Characterization of a New Mouse Model of Empyema and the Mechanisms of Pleural Invasion by *Streptococcus pneumoniae*

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Although empyema affects more than 65,000 people each year in the United States and in the United Kingdom, there are limited data on the pathogenesis of pleural infection. We investigated the pathogenesis of empyema using animal and cell culture models of *Streptococcus pneumoniae* infection. The pathological processes during the development of empyema associated with murine pneumonia due to *S. pneumoniae* (strain D39) were investigated. Lungs were examined using histology, and pleural fluid and blood bacterial colony-forming units, cytokine levels, and cellular infiltrate were determined over time. Bacterial migration across mesothelial monolayers was investigated using cell culture techniques, flow cytometry, and confocal microscopy. After intranasal inoculation with 10⁷ *S. pneumoniae* D39 strain, mice developed pneumonia associated with rapid bacterial invasion of the pleural space; raised intrapleural IL-8, VEGF, MCP-1, and TNF- α levels; and caused significant intrapleural neutrophilia followed by the development of fibrinous pleural adhesions. Bacterial clearance from the pleural space was poor, and *in vitro* assays demonstrated that *S. pneumoniae* crossed mesothelial layers by translocation through cells rather than by a paracellular route. This study describes key events during the development of *S. pneumoniae* empyema using a novel murine model of pneumonia-associated empyema that closely mimics human disease. The model allows for future assessment of molecular mechanisms involved in the development of empyema and evaluation of potential new therapies. The data suggest that transmigration of bacteria through mesothelial cells could be important in empyema development. Furthermore, upon entry the pleural cavity offers a protected compartment for the bacteria.

Keywords: *Streptococcus pneumoniae*; empyema; animal models; translocation; mesothelial cells

CLINICAL RELEVANCE

The incidence of pleural infection continues to increase worldwide, affecting more than 65,000 patients each year in the United States and in the United Kingdom, and is associated with significant morbidity and mortality. Despite recent advances, our current understanding of empyema disease pathogenesis remains limited, mainly due to the lack of appropriate animal models. We have developed a mouse model of *Streptococcus pneumoniae* empyema developing as a complication of pneumonia. The model will allow testing of potential therapeutic agents in a model that closely mimics human disease. Using this model and *in vitro* tissue culture experiments, we demonstrate that invasion of the pleural space is very rapid during *S. pneumoniae* pneumonia and involves translocation through mesothelial cells.

Pleural infection is a global problem that affects over 65,000 patients each year in the United States and in the United Kingdom and is associated with significant morbidity and mortality (1–5). Empyema is defined by the presence of bacteria or pus in the pleural cavity and usually develops as a complication of pneumonia. Important questions regarding the disease pathogenesis remain unanswered, which may in part account for the lack of recent therapeutic advances. A key limiting factor in this area of research is that there is no suitable preclinical animal model of empyema. Studying the evolution of pleuropulmonary infection ideally involves repeated sampling of the lung and pleural cavity, which is not feasible in patients with pneumonia, and the animal models used to date have significant limitations (6). Published *in vivo* models rely on intrapleural delivery of bacteria to create localized pleural infection without concurrent pneumonia and many use species-specific microorganisms, such as *Pasteurella multocida* (7–9), with limited relevance to humans. These models require concomitant administration of systemic antibiotics to control infection and fatalities (8), further limiting their usefulness for evaluating novel therapies. The pleural cavity is lined by a monolayer of mesothelial cells that have proven biological roles against bacterial invasion (10–12), but the mechanisms by which bacteria cross the mesothelial layer and antibacterial and proinflammatory mesothelial cell responses to infection are poorly understood. An improved model of pleural infection that overcomes the limitations of existing infection models could, in combination with mesothelial cell culture models, allow detailed study of the evolution of pleural infection associated with pneumonia.

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Streptococcus pneumoniae is the leading pathogen causing pediatric empyema and approximately 20% of adult cases (13). The aim of this study was to develop a novel *in vivo* model of *S. pneumoniae* pleural infection arising from pneumonia that resembles human disease. Using this model, we describe for the first time the evolution of pneumonia-associated empyema and the path of bacterial invasion of the pleural cavity. Furthermore, we have used *in vitro* cell culture techniques to determine the role that the mesothelial cells may be playing in this process.

