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# M Protein, a Classical Bacterial Virulence Determinant, Forms Complexes with Fibrinogen that Induce Vascular Leakage

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## Summary

**Increased vascular permeability is a key feature of inflammatory conditions. In severe infections, leakage of plasma from the vasculature induces a life-threatening hypotension. *Streptococcus pyogenes*, a major human bacterial pathogen, causes a toxic shock syndrome (STSS) characterized by excessive plasma leakage and multi-organ failure. Here we find that M protein, released from the streptococcal surface, forms complexes with fibrinogen, which by binding to $\beta_2$ integrins of neutrophils, activate these cells. As a result, neutrophils release heparin binding protein, an inflammatory mediator inducing vascular leakage. In mice, injection of M protein or subcutaneous infection with *S. pyogenes* causes severe pulmonary damage characterized by leakage of plasma and blood cells. These lesions were prevented by treatment with a $\beta_2$ integrin antagonist. In addition, M protein/fibrinogen complexes were identified in tissue biopsies from a patient with necrotizing fasciitis and STSS, further underlining the pathogenic significance of such complexes in severe streptococcal infections.**

## Introduction

Polymorphonuclear neutrophils (PMNs) are part of the first line of defense against bacterial infections. Under physiological conditions nonactivated PMNs circulate in the bloodstream. However, once activated by a chemotactic signal, the PMNs become adherent and attach firmly to the endothelium. The cells then start to extravasate into the infected tissue where they phagocytose invading bacteria. These adhesion processes involve the sequential engagement of a number of different adhesion molecules both on PMNs and the endothelium, including selectins, integrins, and members of the family of intercellular adhesion molecules (ICAM) (for a review, see Ley, 2002).

Members of the $\beta_2$ integrin family (CD11a,b,c,d/CD18) are the most abundant integrins expressed by PMNs. In nonactivated PMNs, the majority of these integrins is stored in intracellular granules, mainly in specific granules and secretory vesicles, and only a minor portion is found attached to the plasma membrane (Dib and Andersson, 2000). However, following stimulation specific granules and secretory vesicles may fuse with the plasma membrane and induce an upregulation of the $\beta_2$ integrins at the cell surface. In resting cells, surface-exposed integrins remain in a low affinity state until they become activated by an "inside-out signaling" mechanism, resulting in a conformational change in the receptor and increased ligand binding. The number of $\beta_2$ integrin binding proteins is large and includes ligands that mediate firm adhesion to the endothelium, such as the ICAMs, but also various plasma proteins including $C_3b_i$, factor X, and fibrinogen (for a review, see Crockett-Torabi, 1998). The interaction between fibrinogen and $\beta_2$ integrins is regulated by divalent cations (Yan et al., 1995), and whereas soluble fibrinogen binds only to activated PMNs, immobilized fibrinogen can also interact with unstimulated PMNs (Yan et al., 1995).

*Streptococcus pyogenes* is a significant human pathogen giving rise to a wide spectrum of diseases ranging from uncomplicated infections, such as pharyngitis, impetigo, and erysipelas, to life-threatening conditions associated with shock and organ failure. *S. pyogenes* expresses substantial amounts of M protein, $\alpha$-helical coiled-coil surface proteins (for a review, see Fischetti, 1989), which represents one of the classical virulence determinants of *S. pyogenes* promoting the survival of the bacterium in human blood (Lancefield, 1969). Apart from being associated with the bacterial cell wall, M protein is also released from the surface by the action of a cysteine proteinase secreted by the bacteria (Berge and Björck, 1995). Several reports have shown that M protein binds fibrinogen (Kantor, 1965) with high affinity (Åkesson et al., 1994). Here, we find that M protein/fibrinogen complexes interact with $\beta_2$ integrins and activate PMNs. This results in a massive inflammatory response with profound pathophysiological consequences, which helps to explain the symptoms of streptococcal toxic shock syndrome (STSS), one of the most acute and severe forms of septic shock.

## Results

### Neutrophil Proteinases Release M1 Protein from the Surface of *S. pyogenes*

To test whether M1 protein is released from the streptococcal surface following treatment with human neutro-

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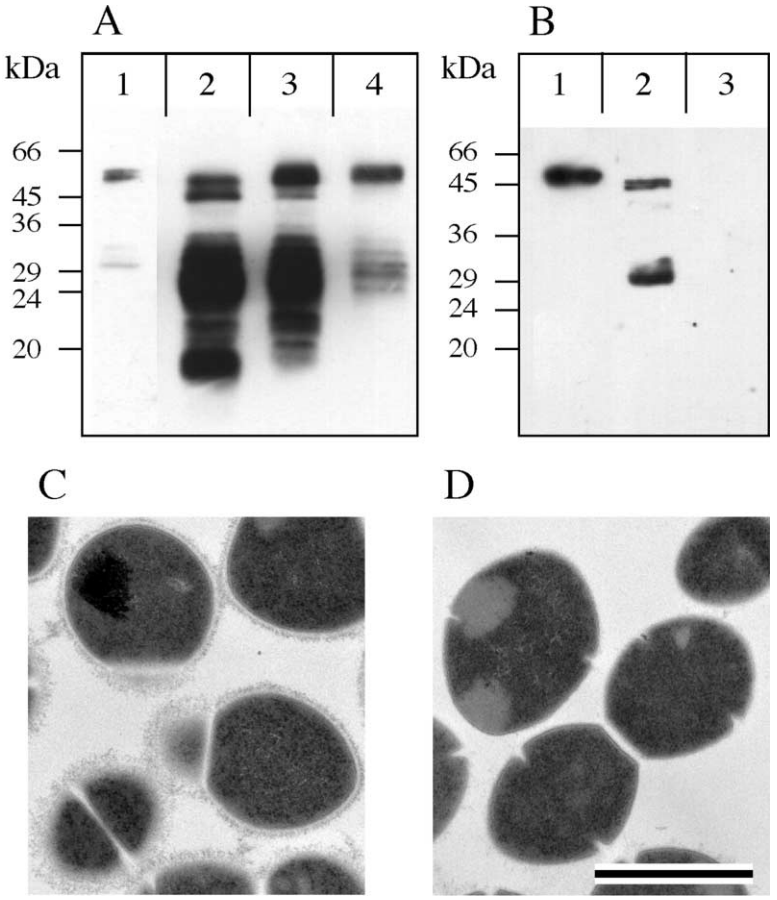



Figure 1. Release of M1 Protein from the Streptococcal Surface Following Treatment with Supernatants from Stimulated PMNs

(A) AP1 bacteria ($2 \times 10^9$ bacteria/ml) were incubated with a serial dilution (100 μl, 10 μl, or 1 μl; lanes 2–4) of exudate from stimulated PMNs ($2 \times 10^6$ cells/ml, see also Experimental Procedures) for 2 hr at 37°C. As a control, the bacteria were incubated with buffer alone (lane 1). Bacteria were centrifuged and the resulting supernatants were run on SDS-PAGE. Separated proteins were transferred onto nitrocellulose and probed with antibodies to M1 protein. Bound antibodies were detected by peroxidase-conjugated secondary antibodies to rabbit immunoglobulin.

(B) 10 ng purified M1 protein (lane 1), AP1 surface proteins released with 100 μl neutrophil secretion products (lane 2), and 10 ng purified protein H (lane 3) were subjected to SDS-PAGE. After transfer onto nitrocellulose membranes, the membranes were incubated with fibrinogen (2 μg/ml) followed by immunodetection with rabbit antibodies to human fibrinogen and a peroxidase-conjugated secondary antibody against rabbit immunoglobulin.

(C) Transmission electron microscopy of thin sectioned AP1 bacteria before incubation with exudate from stimulated PMNs.

