sites containing bacteria and fibrin by degrading N-formylated peptides released by the bacteria.

N-formylated peptides are generated by a wide variety of bacteria (18, 46). The lumen of the human intestine contains ~10⁻⁷ M N-formylated peptides (18). Regardless of whether fMLP was placed in the bottom compartment of chemotaxis chambers containing fibrin gels or in the fibrin gels themselves, 10⁻⁶ M fMLP was required to fully block PMN migration and bacterial killing in response to the gradients of C5a generated in these experiments (Figs. 4 and 5), and to block PMN chemotaxis in response to 10⁻⁷ M LTB₄ in previous experiments (6). However, the presence of 10⁻⁷ M or 10⁻⁸ M fMLP within fibrin gels was sufficient to block bacterial killing by ~80 or ~30%, respectively (Fig. 6). Thus, concentrations of fMLP similar to those found in the normal colon in vivo (18) are more than sufficient to exert a strong inhibitory effect on PMN killing of bacterial pathogens in fibrin-containing matrices. Therefore, we suggest that in the presence of fibrin fMLP is a bacterial virulence factor.
Mechanisms by which LTB4 promotes and fMLP inhibits C5a-stimulated PMN chemotaxis through fibrin gels

Our studies show that fMLP and LTB4 exert qualitatively different effects on chemotaxis of PMN through fibrin gels (6), activation of PMN integrins (47), and production of H2O2 by PMN (48). FMLP and LTB4 signal via heptahelical receptors coupled to Gq,-containing heterotrimeric G proteins. Pertussis toxin, which inactivates Gq, blocks all PMN functions stimulated by fMLP and LTB4 (48). Given their apparent similarities in signaling mechanisms, how might these receptors activate qualitatively distinct PMN effector functions?

One possibility is that while fMLP and LTB4 receptors use the same Gq subunits, they might activate different Gi/By subunits. Five different Gi subunits and 12 different Gq subunits have been identified so far (49). Studies ongoing in our laboratory show that PMN contain several isoforms of Gi subunits. Different heptahelical receptors have been shown to activate different Gi or Gq subunits. For example, both muscarinic M4 and somatostatin receptors couple to Gq but to different Gi/By subunits (50, 51). Azpiroz et al. (52) have shown that molecular contacts between Gq subunits and heptahelical receptors are necessary for G protein activation, indicating that heptahelical receptors could specifically bind particular Gq subunits. There is also evidence that specific Gi/By subunits are responsible for different effector functions and signaling mechanisms. For example, a Gi3 mutant enhances chemotaxis of human PMN in response to IL-8 and fMLP (53, 54) but has no effect on superoxide production stimulated by either ligand. Activation of phosphoinositide 3-kinase and phospholipase C has been shown to be mediated by different Gi/By subunits (55).

A second possibility derives from the observation that heptahelical receptors can directly couple to cytoplasmic effector proteins other than heterotrimeric G proteins. For example, Luttrell et al. (56) and McDonald et al. (57) have shown that following G protein activation, specific ß-arrestins bind to ß-adrenergic receptors, thereby activating Src and mitogen-activated protein kinases; and Neptun et al. (58) have reported that it is the heptahelical receptor, and not the Gq subunit, which determines the cellular function that is stimulated when the receptor is activated by its cognate ligand. Thus, at least two mechanisms have been described by which different heptahelical receptors that activate the same Gq subunits could nonetheless stimulate qualitatively distinct signal cascades and cellular functions.

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References


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