MATERIALS AND METHODS

Murine Models of Pleural Infection

All murine experiments were approved by the University College London Animal Use Ethics Committee and used the capsular serotype 2 D39 *S. pneumoniae* strain (14–16) and outbred CD1 mice. Mice were inoculated with 0.5 to 1.0×10^7 (7) colony-forming units (CFU) by intranasal, intrapleural (both under halothane anaesthesia), or intravenous routes (17, 18). For intrapleural injections, an insulin syringe needle was placed at a shallow angle between the left second and third ribs; injection of trypan blue confirmed that this accurately inoculates the pleural space. Mice were killed, and bacterial CFU were quantified by plating serial dilutions of homogenized left lungs, blood, and pleural lavage fluid (PLF) (obtained by transdiaphragmatic injection of $600 \mu\text{l}$ of PBS) (14, 18). Right lungs were fixed in formaldehyde for immunohistochemical studies. Blood and PLF were analyzed for leukocyte counts by microscopy and cytokine levels using ELISAs. *S. pneumoniae* localization was determined by immunohistochemistry using antibody to capsular polysaccharide as described in the online supplement.

Human Pleural Samples

Pleural fluid samples were collected in EDTA tubes and centrifuged, and the supernatants were stored at -80°C until assayed (regional ethic committee approvals 07/Q1607/20 and 04-MRE5/53). Pleural infection was defined as an effusion in a patient with pneumonia that contained frank pus or had a $\text{pH} < 7.20$ (19, 20). Transudative effusions were defined by Light's criteria in patients with a clinical diagnosis of congestive heart failure.

Human Met5A and Primary Mesothelial Cell Infection Experiments

Detailed cell culture methodologies are described in the online supplement. Briefly, Met5A and primary mesothelial cells obtained from a human lung were seeded into top chambers of Transwell filter inserts and cultured for 35 days until a confluent mesothelial layer was achieved. The upper chamber was inoculated with 1×10^7 or 5×10^7 CFU serum-opsonized D39 (multiplicity of infection [MOI] 10:1 or 50:1), and bacterial migration was assessed by plating serial dilutions of lower-chamber medium. Cell viability was determined by a colorimetric assay measuring supernatant lactate dehydrogenase (LDH) and a TUNEL assay (Roche, Welwyn Garden City, UK). Transepithelial electrical resistance (TEER) was measured using a VoltOhm meter resistance reader (World Precision Instruments, Stevenage, Herts, UK). Mesothelial layer permeability was determined by measuring the fluorescence of the medium from the lower chamber 90 minutes after the addition of 0.01 mM 4-kD FITC-dextran to the upper chamber at an excitation wavelength of 490 nm and an emission wavelength of 520 nm . Scanning electron microscopy was performed on infected washed and fixed Met5A mesothelial layers using conventional methods as described in the online supplement. Some Transwell experiments were preceded by the addition of $1 \mu\text{M}$ colchicine, $1 \mu\text{M}$ nocodazole, or $1 \mu\text{M}$ cytochalasin D to cells for 1 hour before infection.

D39 invasion of Met5A cells was investigated using confluent mesothelial layers grown in 24-well plates. Antibiotic protection assays were performed as described (21) using Met5A cells infected with D39 (MOI 50:1). Cells were trypsinized and lysed by 2% saponin solution, and serial dilutions of the supernatant were plated to obtain bacterial CFU with and without the addition of medium containing penicillin G ($10 \mu\text{g/ml}$) and gentamicin ($200 \mu\text{g/ml}$) for 1 hour before plating.

Additional experiments used FACS and confocal microscopy to identify intracellular bacteria as described in the online supplement (15).

RESULTS

Murine Model of Pleuro-Pulmonary Infection

Using an established murine model of *S. pneumoniae* pneumonia we noticed that mice developed pleural pus, suggesting significant pleural infection. To characterize pleural infection in this model in more detail, female CD1 mice were inoculated intranasally with 10^7 CFU of D39 (a dose previously shown to cause pneumonia) (17, 18) and killed 48 ($n = 5$) or 72 hours ($n = 6$) postinoculation. By 48 hours, most mice had developed macroscopic pleural adhesions (3/5 mice) and pus (5/5 mice). Attempts to prolong survival and control systemic sepsis using intraperitoneal administration of penicillin G (2.5 mg/kg) to mice at 48 hours postinoculation ($n = 4$) or at 24 and 48 hours postinoculation ($n = 4$) had no effect or resulted in the clearance of bacteria from the lungs and no detectable pleural infection (data not shown). Infective doses of less than 10^6 CFU failed to cause significant infection in most mice (data not shown). Therefore, inoculation with 10^7 CFU per mouse and a 48-hour time-point were chosen as the optimal experimental conditions for the development of pleural infection associated with pneumonia.