(D) AP1 bacteria after incubation with $2 \times 10^8$ bacteria in 100 μl PMN exudate. Bar is equal to 1 μm.

phil proteinases, bacteria of the M1 serotype (AP1) were incubated with serial dilutions of secretion products from PMNs stimulated by antibody-crosslinking of CD11b/CD18. As described previously, activation of the $\beta_2$ integrins by antibody-crosslinking mimics adhesion-dependent receptor engagement and induces the release of neutrophil elastase, cathepsin G, and proteinase 3 (Gautam et al., 2000). This was confirmed in the present study using an indirect ELISA (data not shown). Incubation of AP1 bacteria with neutrophil exudates results in the release of several streptococcal surface proteins as determined by SDS-PAGE (data not shown). The presence of M1 protein was analyzed by Western blot analysis using a polyclonal antiserum against M1 protein. Figure 1A shows that in the absence of secreted neutrophil components, only small amounts of M1 protein are released from the bacterial surface, whereas larger quantities of M1 protein fragments with different molecular masses were detected when bacteria were incubated with increasing volumes of neutrophil secretion products. A size-comparison of the largest M1 protein fragment with intact M1 protein suggests that it covers most, if not all, of the extracellular part of the molecule. With increasing concentrations of neutrophil secretion products, M1 protein was further degraded (Figure 1A). In the absence of bacteria, neutrophil secretion products probed with antiserum against M1 protein failed to show immunoreactivity, demonstrating the specificity of the antibody (data not shown). As mentioned above, M protein has an affinity for fibrinogen and in the AP1 strain, the M1 protein is the only fibrinogen binding molecule (Berge and Björck, 1995; Herwald et al., 2003; Kihlberg et al., 1995). To test whether the generated M1 protein fragments were still capable of binding fibrinogen, streptococcal proteins released by neutrophil exudate were run on SDS-PAGE and transferred onto nitrocellulose. Filters were probed with fibrinogen followed by immunodetection with specific antibodies against fibrinogen. Whereas M1 protein binds fibrinogen, the closely related protein H does not (Åkesson et al., 1994). This is demonstrated in Figure 1B, which also shows that treatment with secreted neutrophil proteinases releases several fibrinogen binding peptides from AP1 bacteria (Figure 1B, lane 2). The molecular masses of these peptides correlate well with the M1 protein fragments seen in Figure 1A. Identical membranes that were incubated with the antifibrinogen antibodies alone failed to give any reactivity, showing that the antibodies do not crossreact with neutrophil or streptococcal components (data not shown). Transmission electron microscopy analyses of thin-sectioned AP1 bacteria before and after incubation with neutrophil exudates, revealed that the secreted proteinases efficiently remove the fibrous surface proteins of AP1 bacteria (Figures 1C and 1D). In the AP1 strain, these hair-like structures represent M1 protein and protein H (Kihlberg et al.,

1995), and the results show that neutrophil proteinases release M1 protein fragments with retained fibrinogen binding activity from the bacterial surface.

### M1 Protein Triggers the Release of Heparin Binding Protein (HBP) from PMNs in Human Blood

The only blood cells that were reported to produce the inflammatory mediator HBP are PMNs (Edens and Parkos, 2003). The observation that fragments of M1 protein were released by neutrophil proteinases raised the question whether these fragments and/or other *S. pyogenes* components could enhance the inflammatory response by releasing HBP from PMNs. Soluble streptococcal components were therefore added to human whole blood and the HBP release was measured. Figure 2A shows that approximately 60% of the HBP stored in PMNs is mobilized when M1 protein (1 μg/ml final concentration) is added to blood (diluted 1/10). Interestingly, both lower and higher concentrations of M1 protein resulted in less efficient HBP release. Apart from M1 protein, formyl-methionyl-leucyl-phenylalanine (fMLP) and to a lesser extent lipoteichoic acid (LTA) and protein H, also evoked secretion of HBP. Hyaluronic acid (HA), which is part of the streptococcal capsule, and the secreted streptococcal proteins SpeB and protein SIC, did not induce HBP secretion. In order to exclude the possibility that the HBP release is caused by LPS contamination, purified LPS was tested as a control. However, even at a high concentration (10 μg/ml) LPS failed to induce the release of HBP from PMNs.

To localize the region in the M1 protein that triggers secretion of HBP from PMNs, fragments A-S and S-C3 of M1 protein (Åkesson et al., 1994) were tested. Figure 2B shows that fragment A-S mobilizes HBP whereas fragment S-C3 has no effect, demonstrating that the $NH_2$-terminal part of the M1 protein is sufficient for HBP release. Previous studies have mapped fibrinogen binding to the B domains of fragment A-S, albumin binding sites to the C repeats of S-C3, and IgG Fc binding activity to the S region, which is present in both fragments (Åkesson et al., 1994; Ringdahl et al., 2000). The M1 protein and its two fragments (A-S and S-C3), used for the stimulation of PMNs, are recombinant proteins produced in *E. coli*. However, M1 protein produced by *S. pyogenes* also releases HBP, as shown with an isogenic AP1 mutant strain, termed MC25, expressing a truncated M1 protein lacking the COOH-terminal cell-wall-anchoring motif. This strain has no surface bound M1 protein, but produces an M1 protein fragment covering the surface-exposed part of the molecule, including the fibrinogen binding B domains, that is secreted into the growth medium (Collin and Olsén, 2000). Figure 2C shows that supernatants of an overnight culture from MC25 bacteria when added to human blood, trigger the release of HBP, while culture supernatants from AP1 bacteria or growth medium alone, did not have this effect. The results demonstrate that soluble M1 protein, produced by *E. coli* or *S. pyogenes*, induces HBP release in human blood.

### The Release of HBP from Human Blood PMNs Is Modulated by Signal Transduction Mediators and Extracellular Divalent Metal Ions

PMNs release their granular content upon cell lysis or by regulated secretory mechanisms involving sophisticated signal transduction machinery (Borregaard and Cowland, 1997). To investigate by which mechanism M1 protein induces mobilization of HBP in human blood, the influence of signal transduction inhibitors on HBP release was analyzed. Theoretically, fMLP contamination of the M1 protein preparation could cause activation of PMNs. Therefore the first substances tested were t-boc-MLP (an fMLP antagonist) and pertussis toxin. This toxin is an antagonist of $G_i$ protein-coupled seven membrane-spanning receptors, and fMLP receptors also belong to this group of receptors. As shown in Table 1, neither of the two components inhibited the release of HBP in blood, implicating that fMLP was not present in the M1 protein preparation and that M1 protein does not act as an fMLP receptor agonist. The next signal transduction inhibitors to be tested were genistein, a tyrosine kinase inhibitor, and wortmannin, a phosphatidylinositol 3-kinase inhibitor. These inhibitors abrogate downstream effects of $\beta_2$ integrin-triggered PMN signaling (Axelsson et al., 2000), and both substances blocked the release of HBP. To study the effect of intracellular and extracellular calcium, BAPTA-AM (complexing intracellular calcium) and EGTA (complexing extracellular calcium) were added to the blood. Like genistein and wortmannin, this treatment inhibited the mobilization of HBP. When EGTA was used in the absence of BAPTA-AM, it also blocked HBP release. These results suggest that the M1 protein-induced release of HBP is dependent on divalent metal ions. Other inhibitors, such as AG1478, a selective inhibitor of EGF receptor tyrosine kinase, GF109203, a protein kinase C inhibitor, H-89, an inhibitor of cAMP-dependent protein kinase, PD98059, an inhibitor of the MAPK pathway, and U-73122, a phospholipase C inhibitor, did not interfere with the secretion of HBP. Taken together, the results suggest that the release of HBP in human blood induced by M1 protein is dependent on the interaction with a receptor-like structure at the neutrophil surface. The data also demonstrate that this interaction is dependent on extracellular divalent metal ions.

### M1 Protein Precipitates Fibrinogen in Plasma

To identify a putative neutrophil receptor mediating the release of HBP in blood, binding of $^{125}$I-M1 protein to purified PMNs was tested. No binding to the PMNs was detected, suggesting that the interaction requires a cofactor, presumably a plasma protein. However, one of our initial observations was that the addition of M1 protein (at a concentration of 1 μg/ml) to plasma (diluted 1/10) provoked a visible precipitate, while at other concentrations of M1 protein no precipitate was formed in the plasma sample. The results of Figure 3A show that $^{125}$I-M1 protein is coprecipitated when added together with unlabeled M1 protein to plasma at the critical concentration. Maximal release of HBP from PMNs was also recorded at an M1 protein concentration of 1 μg/ml in blood diluted 1/10 (Figure 3B), suggesting that M1 precipitation and HBP release are correlated. The observation that M protein forms insoluble complexes in human plasma was reported already in 1965, and was found to be the result of interactions between M protein

