Neutrophil degranulation mediates severe lung damage triggered by streptococcal M1 protein

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Short title: PMN degranulation initiates lung damage

List of abbreviations: BAL, bronchioalveolar lavage; HBP, heparin binding protein; MCP-1, monocyte chemotactic protein 1; MMP-9, matrix metalloproteinase 9; MPO, myeloperoxidase; STSS, streptococcal toxic shock syndrome.
Abstract

*Streptococcus pyogenes* of the M1 serotype can cause streptococcal toxic shock syndrome commonly associated with acute lung injury. The aim of the study was to investigate the role of neutrophils and their secretion products in M1 protein-induced lung damage.

The degranulation of neutrophils by M1 protein was studied in whole blood using marker analysis for individual granule subsets. In mice, M1 protein was injected intravenously and the lung damage was assessed by histology, electron microscopy, cell count in the bronchoalveolar lavage fluid and analysis of lung vascular permeability. Comparisons were made in mice with intact white blood count, neutropenic mice and neutropenic mice injected with the secretion of activated neutrophils.

In whole blood, M1 protein forms complexes with fibrinogen which bind to β2-integrins on the neutrophil surface resulting in degranulation of all four subsets of neutrophil granules. Intravenous injection of M1 protein into mice induced neutrophil accumulation in the lung, increase in vascular permeability and acute lung damage. Depletion of neutrophils from the circulation completely abrogated lung injury and vascular leakage. Interestingly, the lung damage was restored by injecting neutrophil secretion.

Our data suggest that neutrophil granule proteins are directly responsible for lung damage induced by the streptococcal M1 protein.

Key words: lung injury, M1 protein, neutrophil granule proteins, *streptococcus pyogenes*
Introduction

Polymorphonuclear leukocytes (PMN) are the earliest immune cells to be recruited to the site of injury or infection and release a wide array of granule proteins which contribute to host defence and tissue repair [1]. However, in some situations there is a misdirected activation of the immune system which may in itself give rise to host tissue damage. For example, bacterial infections and septicaemia involve immune cell activation that could potentially lead to lung injury. The contribution of PMN to the vascular dysfunction in response to bacterial infections is controversial, and may depend on the specific pathogen involved. However, in experimental mouse models of septicaemia, neutropenic mice often show reduced lung damage compared to normal mice, and inhibitors to neutrophil components or knock-out mice of PMN granule proteins point at the involvement of PMN and their secretion products in the initiation and progress of the lung injury [2, 3].

*Streptococcus pyogenes* is a significant human pathogen causing a wide panoply of diseases, from uncomplicated infections to life-threatening conditions such as the streptococcal toxic shock syndrome (STSS), characterized by hypotension and multiple organ failure. The M protein is a major surface protein and, due to its anti-phagocytic function, virulence factor of *S. pyogenes*. Of the over 80 serotypes, the M1 serotype is predominantly associated with the fatal STSS [4]. We have recently reported that M1 protein released from the bacterial surface - spontaneously or by the action of proteases [5] - forms complexes with fibrinogen, which activate PMN to liberate heparin-binding protein (HBP). Notably, our previous studies have shown that HBP is a crucial mediator of PMN-induced permeability increase in inflammation [6] and hence, it is tempting to speculate that HBP is critically involved in the *S. pyogenes* induced lung injury. Recent studies revealed further virulence mechanisms
of M1 protein, all of which may contribute to the lung damage observed in the STSS. These mechanisms include cytokine [7] and tissue factor [8] release from monocytes as well as chemokine expression in epithelial cells [9]. Moreover, M1 protein was shown to induce activation of T cells [10] and platelets [11] which results in thrombus formation.