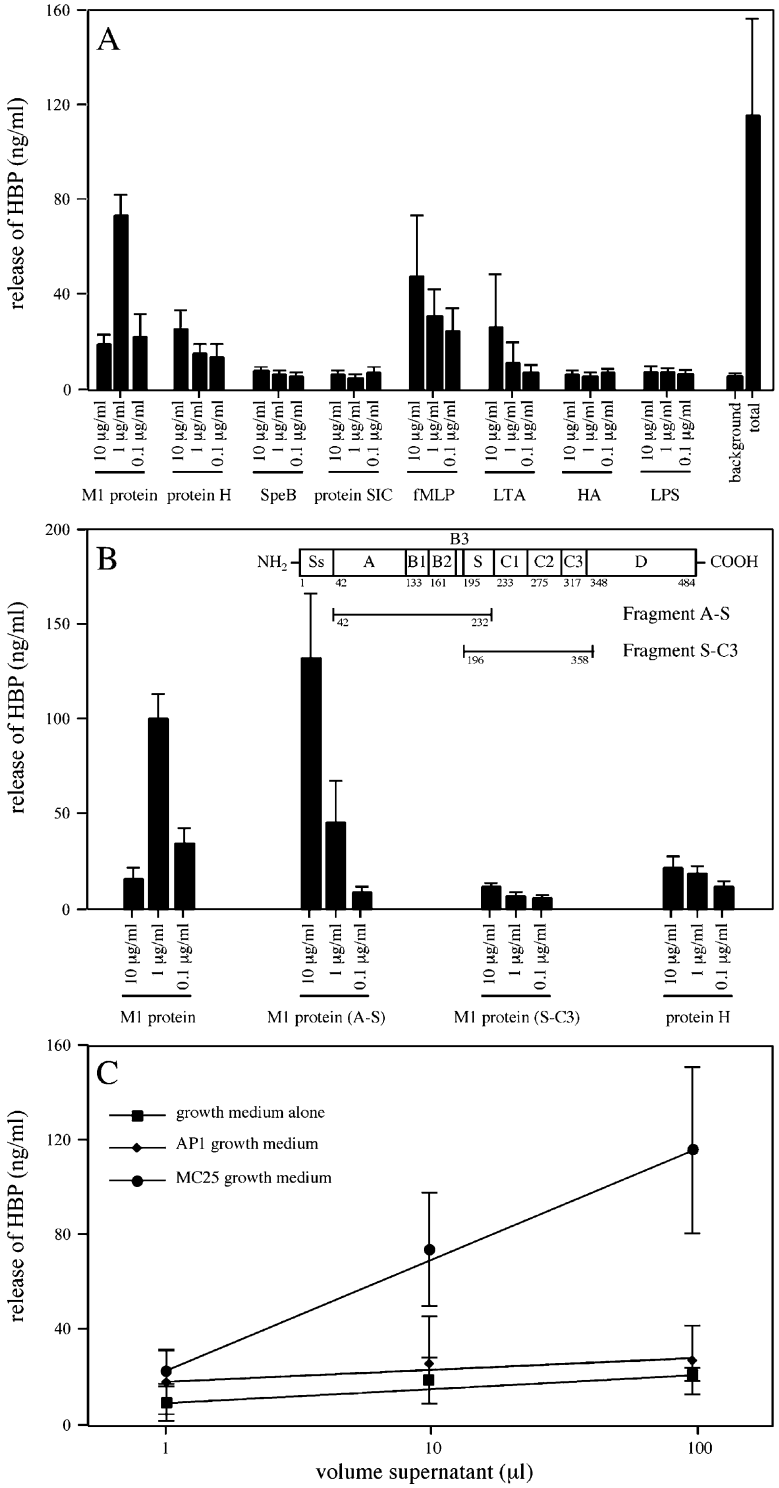



Figure 2. Release of HBP in Human Blood

(A) Human blood was incubated with M1 protein, protein H, SpeB, protein SIC, fMLP, lipoteichoic acid (LTA), hyaloronic acid (HA), or LPS for 30 min at 37°C. Cells were pelleted and the concentration of HBP in the supernatants was determined by ELISA. The total amount of HBP in blood was determined by lysing cells with Triton X-100, and the amount of HBP released after incubation for 30 min at 37°C without stimulation, was considered as background. The figure presents the mean ± SD of three independently performed experiments, each done in duplicate.

(B) M1 protein, M1 protein fragments A-S and S-C3 (schematically depicted at the top), or protein H, was added to human blood, followed by incubation for 30 min at 37°C. Cells were pelleted and the concentration of HBP in the supernatants was determined by ELISA. The figure presents the mean ± SD of three independently performed experiments, each done in duplicate.

(C) Serial dilutions of supernatants from overnight cultures of strains AP1 and MC25, or growth medium alone, were added to human blood and the release of HBP was determined.

and fibrinogen (Kantor, 1965). The interaction between purified M1 protein and purified fibrinogen was therefore investigated, and also in buffer precipitates were formed at the same concentrations of M1 protein and fibrinogen as in plasma (Figure 3C). In contrast, no precipitation occurred when M1 protein was added to fibrinogen-deficient plasma (data not shown). The presence of serine proteinase inhibitors did not influence M1 protein-induced precipitation, indicating that a thrombin-like cleavage of fibrinogen did not cause the precipitation (data not shown). Scanning electron microscopy revealed amorphous aggregates (Figure 3D), whereas plasma clots induced by thrombin showed networks of fibrin fibrils similar to those described previously (Her-

Table 1. Influence on M1 Protein-Induced Release of HBP in Human Blood by Various Signal Transduction Inhibitors

| Inhibitor | Target | M1 protein-induced release (percent ± SEM)[a] |
|---|---|---|
| t-boc-MLP | fMLP receptor | 110,7 ± 6,2 |
| pertussis toxin | $G_i$ protein-coupled seven membrane spanning receptors | 135,7 ± 5,1 |
| genistein | tyrosine kinases | 12,7 ± 8,4 |
| wortmannin | phosphatidylinositol 3-kinase | 3,0 ± 1,3 |
| BAPTA and EGTA | intra- and extracellular calcium | 1,6 ± 4,0 |
| EGTA | extracellular calcium | 1,3 ± 4,0 |
| AG1478 | EGF receptor tyrosine kinase | 103,0 ± 8,7 |
| GF109203 | protein kinase C | 116,0 ± 5,3 |
| H-89 | cAMP-dependent protein kinase | 137,7 ± 9,8 |
| PD98059 | MAPK pathway | 108,0 ±20,0 |
| U-73122 | phospholipase C | 83,7 ± 22,4 |

[a]The release induced by M1 protein corresponds to 100 percent.

wald et al., 2003). The results demonstrate that M1 protein, when added to human plasma within a narrow concentration range, has the potential to induce the formation of M1 protein/fibrinogen precipitates. These precipitates are morphologically different from physiological clots induced by thrombin.

### M1 Protein/Fibrinogen Complexes Activate PMNs

In another set of experiments, we analyzed the interaction between M1 protein/fibrinogen complexes and PMNs by scanning electron microscopy. Figure 4A shows that PMNs reconstituted with a mixture containing M1 protein and plasma form aggregates that are covered with an amorphous protein layer (Figure 4A, upper left), similar to the M1 protein/fibrinogen precipitates seen in Figure 3D. No protein precipitates or cell aggregates were found when PMNs were reconstituted with plasma in the absence of M1 protein (Figure 4A, upper right), or when PMNs were treated with M1 protein dissolved in buffer instead of plasma (Figure 4A, lower left). Purified PMNs incubated with buffer alone served as a control (Figure 4A, lower right). Additional experiments with normal and fibrinogen-deficient plasma revealed that the aggregation of PMNs in the presence of M1 protein is fibrinogen-dependent (data not shown). The results indicate that the interaction between PMNs

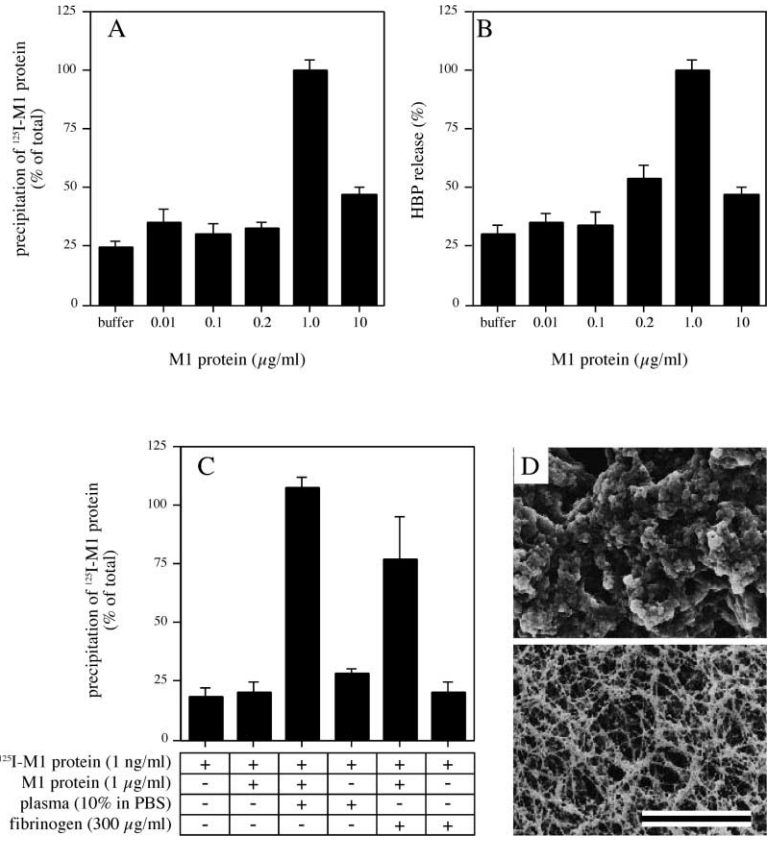



Figure 3. M1 Protein-Induced Release of HBP Correlates with M1 Protein-Induced Precipitation of Plasma

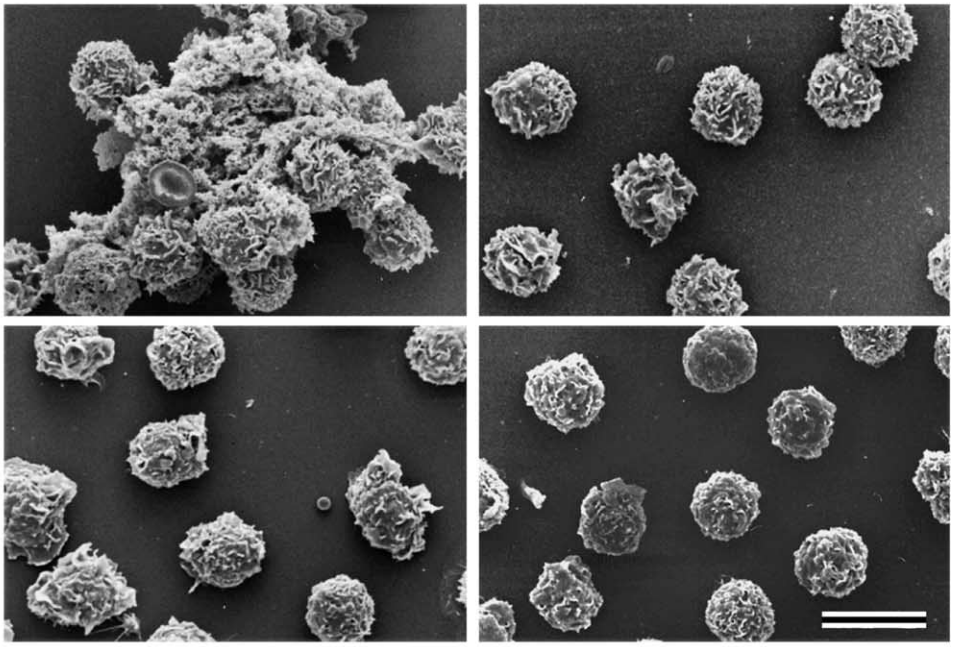
(A) Samples of 10% human plasma in PBS (1 ml) were incubated with $^{125}$I-M1 protein ($10^5$ cpm/ml, approximately 1 ng) in the absence or presence of nonlabeled M1 protein for 30 min at 37°C. Samples were centrifuged and the radioactivity of the pellets was measured. The percentage of added total radioactivity measured in the pellets is given and the figure shows the mean ± SD of three independent experiments, each done in duplicate.

(B) Human whole blood was treated with M1 protein for 30 min at 37°C. Cells were centrifuged and the amount of HBP in the supernatants was determined.

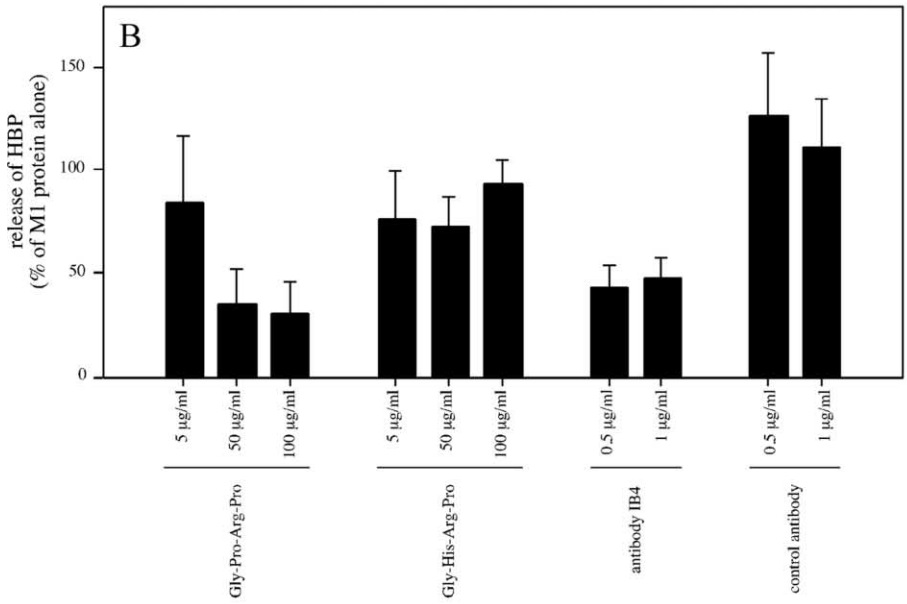
(C) One ml samples of human plasma (10% in PBS) or fibrinogen (300 μg/ml in PBS) were incubated with $^{125}$I-M1 protein ($10^5$ cpm/ml, approximately 1 ng) in the absence or presence of nonlabeled M1 protein. After 30 min of incubation at 37°C, samples were centrifuged and the radioactivity of the pellets was measured. Results are presented as percentage of added total radioactivity. The figure presents the mean ± SD of three independent experiments, each done in duplicate.

(D) Scanning electron microscopy of plasma clots induced by the addition of M1 protein (top) or thrombin (bottom). Bar is equal to 5 μm.

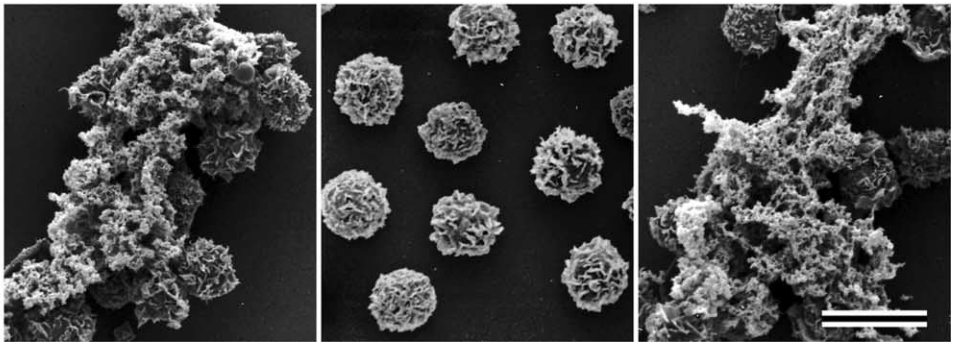
A



B


release of HBP
(% of M1 protein alone)
150
100
50
0
5 µg/ml
50 µg/ml
100 µg/ml
Gly-Pro-Arg-Pro
5 µg/ml
50 µg/ml
100 µg/ml
Gly-His-Arg-Pro
0.5 µg/ml
1 µg/ml
antibody IB4
0.5 µg/ml
1 µg/ml
control antibody

C



and M1 protein/fibrinogen complexes activates the cells, which causes HBP release. The data described above show that M1 protein/fibrinogen complexes bind to PMNs and induce their aggregation and activation, resulting in HBP release.

### M1 Protein-Induced HBP Release Is Blocked by a $\beta_2$ Integrin Antagonist

Human fibrinogen binds to PMNs via $\beta_2$ integrins (Altieri, 1999) and for CD11c/CD18 the binding site was mapped to the $NH_2$-terminal region of the A$\alpha$ chain of fibrinogen. A peptide derived from this region (Gly-Pro-Arg-Pro) has been shown to block adherence of TNF-stimulated PMNs to fibrinogen-coated surfaces, while other peptides from the same region, including Gly-His-Arg-Pro, had no effect (Loike et al., 1991). Furthermore, it was demonstrated that antibodies against $\beta_2$ integrins inhibit the binding of fibrinogen to activated PMNs, and among those antibodies, a monoclonal antibody (IB4) directed against the common $\beta$-chain of integrins, was the most potent (Loike et al., 1991). Platelet-induced activation of PMNs was also found to be dependent on the interaction between CD11c/CD18 and the A$\alpha$ chain of platelet-expressed fibrinogen (Ruf and Patscheke, 1995). As shown for the binding of fibrinogen to PMNs, platelet-induced activation of PMNs was also inhibited by the Gly-Pro-Arg-Pro peptide and by antibodies to CD11c, whereas the Gly-His-Arg-Pro peptide had no effect (Ruf and Patscheke, 1995). These reports demonstrate that the binding of PMNs to immobilized fibrinogen (for instance on coverslips or platelets) involves $\beta_2$ integrins leading to an activation of PMNs. We therefore tested the Gly-Pro-Arg-Pro and Gly-His-Arg-Pro peptides, as well as an antibody against the $\beta_2$ integrins (IB4), for their ability to interfere with the M1 protein-induced release of HBP. As shown in Figure 4B, the addition of Gly-Pro-Arg-Pro to human blood blocked the mobilization of HBP by M1 protein in a dose-dependent manner, and the IB4 antibody directed against the common $\beta$-chain of integrins also impaired HBP release. The control substances, Gly-His-Arg-Pro and an unrelated antibody against H-kininogen, did not influence HBP secretion (Figure 4B). As an additional control, antibodies against complement receptors 1 and 3, and the complement inhibitor compstatin were tested. No interference with HBP release was recorded (data not shown), suggesting that complement does not participate in the release process. The effect of Gly-Pro-Arg-Pro on M1 protein-induced PMN aggregation was analyzed by scanning electron microscopy. As shown in Figure 4C (middle image), Gly-Pro-Arg-Pro inhibited the aggregation of PMNs in a mixture of plasma and M1 protein. In contrast, Gly-His-Arg-Pro did not influence the aggregation of PMNs. These results support the notion that M1 protein/fibrinogen complexes activate PMNs through $\beta_2$ integrin crosslinking, which triggers the release of HBP. This mechanism appears to be similar to the previously described antibody-mediated crosslinking of CD11b/CD18 that mimics adhesion-dependent receptor engagement and causes a massive release of HBP from PMNs (Gautam et al., 2000, 2001).