This study was undertaken to investigate the importance of the PMN activation in response to M1 protein/fibrinogen complexes in the pathogenesis of M1 protein-induced lung damage. Our data indicate direct proof for the almost exclusive role of PMN degranulation in the onset of M1 protein-mediated lung injury which may serve as primary therapeutical target.
Materials and Methods

PMN activation by M1 protein

M1 protein (1 µg/ml) generated as described [5] was added to human whole blood in the presence or absence of the peptides Gly-Pro-Arg-Pro or Gly-His-Arg-Pro (1 mM, Bachem) or the CD18 antibody IB4 (10 µg/ml). Some samples were treated with protein H (1 µg/ml) from *S. pyogenes* instead of M1 protein. After incubation at 37 °C for 30 min the samples were centrifuged (300 g, 15 min) and the supernatant was analysed for MPO and MMP-9 as stated below. PMN in the pellet were stained with antibodies to CD16 (marker for secretory vesicles, BD), CD11b (marker for secretory vesicles and tertiary granules, Pharmingen), CD66b (marker for secondary granules, Immunotools), and CD63 (marker for primary granules, Eurobiosciences) and analysed by FACS.

Animal experiments

Balb/c mice of either sex (approx. 20 g) were used for in vivo experiments. Mice were anesthetized with Isoflurane inhalation followed by ketamine/xylazine i.p., and a catheter was placed in the left jugular vein. Mice were intravenously injected with 15 µg M1 protein in PBS, and were sacrificed 30 min or 4 h later by an overdose of anaesthesia. PMN depletion was induced through i.p. treatment with mAb RB6-8C5 (250 µg/mouse) 12 h before injection of M1 protein [12]. Neutropenia was confirmed on the day of experiment by manual blood count. Monocytes were eliminated 18 h prior to experimentation by i.v. injection of 0.2 ml of clodronate liposomes into the lateral tail vein, as described previously [13]. Clodronate was a gift from Roche (Mannheim) and was incorporated into liposomes as previously described.
Depletion of monocytes was monitored by FACS analysis using antibodies to Gr1 and F4/80. To substitute for PMN granule products in neutropenic animals, 300 µl of human PMN secretion were injected in parallel with M1 protein. This number was based on the assumption that the average mouse blood contains about $3 \times 10^6$ PMN which would be activated in the presence of M1 protein.

In separate experiments, M1 protein/fibrinogen precipitate was formed ex vivo and injected intravenously. 20 µg M1 protein were added to 6 mg fibrinogen in distilled water and incubated for 10 min. After centrifugation, the pellet was resuspended in 100 µl PBS and injected into the jugular vein. Mice stimulated in this way were subjected to similar treatments as those directly injected with M1 protein. All animal experiments were approved by the local ethical committee for animal experimentation.

PMN secretion

Human PMN were isolated from fresh blood of healthy donors using polymorphprep (Nycomed Pharma) according to the manufacturer’s instructions. PMN were resuspended in DMEM medium at $10^6$/ml and PMN secretion was obtained by antibody cross-linking of CD18 as described before [15].

In separate experiments murine whole blood was obtained by cardiac puncture and PMN were isolated using NycoPrep Animal (Nycomed Pharma). PMN were resuspended in DMEM and incubated for 30 min at 37 °C. Cells were spun down and the supernatant was used as ctrl secretion. Thereafter, PMN were again resuspended in DMEM and incubated with M1 protein/fibrinogen precipitate. After 30 min, PMN were spun down and the supernatant was used as M1 secretion.
Bronchioalveolar lavage (BAL) and vascular permeability assay

After exsanguination via *V. cava inferior*, the trachea was catheterised and the left lung was lavaged three times with 500 µl PBS. Leukocytes in the BAL fluid were manually counted, and the protein concentration was assessed using a standard protein assay (BioRad). BAL protein concentration and the wet weight/dry weight ratio were used as indicators of plasma exudation. To obtain the wet weight/dry weigh ration, excised lungs were weight, dried over night at 60 °C, and weight again. In mice subjected to injection of preformed M1 protein/fibrinogen precipitate, Evans Blue (EB) dye was used to assess vascular leakage [16]. EB (50 mg/kg) was administered i.v. and dye extravasation used to assess change in vascular permeability. At the end of the experiment, the pulmonary circulation was flushed with PBS and EB was extracted from homogenized lung tissue by incubating in formamide for 24h at 60 °C. The optical density of the supernatant and of serum was measured at 620 nm and EB-albumin extravasation was expressed as µl serum equivalents per g lung tissue.