### Intravenous Injection of M1 Protein into Mice Causes Severe Lung Lesions that Are Prevented by the Administration of a $\beta_2$ Integrin Antagonist

Previous work has identified HBP in humans and pigs (Flodgaard et al., 1991). Before mouse experiments were performed, we investigated if mice also produce a homolog of HBP. To this end, bone marrow cells from mice were isolated and the existence of such an HBP homolog was demonstrated by RT-PCR analysis using a primer set derived from human HBP and by Western blot analysis using antibodies against human HBP (data not shown). A series of animal experiments was now conducted to determine if the effects observed in vitro could relate to pathophysiological conditions reported during severe infection. Mice (three animals per group) received M1 protein i.v. (15 $\mu$g/animal), a mixture of M1 protein (15 $\mu$g/animal) and peptide Gly-Pro-Arg-Pro (400 $\mu$g/animal), or a mixture of M1 protein (15 $\mu$g/animal) and peptide Gly-His-Arg-Pro (400 $\mu$g/animal). Alternatively, animals were treated with protein H (15 $\mu$g/animal) or vehicle alone. Thirty minutes after administration the breathing of mice injected with M1 protein or M1 protein plus peptide Gly-His-Arg-Pro was clearly affected as compared to the other groups of mice. Animals were sacrificed and the lungs were removed, stained with hematoxylin and eosin and subjected to light microscopy, or analyzed by scanning electron microscopy. Figure 5A depicts a representative lung sample from a mouse injected with buffer only, showing intact lung tissue. Lung sections from mice injected with M1 protein, however, demonstrate severe hemorrhage and tissue destruction (Figure 5B). These lesions were almost completely prevented when M1 protein was injected together with Gly-Pro-Arg-Pro, even though the tissue was slightly swollen, indicating an inflammatory reaction (Figure 5C). By contrast, application of Gly-His-Arg-Pro could not prevent the M1 protein-induced bleeding and tissue destruction (Figure 5D). Protein H was injected as a control and analysis of the lung tissue revealed no

Figure 4. Binding of M1 Protein/Fibrinogen Complexes to $\beta_2$ Integrins Activates PMNs

(A) PMNs preincubated with a mixture of M1 protein (1 $\mu$g/ml) and human plasma (10% in PBS) were analyzed by scanning electron microscopy (upper left). Purified PMNs (upper right), PMNs incubated with plasma (lower left) or M1 protein alone (lower right), are shown. Bar is equal to 10 $\mu$m.

(B) M1 protein was added to whole human blood (1 $\mu$g/ml) followed by the addition of different amounts of Gly-Pro-Arg-Pro, Gly-His-Arg-Pro, monoclonal antibody IB4 to CD18, or antibody AS88 (directed against human H-kininogen). After 30 min of incubation at 37°C, cells were spun down and the amount of HBP in the supernatants was determined. Data are expressed as percent of HBP release in blood induced by M1 protein alone, and the bars represent means ± SD of three experiments, each done in duplicate.

(C) Electron microscopy of purified PMNs in a mixture of plasma and M1 protein (left image). In the other images, fibrinogen-derived peptides Gly-Pro-Arg-Pro (middle image) or Gly-His-Arg-Pro (right image), were added to the mixture of plasma and M1 protein, prior to the incubation with PMNs. Bar is equal to 10 $\mu$m.

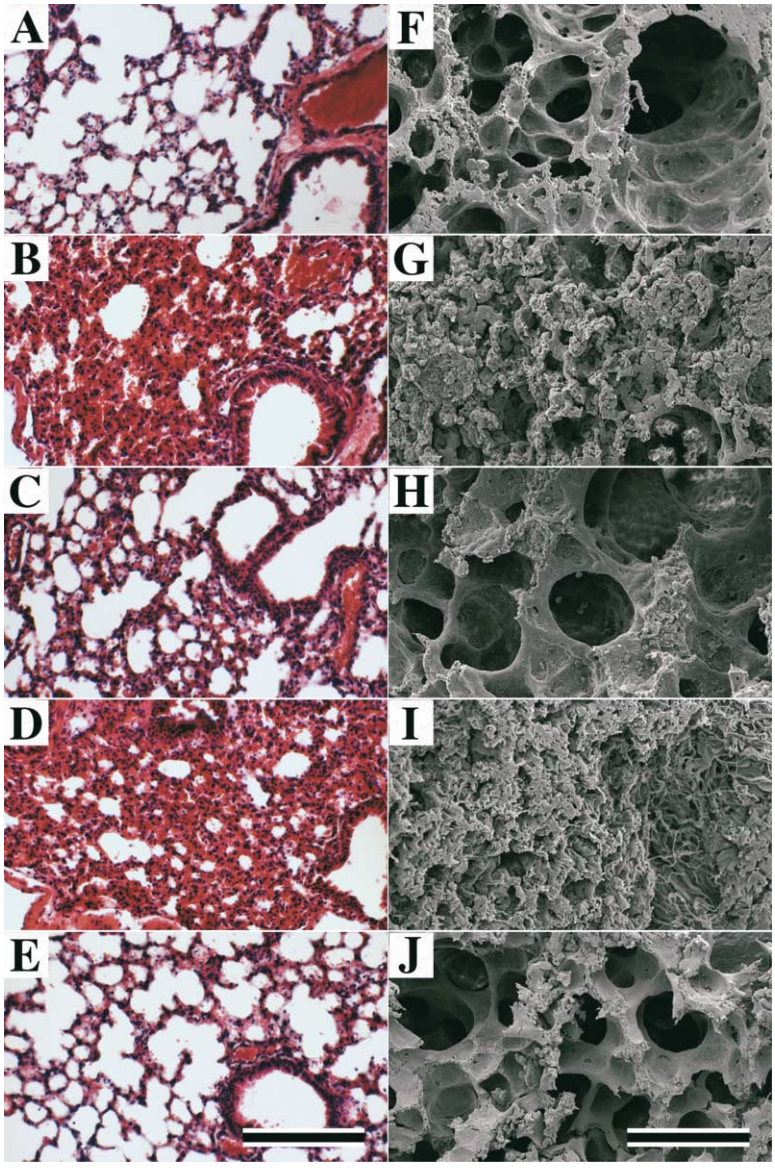



Figure 5. M1 Protein Induces Severe Lung Lesions in Mice

Light microscopy (A–E) and scanning electron microscopy (F–J) of murine lung tissue sections are presented. The figure shows representative micrographs of lungs from mice injected i.v. with buffer alone (A and F), M1 protein (B and G), M1 protein and peptide Gly-Pro-Arg-Pro (C and H), M1 protein and peptide Gly-His-Arg-Pro (D and I), or with protein H (E and J). Bars represent 50 μm (A–E) and 5 μm (F and J).

hemorrhage and the alveoli were less swollen in these mice (Figure 5E). In order to investigate lung tissue at higher magnification, tissue sections were also analyzed by scanning electron microscopy. Figure 5F shows a lung section from a PBS-treated mouse with no signs of pulmonary damage. However, injection of M1 protein resulted in severe leakage of erythrocytes as seen before, but also in the deposition of proteinous aggregates (Figure 5G). The morphology of the aggregates resembles the M1 protein-induced amorphous plasma precipitates seen in Figure 4C (left image). The lungs of mice injected with M1 protein and Gly-Pro-Arg-Pro contained no precipitates, but some alveolar swelling and minor leakage of erythrocytes were observed indicating an inflammatory reaction (Figure 5H). In contrast, treatment with Gly-His-Pro-Arg did not influence M1 protein-induced lung damage (Figure 5I). The injection of protein H did neither cause serious bleeding nor did the tissue appear to be inflamed (Figure 5J). The animal experiments suggest that M1 protein/fibrinogen complexes activate PMNs via the $\beta_2$ integrins, which causes massive vascular leakage and deposition of protein aggregates in the lung tissue. The results also show that the pathophysiological effect of the complexes is blocked when fibrinogen-induced crosslinking of $\beta_2$ integrins is prevented by the Gly-Pro-Arg-Pro peptide.