Histology and electron microscopy

After completion of the experiment, one part of the right lung was fixed in formalin, embedded in paraffin and stained with Mayer’s hematoxylin and eosin for histological examination [5]. Another part of the lung was prepared for scanning electron microscopy as described [5].

Analysis of PMN degranulation

The release of the primary granule specific enzyme MPO from PMN after incubation of whole blood with M1 protein was quantified as previously described by Suzuki *et al.* [17].
Qualitative release of the secondary and tertiary granule enzyme MMP-9 from PMN was analysed by Western Blot as described before [18]. MMP-9 activity was quantified using the SensoLyte MMP-9 assay kit (Anaspec). The fluorescent product was measured at 520 nm using a fluorescence plate reader (Fluoroskan Ascent, Labsystems).

Statistics

All statistical calculations were performed using Statistica 8.0 (Statsoft Inc., Tulsa, OK, USA). Data were analyzed with one-way analyzes of variance (ANOVA) followed by Tukey’s HSD test if the overall $F$-ratio was significant. The results are presented as individual values or means and standard deviation. A $P$-value < 0.05 was considered significant.
Results

*M1 protein/fibrinogen complexes induce degranulation of PMN*

Previous work has shown that, M1 protein, when added to human blood, forms complexes with fibrinogen [5]. These complexes in turn are capable of activating PMN and thereby induce the release of granule proteins from internal stores [5]. To assess whether all four PMN granule subsets are liberated in response to M1 protein, we incubated human whole blood with M1 protein. Mobilization of PMN granules was recorded by FACS analysis, gating on the PMN in the forward/side scatter allowing the specific analysis of upregulation of marker proteins of granule subsets. In the presence of M1 protein we found an upregulation of CD16, Cd11b, CD66b, and CD63 indicative of the mobilization of secretory vesicles, tertiary, secondary, and primary granules. Similarly, we could detect a strong increase in the primary granule marker protein MPO, and the secondary and tertiary granule marker MMP-9 in the plasma (Figure 1 A-C). Fibrinogen binds to PMN via ß2-integrins and it has been shown that the Gly-Pro-Arg-Pro peptide effectively blocks adhesion of activated PMN to fibrinogen [19]. Thus, experiments were performed in the presence of the Gly-Pro-Arg-Pro peptide or the control peptide Gly-His-Arg-Pro. We found that this treatment blocks the release all marker proteins from M1 protein-stimulated PMN (Figure 1 A-C), while the control peptide had no effect. The ß2-integrin antibody IB4 had similar effects as the Gly-Pro-Arg-Pro further supporting the crucial role of ß2-integrins in PMN degranulation. *S. pyogenes* not only sheds M1 protein but also other surface proteins. We investigated the specificity of the proposed mechanism and incubated whole blood with protein H, another surface protein
isolated from the M1 serotype of *S. pyogenes*. However, protein H did not result in release of MPO or MMP-9 from PMN (Figure 1 B,C).

*Intravenous injection of M1 protein into mice causes neutrophil-dependent lung damage*