### The Gly-Pro-Arg-Pro Peptide Prevents Vascular Leakage and Lung Damage in Mice Infected with M1 Protein-Expressing *S. pyogenes* Bacteria

In a second series of animal experiments, nine mice were subcutaneously infected with M1 protein-expressing *S. pyogenes* bacteria in PBS. Three mice were treated with either peptide Gly-Pro-Arg-Pro or Gly-His-Arg-Pro as described in Experimental Procedures, while three mice received only the bacterial suspension. As a negative control, three mice were given a subcutaneous injection of PBS. Six hours after infection, animals were sacrificed, blood samples were taken, and lungs were removed and examined by scanning electron microscopy.

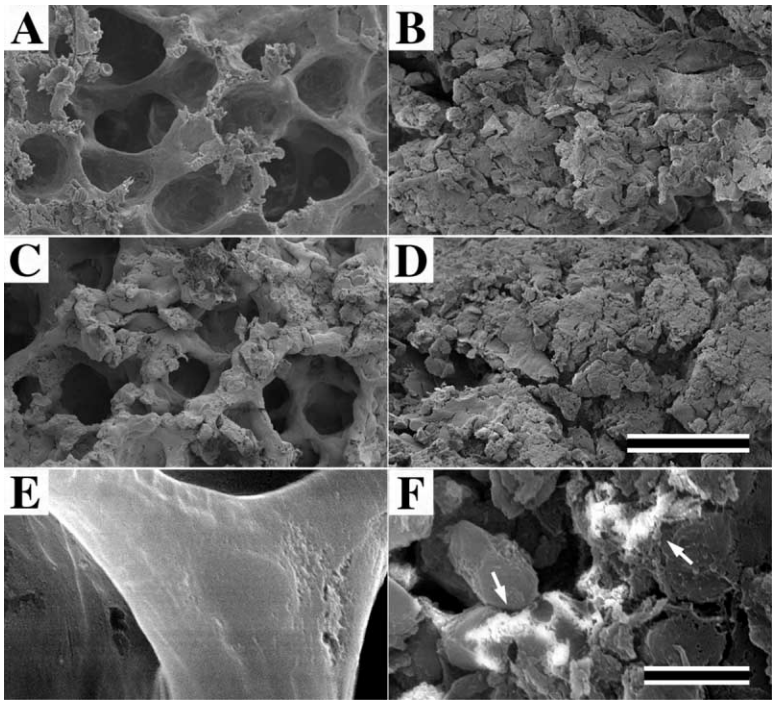



Figure 6. Analysis of Lung Tissue from Mice Subcutaneously Infected with *S. pyogenes*

Scanning electron microscopy of lung sections derived from mice subcutaneously injected with PBS (A), infected with *S. pyogenes* without treatment (B), or treated with Gly-Pro-Arg-Pro (C) or Gly-Pro-Arg-Pro (D). Immunodetection with anti-M1 protein antibodies in lung tissue from mice injected with PBS (E) or *S. pyogenes* bacteria (F). Bright areas containing gold-labeled antibodies are marked by arrows. Bars represent 5 μm (A–D) and 25 μm (E and F).

No bacterial growth was recorded in the blood samples from the animals, indicating that during this 6 hr period the bacteria had not yet disseminated from the local site of infection. Figures 6A–6D show electron micrographs of representative lung tissue sections from these animals. Recovered lungs from mice that received buffer alone (Figure 6A) showed no signs of pulmonary damage. However, mice that were infected with streptococci were suffering from severe lung lesions characterized by protein deposits (Figure 6B). When infected animals were treated with Gly-Pro-Arg-Pro, the lung tissue was much less affected (Figure 6C), whereas treatment with Gly-His-Arg-Pro failed to prevent pulmonary lesions (Figure 6D). Lung tissue from these mice was also analyzed by immunostaining and scanning electron microscopy using antibodies against M1 protein which were detected by gold labeled secondary antibodies. Figure 6F shows that M1 protein is present in the infiltrated tissue as indicated by colloidal gold staining of fibrin deposits, which appear as bright areas. In contrast, no M1 protein was detected in lungs from noninfected animals (Figure 6E). Finally, no bacteria were observed in the lungs of infected or noninfected mice. The results demonstrate that before the infection has disseminated from its local subcutaneous site, protein complexes containing M1 protein are transported to the lungs, where they induce severe vascular leakage.

### M1 Protein/Fibrinogen Precipitates Are Formed in a Patient with Necrotizing Fasciitis and Streptococcal Toxic Shock Syndrome (STSS)

STSS is a clinical condition associated with high morbidity and mortality (for a review, see Stevens, 2003). Cardinal symptoms of STSS are hypovolemic hypotension and respiratory failure. The syndrome is commonly preceded by a focal infection and in some cases by necrotizing fasciitis, a deep and extensive soft tissue infection resembling the subcutaneous murine model of infection described above. We therefore investigated whether M1 protein/fibrinogen complexes could be detected in biopsies from infected soft tissue obtained from a patient with necrotizing fasciitis and STSS. Tissue sections were analyzed by confocal immunofluorescence microscopy using antibodies against M1 protein and fibrinogen. Numerous streptococci are found at the epicenter of the infection, and although some M1 protein is still associated with the bacteria, most has been released into the tissue (Figure 7). Importantly, the shedded M1 protein colocalized with fibrinogen, demonstrating that the amount of M1 protein at the site of infection is sufficient to allow the formation of large M1 protein/fibrinogen complexes. M1 protein was not detected in biopsies from distal and noninfected tissue of this patient (data not shown). These observations in a human patient with severe streptococcal disease underline the potential pathophysiological significance of M1 protein/fibrinogen complexes generated at the infectious focus.

## Discussion

Over the last two decades a worldwide increase in severe infections caused by *S. pyogenes* has attracted considerable concern in the research community. The dramatic nature and the high mortality of these infections have also raised public awareness of these diseases. Severe invasive *S. pyogenes* infections are associated with disturbed microcirculation, hypotension, shock, and organ failure. Several virulence factors of *S. pyogenes*, including pyrogenic exotoxins A, B, and C, and more recently identified superantigens with strong mitogenic activity (for references, see review by Cunningham, 2000), contribute to the pathogenesis of this

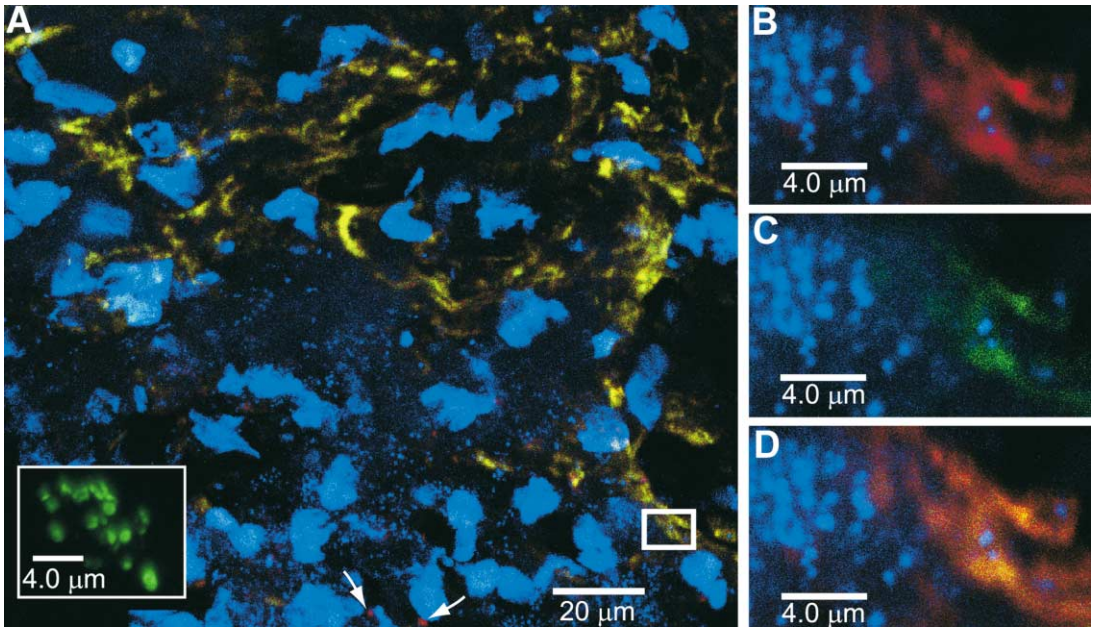



Figure 7. M1 Protein and Fibrinogen Colocalize at the Site of *S. pyogenes* Infection in Biopsies from a Patient with Necrotizing Fasciitis and Toxic Shock Syndrome

Tissue biopsies obtained from a patient with necrotizing fasciitis and STSS caused by an M1 strain, were stained for *S. pyogenes*, the M1 protein and fibrinogen, and analyzed by confocal microscopy as detailed in Experimental Procedures. The figure shows simulated maximum projections of a sequential scan. The M1 protein is shown in red, fibrinogen in green, and yellow stain illustrates areas with colocalized M1 and fibrinogen. Cellular infiltrates are indicated in blue by the nuclear stain DAPI.

(A) Extensive colocalization of M1 and fibrinogen is noted in biopsies collected at the epicenter of infection, which contain high amount of *S. pyogenes* as evident by staining for Lancefield group A carbohydrate (see insert). The arrows indicate M1 protein-coated streptococci of a size of 1–1.3μm. The area indicated by the white rectangle was analyzed at a higher magnification in B–D.

(B) Released M1 protein (red) localized next to an area of streptococci stained in blue by the DNA binding stain DAPI. The size of the blue-stained bacteria, 0.8 μm, corresponds to that reported for streptococci.

(C) Fibrinogen (green) and (D) colocalization of M1 and fibrinogen (yellow areas).

condition designated streptococcal toxic shock syndrome, STSS (Cone et al., 1987), by triggering the induction of proinflammatory cytokines. It was demonstrated that the propensity of the host to mount a high or low cytokine response to superantigens was directly and significantly correlated to the disease severity (Norrby-Teglund et al., 2000). Interestingly, STSS is associated with specific HLA class II haplotypes, explaining why some individuals develop lethal *S. pyogenes* disease, whereas other are barely affected when infected with the same streptococcal subclone (Kotb et al., 2002).

A multitude of host-microbe interactions determine the outcome of *S. pyogenes* infections and considerable scientific effort has been investigated to unravel the complexity of this molecular relationship. For instance, recent work has identified an important cytolysin-mediated secretion system in *S. pyogenes* (Madden et al., 2001), whereas another study described a sophisticated mechanism for tissue invasion by the bacteria through CD44-mediated cell signaling (Cywes and Wessels, 2001). As mentioned, the M protein is one of the classical virulence determinants of *S. pyogenes*. M proteins form α-helical coiled-coil dimers, which appear as hair-like structures at the bacterial surface. Based on extensive sequence variation in the $NH_2$-terminal region extending from the bacterial surface, isolates of *S. pyogenes* can be divided into more than 80 serotypes. In the absence of type-specific anti-M protein antibodies, M protein-expressing *S. pyogenes* survive and grow in human blood (Lancefield, 1969). In cases of STSS, the M1 serotype is the most prevalent, and a particular subclone of M1 has globally disseminated and been the dominating strain in these infections for more than twenty years (Davies et al., 1996). Moreover, patients dying from STSS caused by M1 strains were found to have low antibody titers toward the M1 protein (Holm et al., 1992). These observations all suggest that the M1 protein participates in molecular events, which contribute to this devastating clinical condition. However, it should be stressed that the overwhelming majority of *S. pyogenes* infections, including those caused by M1 strains, are noncomplicated throat and skin infections, indicating that several molecular events have to act in concert for STSS to develop.

The gene encoding the M1 protein has been sequenced in different M1 isolates and a comparison showed more than 95 percent sequence identity (Åkesson et al., 1994; Harbaugh et al., 1993). The binding of fibrinogen to different M1 strains underlines this structural conservation. Thus, when 43 M1 isolates were tested, including isolates from patients with STSS, all showed high binding of radiolabeled fibrinogen (L.B. and I.-M. Frick, unpublished data). The affinity between M1 protein and fibrinogen has been determined and the

equilibrium constant is high; $1.1 \times 10^{10}$ $M^{-1}$ (Åkesson et al., 1994). This fact and the presence of two fibrinogen binding so-called B domains in the M1 protein (Åkesson et al., 1994; Ringdahl et al., 2000), explain the rapid formation of M1 protein/fibrinogen complexes in plasma described here. Apart from isolates of the M1 serotype, M3 strains are the most common in patients with STSS, and it is notable that the M3 protein also has two repeats, which are homologous to the B domains of M1 protein, and that M1 and M3 strains bind fibrinogen to the same extent (L.B. and H.H., unpublished data).

In the present study, M1 protein/fibrinogen complexes were found to activate neutrophils leading to HBP release, presumably by crosslinking $\beta_2$ integrins. M proteins are typically associated with the bacterial cell wall, but at least in the case of M1 protein, several mechanisms have been reported that cause the release of the protein. For instance, *S. pyogenes* secretes a cysteine proteinase (SpeB), which efficiently solubilizes fibrinogen binding fragments of the M1 protein (Berge and Björck, 1995). Moreover, when grown under conditions not allowing the production of SpeB, substantial amounts (0.4–0.6 mg/l) of M1 protein can be isolated from the culture medium (Åkesson et al., 1994). Finally, this work demonstrates that PMN-derived proteinases cleave and release fibrinogen binding M1 protein fragments, and shedded M1 protein was also found in the infected soft tissue of a patient suffering from necrotizing fasciitis and STSS. Compared to most other human bacterial pathogens, *S. pyogenes* infections give rise to an intense local inflammation, which will attract PMNs and induce plasma exudation at the site of infection. This will in turn, promote the formation of M1 protein/fibrinogen complexes and further enhance the local inflammatory response. Via the bloodstream such complexes and/or bacteria with surface bound fibrinogen could be deposited in various organs and induce plasma leakage by local activation of PMNs and release of HBP.

Increased vascular permeability is a fundamental pathophysiological mechanism in shock. In STSS, the leakage of plasma from the capillaries into the tissue is excessive and these patients require massive amounts (10–20 liters/day) of intravenous fluids. Several previous investigations have shown (for a review, see Edens and Parkos, 2003) that activated PMNs induce vascular leakage, and a recent study demonstrated that among the various substances released by activated PMNs, HBP is entirely responsible for this effect (Gautam et al., 2001). As shown here, crosslinking of $\beta_2$ integrins by M1 protein/fibrinogen complexes, results in the activation of PMNs followed by the release of HBP. Moreover, after i.v. injection of M1 protein into mice and subcutaneous infection with *S. pyogenes*, microscopical analysis revealed the presence of blood cells and protein aggregates in the lung tissue. This resembles a mechanism referred to as "frustrated phagocytosis" where PMNs are activated at a surface, or they are attempting to phagocytose a particle that is too large. As a consequence, these immobilized and activated PMNs will release leakage-inducing HBP and other proinflammatory mediators, such as oxidative metabolites and proteolytic enzymes, which will further increase the tissue damage. The significance of PMNs in the development of STSS is further underlined by the rapid turnover of these cells in STSS patients, who exhibit a strikingly high percentage (40%–50%) of immature PMNs (Stevens, 1995).

Intravenous injection will target a substance to the lungs, and small amounts of M1 protein administrated i.v. to mice induced severe lung lesions in the animals. Observations made in our murine model of subcutaneous *S. pyogenes* infection, demonstrate that M1 protein is present in the lungs and that vascular leakage is induced before the bacteria can be found in the bloodstream or in the lungs. This and the finding that M1 protein/fibrinogen complexes are detected in infected tissue from a patient with necrotizing fasciitis, further support a role for these protein complexes in the induction of pulmonary vascular leakage. Endothelial cells lining the vasculature form a fragile barrier between the blood and the interstitium of the lung. During an inflammatory disease state, extravascular leakage of fluids and macromolecules, fibrin deposition, and infiltration of red and white blood cells, can result in a functional reduction of the alveolar space, eventually leading to respiratory failure. In particular, activated PMNs are believed to contribute to the leakage and vascular injury in this condition, which in severe cases is connected with high mortality rates. In relation to the M1 protein-induced lung lesions described here, it is noteworthy that severe pulmonary hemorrhages have been reported in patients with fulminant *S. pyogenes* infections (Ooe et al., 1999), and that respiratory failure is a major and common complication in cases of STSS (Stevens, 1995).