To characterize the role of PMN activation in the responses to M1 protein/fibrinogen complexes *in vivo*, mice were injected with M1 protein i.v. (15 µg/mouse) and followed for 30 min. After exsanguination, the lungs were removed and analysed by light microscopy and scanning electron microscopy. Compared to mice treated with vehicle only, M1 protein injection induced severe lung damage, depicted by haemorrhage, deposition of fibrinogen aggregates, and swelling of the alveolar membrane (Figure 2). Moreover, bronchioalveolar lavage (BAL) was carried out to monitor the inflammatory response. To this end, when BAL was analyzed with respect to leukocyte and protein content, the number of cells in the BAL fluid, most of which were PMN, was clearly increased 30 min after injection of M1 protein. This suggests an involvement of these cells in the response to M1 protein (Figure 3 A). Moreover, we assessed the protein concentration in the BAL fluid and the wet weight/dry weight ratio of the lungs. Both parameters, when enhanced, indicate an increase in vascular permeability in the pulmonary circulation. Intravenous injection of M1 protein significantly increased these two values (Figure 3 B/C). Thus, the extravasation of PMN to the alveolar space was associated with leakage of plasma from the lung vasculature which conforms with the documented link between neutrophil recruitment to inflammatory loci and increase in vascular permeability [20].
In the next series of experiments we wished to analyze the contribution of PMN to M1 protein-induced lung damage by removing PMN from mice upon i.p. treatment with mAb RB6-8C5. Antibody injection resulted in a total neutropenia (<500 cells/µl and less than 20% of basal PMN count) which was sustained throughout the experimental procedure. In PMN-depleted mice, no destruction of the lung tissue was seen after M1 protein injection (Figure 2). Moreover, the lung vascular permeability was not significantly altered as compared with the control mice (Figure 3 B/C). Similar results were found when mice were treated with M1 protein for 4 h (Figure 3 D-F), suggesting that PMN may not only be involved in the immediate response to M1 protein but also in the sustained lung destruction. These relationships were further established in a second set of experiments where preformed M1 protein/fibrinogen complexes were injected. Injection of the M1 protein/fibrinogen precipitate resulted within 30 min in enhanced vascular permeability. As for the response to injection of M1 protein alone, this permeability increase is dependent on the presence of PMN as enhanced protein extravasation was largely prevented in PMN depleted mice (Figure 4). Interestingly, it is suggested from these data that the M1 protein/fibrinogen complex rather than M1 protein in itself is central to the pathogenesis of the vascular derangement following S. pyogenes infection. Collectively, these observations clearly imply an imperative role of PMN in the lung injury caused by M1 protein.

It has previously been shown that M1 protein activates monocytes to express pro-inflammatory cytokines and tissue factor. To address the possible contribution of monocytes in the M1 protein-induced lung damage, we depleted monocytes by intravenous application of clodronate liposomes. Instillation of M1 protein in these
mice did not reduce the lung damage compared to mice with intact white blood count, pointing at a minor contribution of monocytes (Figures 2-3).

*PMN contribute to lung injury via release of PMN granule proteins*

PMN may contribute to lung tissue dysfunction and altered vascular permeability through different mechanisms, e.g. release and production of cytokines [21], generation of reactive oxygen species (ROS) [22], or exocytosis of preformed granule proteins [3, 6]. Since our results demonstrate that the M1 protein/fibrinogen complex is a powerful inducer of PMN degranulation it was of interest to investigate the impact of PMN secretion products on lung vascular function. PMN secretion (300 μl/mouse) obtained from human PMN after antibody cross-linking of CD18 was injected i.v. into neutropenic mice together with M1 protein or M1 protein/fibrinogen precipitate. Injection of the PMN secretion caused a similar deleterious lung injury and enhanced permeability in neutropenic mice as seen after injection of M1 protein or M1 protein/fibrinogen precipitate in mice with intact WBC (Figures 2-4). A similar response was found when PMN secretion was injected in the absence of M1 protein (Figures 2-3). In further experiments we injected murine PMN secretion obtained from isolated blood PMN activated with M1 protein/fibrinogen complexes. The murine PMN secretion induced vascular leakage to a similar extent as the human secretion (Figure 5). Since injection of the PMN secretion mimicked the lung damage induced by M1 protein completely it is suggested that PMN degranulation constitutes a final critical step within the chain of events triggered by M1 protein that eventually leads to lung injury. Western blot analysis revealed the presence of elastase, LL-37, MMP-9, and albumin indicative of the release of primary, secondary, and tertiary granules as well as secretory vesicles in the PMN secretion [23]. We were unable to
detect ROS in the PMN secretion which is also to be expected in view of the short life-span of these elements. Cytokines and chemokines are not stored in PMN granules [24], and thus, are not supposed to be present in the PMN secretion. In line with this, we were unable to detect TNF, IFN\(\gamma\), IL-8 or MCP-1 in the PMN secretion used (data not shown). On the other hand, once the PMN have extravasated a second burst of transcriptional activity is launched resulting in production of IL-1, TNF, interleukin-8, and MCP-1 [25].
Discussion