The formation of M1 protein/fibrinogen complexes, their interaction with $\beta_2$ integrins resulting in the activation of PMNs, and the subsequent release of leakage-inducing HBP, represent a chain of events which may explain the extremely rapid progress of STSS and the severity of the syndrome. The identification of this potentially important pathophysiological pathway and the observation that a peptide interfering with the interaction between fibrinogen and $\beta_2$ integrins, prevents M protein-induced lung lesions, suggest that the data described here could be helpful in the development of therapeutic strategies in STSS. The fact that 30–70 percent of patients suffering from this condition die in spite of aggressive modern therapy (Stevens, 1995), emphasizes the urgent need for more efficient treatments.

## Experimental Procedures

### Reagents

Neutrophil Isolation Medium (NIM) was purchased from Cardinal Associates Inc. (Santa Fe, NM). RPMI 1640 medium with Glutamax I, Minimum Essential Medium (MEM) with Earle's salts and L-glutamine, fetal bovine serum, and penicillin/streptomycin solution were purchased from Life Technologies (Täby, Sweden). Ionomycin and formyl-methionyl-leucyl-phenylalanine were obtained from Calbiochem (La Jolla, CA). The acetoxymethyl ester of N,N′-(1,2-ethanediylbis(oxy-2,1-phenylene))bis(N-(carboxymethyl)) (BAPTA), and ProLong Antifade Kit were from Molecular Probes (Eugene, OR). 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) was from Merck (Whitehouse Station, NJ). Streptococcal cysteine proteinase (SpeB) zymogen was purified as described earlier (Berge and Björck, 1995). Recombinant M1 protein, fragments A-S and S-C3, and protein H were expressed in *E. coli* and purified (Åkesson et al., 1994), and recombinant human HBP was also produced and purified as previously described (Rasmussen et al., 1996). Lipoteichoic acid (LTA), hyaluronic acid (HA), and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Mouse mAB

2F23C3 and rabbit antiserum (409A) to recombinant HBP were prepared and purified as described earlier (Lindmark et al., 1999) and peroxidase-conjugated goat antirabbit IgG was from Bio-Rad Laboratories (Richmond, CA). Peptides Gly-Pro-Arg-Pro and Gly-His-Arg-Pro were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Fluanison/fentanyl and midazolam were from Janssen Pharmaceutica, Beers, Belgium and Hoffman-La Roche, Basel, Switzerland.

### Cell Culture, Neutrophil Isolation, and Stimulation of Cells

Human PMNs were isolated from fresh heparinized blood of healthy volunteers using NIM, a single step density gradient medium, according to the instructions supplied by the manufacturer. Neutrophil proteinase release was induced by PMN activation through antibody crosslinking of CD11b/CD18 as described previously (Gautam et al., 2000).

### Bacterial Strains

The AP1 strain used in this study is the 40/58 *S. pyogenes* strain from the WHO Collaborating Centre for references and Research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czech Republic. The MC25 strain is an isogenic AP1 mutant strain, devoid of surface-associated M1 protein (Collin and Olsén, 2000).

### Enzymatic Treatment of *S. pyogenes*

*S. pyogenes* bacteria (strain AP1) were grown in Todd-Hewitt broth (Difco, Detroit, MI) at 37°C for 16 hr and harvested by centrifugation at 3000 × g for 20 min. The bacteria were washed twice in PBS and resuspended in PBS to $2 \times 10^{10}$ cells/ml. Various amounts of secretion products from PMNs were added to bacterial suspensions followed by incubation for 2 hr at 37°C. Bacteria were spun down at 3000 × g for 20 min, and the resulting pellets and supernatants were saved. Digestions were terminated by the addition of SDS sample buffer containing 5% 2-mecaptoethanol.

### SDS-Polyacrylamide Gel Electrophoresis, Western Blotting, and Immunoprinting

Proteins were separated by 12.5% (w/v) polyacrylamide gel electrophoresis in the presence of 1% (w/v) SDS (Laemmli, 1970). Proteins were transferred onto nitrocellulose membranes for 30 min at 100 mA. The membranes were blocked with PBS containing 5% (w/v) dry milk powder and 0.05% (w/v) Tween-20, [pH 7.4]. Membranes were probed with a polyclonal antibody against M1 protein, diluted 1:50,000 in the blocking buffer, was used. Bound antibodies were detected using peroxidase-conjugated secondary antibodies against rabbit IgG (dilution 1:3000) followed by a chemiluminescence detection method. Alternatively, membranes were blocked, incubated with fibrinogen (2 μg/ml), followed by immunodetection with rabbit antibodies to fibrinogen (1:1000) and peroxidase-conjugated secondary antibodies against rabbit immunoglobulin (1:3000 diluted).

### Determination of HBP

100 μl human blood were diluted in PBS to a final volume of 1.0 ml and incubated with various PMNs-activating components for 30 min at 37°C. Cells were centrifuged (300 × g for 15 min) and the resulting supernatants were analyzed by sandwich ELISA. In order to quantify the total amount of HBP in blood, cells were lysed with 0.02% (v/v) Triton X-100, and pelleted as described above. The concentration of HBP in neutrophil exudates was determined by a sandwich ELISA (Tapper et al., 2002).

### Precipitation Assay

Radiolabeled M1 protein ($^{125}$I-M1 protein), approximately 10,000 cpm, was incubated for 30 min with various amounts of nonradiolabeled M1 protein in PBS containing 10% plasma or 300 μg/ml fibrinogen. After centrifugation, the pellets were subjected to γ-counting.

### Scanning Electron Microscopy

Specimen samples were fixed as described (Herwald et al., 2003). For immunolabeling, samples were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in cacodylate buffer for 2 hr at room temperature, washed and preincubated with 0.5% BSA in the same buffer for 1 hr at room temperature. Specimens were subsequently washed, incubated with an antiserum against M1, washed, incubated with a secondary antibody (antirabbit IgG) coupled to 10 nm colloidal gold, followed by a final washing and then fixation step with 2.5% glutaraldehyde in cacodylate buffer. After fixation, samples were washed, dehydrated, critical point dried, sputtered with palladium/gold as described earlier (Herwald et al., 2003). Samples were examined in a Jeol JSM-350 scanning electron microscope. Alternatively, samples for immunolabeling were coated with a thin layer of carbon instead of palladium/gold prior to scanning electron microscopy.

### Animal Experiments

Adult female mice (approximately 20 g) of the Balb/c strain were used. Animals were anaesthetized as described (Persson et al., 2003). Mice were given an intravenous injection of 100 μl of a solution containing 150 μg/ml M1 protein. Alternatively, 100 μl of solutions containing 150 μg/ml M1 protein plus 4 mg/ml Gly-Pro-Arg-Pro or Gly-His-Arg-Pro were injected intravenously. As a control, vehicle alone was injected. Thirty min after injection, mice were sacrificed and the lungs were removed. Alternatively, 100 μl of a bacteria solution ($2 \times 10^9$ AP1 bacteria/ml PBS in the presence or absence of 400 μg Gly-Pro-Arg-Pro or Gly-His-Arg-Pro) were injected together with 0.9 ml of air into the dorsal region of the mouse. After 30 min, mice were given an intraperitoneal injection of 100 μl of a solution containing PBS or 2 mg/ml Gly-Pro-Arg-Pro or Gly-His-Arg-Pro, respectively. Six hours after infection, mice were sacrificed and the lungs were removed.

### Histochemistry

Mice were sacrificed, lungs rapidly removed and fixed at 4°C for 24 hr in buffered 4% formalin (pH 7.4; Kebo). Tissues were dehydrated and imbedded in paraffin (Histolab Products AB), cut into 4 μm sections, and mounted. After removal of the paraffin, tissues were stained with Mayers hematoxylin (Histolab Products AB) and eosin (Surgipath Medical Industries, Inc.).

### Immunofluorescence and Confocal Microscopy

Snap-frozen biopsies of tissue, collected either from the epicenter of infection (fascia) or from a distal site with no evidence of inflammation (muscle), from a patient with necrotizing fasciitis and toxic shock syndrome caused by an M1T1 *S. pyogenes* strain (kindly provided by Prof. Donald E. Low, Mount Sinai Hospital, Toronto, Canada) were cryosectioned, fixed, and immunostained as previously described (Norrby-Teglund et al., 2001). Staining for the M1 protein was achieved by incubation with a polyclonal rabbit antiserum against M1 (diluted 1:10 000), double staining for fibrinogen was obtained through direct labeling of purified rabbit antihuman fibrinogen antibodies diluted to a concentration of 3 mg/ml (Dakocytomation) by Zenon Alexa fluor 532 IgG labeling kit (Molecular Probes). A polyclonal rabbit antiserum against the Lancefield group A carbohydrate was used to detect *S. pyogenes* (Norrby-Teglund et al., 2001). Single stainings were performed to assure specificity of staining patterns. For evaluation, the Leica confocal scanner TCS2 AOBS with an inverted Leica DMIRE2 microscope was used.

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