*S. pyogenes* of the M1 serotype is commonly associated with large outbreaks of invasive streptococcal infections and the development of the STSS. The fatal outcome of the STSS is mainly based on the establishment of an acute lung damage characterized by severe oedema formation. We have previously reported that M1 protein shed from the surface of *S. pyogenes* forms complexes with fibrinogen which induce the activation of PMN [5]. Shortly after, several other virulence mechanisms of M1 protein were reported, which may be involved in the pathogenesis of the acute lung damage in STSS. These comprise the activation of monocytes, T cells, and platelets as well as the secretion of chemokines, cytokines and tissue factor [7-11]. Our study, however, points at an almost exclusive role of the intravascular activation of PMN and the subsequent discharge of granule proteins in the onset of the M1 protein-induced lung oedema and lung damage.

Several granule proteins have been suggested to be critically involved in the progression of the acute lung injury, among them proteases (elastase, gelatinase, cathepsin G, proteinase-3) and defensins [2]. The use of neutralizing agents and gene-targeted mice has indicated the individual contribution of these to the pathology of acute lung injury [26, 27]. Our more general approach clearly demonstrates the significance of granule release in the pathophysiology of the acute lung injury. Similarly interesting is the finding that the M1 protein/fibrinogen complexes activate PMN intravascularly so that a direct interaction between PMN and the endothelium is not necessary for the development of the lung injury.

We have previously shown that intravenous injection of the tetrapeptide Gly-Pro-Arg-Pro ameliorates the M1 protein-induced lung damage [5], identifying PMN β2-integrins as a possible target for interventions in fatal group A streptococcal infections.
infections. However, blockade of integrin function has been largely disappointing in trials in patients exhibiting various forms of inflammatory disease [28]. This study puts neutrophil degranulation as a potential therapeutic target in perspective. Inhibition of neutrophil granule exocytosis may not only interfere with PMN extravasation, but also influence vascular leakage and the second wave of inflammatory cell invasion [29], and thereby improve the outcome of a patient. Recently, a novel inhibitor of degranulation based on interference with myristoylated alanine-rich C kinase substrate has been developed with promising results in vitro [30, 31] and further investigations are needed to proof the effectiveness in vivo. In respect to severe infections with *S. pyogenes*, it is noteworthy to mention that leakage of plasma from the bloodstream into the tissue induces a life-threatening hypovolemic hypotension combined with high morbidity and mortality. Thus, the present study suggests that substances neutralizing the effect of HBP or preventing PMN degranulation are an interesting target for drug development. 

Taken together, our findings demonstrate that complexes formed by M1 protein shed from *S. pyogenes* and fibrinogen stimulate circulating PMN to degranulate. This response induces a rapid increase in lung vascular permeability, haemorrhage, and deposition of fibrinogen precipitates reflecting the whole picture of acute lung damage. Similar pathophysiological connections may exist where intravascular activation of PMN is associated with acute lung damage, such as disseminated intravascular coagulation. These data point at the powerful pernicious effect of PMN granule proteins in the early stages of acute lung injury and may not only provide mechanistic insight but also stimulate therapeutic approaches that target PMN activation and degranulation rather than individual granule components.
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References


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Figure 1

**M1 Protein activates PMN in whole blood via binding to β2 integrin.** Whole blood was incubated with M1 protein (1 µg/ml) in the presence or absence of the peptide Gly-Pro-Arg-Pro (1 mM), Gly-His-Arg-Pro, or mAb IB4 (10 µg/ml) for 30 min. Alternatively, whole blood was incubated with protein H (1 µg/ml). The mobilization of PMN granules was analyzed by surface expression of CD16 (secretory vesicles), CD11b (secretory vesicles and tertiary granules), CD66b (secondary granules), and CD63 (primary granules) after gating on the PMN fraction in the FSC/SSC plot (A). Histograms are representative of three independent experiments. Release of the primary granule marker MPO (B) was assessed by spectrophotometric analysis, and release of the secondary and tertiary granule marker MMP-9 was analysed by fluorometric analysis (C). * indicates significant difference compared to control and to Gly-Pro-Arg-Pro-treated. Data are expressed as mean ± SD. n = 3 for each bar.

Figure 2

**M1 protein-induced lung damage in mice is mediated by PMN granule proteins; morphological data.** Light microscopy (A-F) and scanning electron microscopy (G-L) of murine lung sections are exemplified. The figure shows representative micrographs of lungs from mice injected i.v. with buffer alone (A, G) or M1 protein (30 min, 15 µg) (B, H). In addition, animals were rendered neutropenic (C, I) and injected with PMN secretion (D, J) prior to injection of M1 protein. Moreover, mice were depleted of monocytes before injection of M1 protein (E, K). Finally, mice received PMN secretion (F, L). Bars represent 250 µm for light microscopy and 25 µm for scanning electron microscopy.
Figure 3

M1 protein-induced lung damage in mice is mediated by PMN granule proteins; analysis of BAL fluid and vascular leakage. Mice with intact white blood count were injected i.v. with buffer (ctrl), M1 protein, or PMN secretion. In separate experiments, mice were depleted of PMN (PMN depl) or monocytes (Mono depl) prior to injection of M1 protein, in some experiments also combined with injection of PMN secretion. After 30 min (A-C) or 4 h (D-F) the mice were sacrificed. Charts show individual data points and the horizontal bars indicate the average. A/D: Number of PMN in the cytospin of BAL fluid. B/E: Lung wet weight/dry weight ratio. C/F: Protein concentration in BAL fluid. * indicates significant difference between the groups.

Figure 4

Injection of M1 protein/fibrinogen precipitates causes PMN-dependent increase in vascular permeability in the lung. Complexes of M1 protein and fibrinogen were prepared ex-vivo and injected into mice with intact WBC, neutropenic mice, or neutropenic mice injected with PMN secretion. Vascular protein leakage was assessed by Evans blue dye extravasation 30 min after injection of M1 protein/fibrinogen complexes. Data are presented as mean±SD. Each bar represents 6 mice. * indicates significant difference between the groups.

Figure 5

Murine PMN secretion obtained from PMN activated with M1 protein/fibrinogen precipitates induces lung damage. Supernatant from non-
activated murine PMN or from murine PMN activated with M1 protein/fibrinogen complexes was injected i.v. into mice. After 30 min the mice were sacrificed. Charts show individual data points and the horizontal bars indicate the average. A: Lung wet weight/dry weight ratio. B: Protein concentration in BAL fluid. * indicates significant difference between the groups.
Figure 1

A

CD16

CD11b

CD66b

CD63

ctrl  M1  M1 + Gly-Pro-Arg-Pro  M1 + Gly-His-Arg-Pro
Figure 4

[Bar graph showing Evans blue levels in different conditions: ctrl, PMN depl, PMN depl + PMN sec. M1/fibrinogen precipitate is on the x-axis, and Evans blue levels are on the y-axis. Asterisks indicate significant differences.]
Figure 5

A. Wet weight/dry weight

B. Protein [μg/ml]

* indicates statistical significance between ctrl secretion and M1 secretion.