The model was further characterized using mice inoculated with *S. pneumoniae* ($n = 9$) or with PBS only ($n = 6$). By 48 hours postinoculation, all mice given D39 developed signs of severe infection (marked piloerection and reduced spontaneous mobility), which was fatal in five mice. At necropsy, all mice had macroscopic findings of severe pneumonia, with extensive lung consolidation, congestion, and, in some cases, hemorrhage or necrosis. Empyema was confirmed macroscopically in seven mice, with thick and discolored pleural pus of varying volumes present (Figures 1A and 2A) and 8.78 (SD, 0.48) $\log_{10}$ bacterial CFU/ml in PLF. In some mice, early-stage adhesions that stained with Mason's Trichrome were identified, confirming that they were predominantly fibrinous in structure (Figures 1B–1D). These adhesions were easily separated from the chest wall upon opening of the thoracic cage, preventing accurate quantification. No evidence for parietal pleural thickening or for hemothorax or extrathoracic (e.g., intraperitoneal) infection was observed. Histology confirmed significant consolidation consistent with severe pneumonia involving all lobes, with neutrophilic and red blood cell infiltrates, air space consolidation, and interstitial edema. The pleural cavity contained a pronounced inflammatory cell influx ($3.07 \times 10^7/\text{ml}$ SD 1.42 of white cells versus $1.12 \times 10^6/\text{ml}$ SD 0.62 for PBS controls) (Figures 1C and 1D). PLF cytokine levels had a similar pattern to those found in pleural fluid from human subjects with empyema, with increased levels of MCP-1/CCL2, MIP-2/CXCL2 (mouse homolog of human IL-8), VEGF, and TNF- α compared with controls (Table 1). Overall, the results showed that *S. pneumoniae*-inoculated mice developed pneumonia associated with pleural infection that resembled human empyema phenotypically, histologically, and immunologically.

Time Course of the Development of the Murine Model of Empyema

Using the empyema model, we characterized the key early events in the temporal evolution of pneumonia-associated pleural infection. Although *S. pneumoniae* mice only began to show signs of infection after 24 hours (reduced spontaneous movement, piloerection), histological evidence of inflammation was observed around the main airways at 4 hours postinoculation

Figure 1. Macroscopic and histological appearance of murine empyema 48 hours postinoculation. (A) Macroscopic appearance of lungs and pleural cavity. Arrows indicate fluid and pus accumulation. (B) Montage of a thorax section (original magnification: $\times 4$) stained with Masson's trichrome showing consolidated lung and fibrinous adhesions (arrows). h = heart; l = lung. (C and D) Masson's trichrome staining showing fibrin deposits (arrowheads) and numerous inflammatory cells (arrows). Original magnification: $\times 20$.

(see Figure E1 in the online supplement), and by 24 hours there was extensive lung consolidation. Significant morphologic changes were observed over time in the visceral pleural monolayer. At 4 hours, the mesothelial cells remained flat and normal in appearance, but by 12 hours the consolidation had extended to the lung periphery, and the cells of the visceral pleura had become columnar and raised, and some were rounded (Figure 2). After 24 hours, mesothelial cells had abundant, pale cytoplasm

and pale nuclei, indicative of cell necrosis (Figures 2A–2C). Bacterial PFL CFU and the proportion of mice with pleural infection increased over time. Live bacteria were detected in PLF as early as at 4 hours postinfection in 33% of mice; by 48 hours, all mice had large numbers of *S. pneumoniae* present in PLF (Figure 3A). In contrast, bacterial CFU within the lungs increased by less than 1 $\log_{10}$ from 4 to 48 hours, and bacteremia was only detected after 12 hours (Figures 3B and 3C). For individual mice, bacterial CFU in the PLF correlated closely with blood but not lung homogenate CFU (Figures 3D and 3E). Localization of *S. pneumoniae* using immunohistochemistry and anticapsular antibody revealed a pattern of bacterial migration away from the main airways toward the lung periphery and the visceral pleura over time (Figure 2). By 24 hours, there was a marked increase in bacteria in close proximity to the visceral pleura and the submesothelial cell layer (Figure 2G), coinciding with an increase in median PLF from 3.5 $\log_{10}$ at 12 hours to 7.3 $\log_{10}$ by 24 hours (Figure 3A). Bacterial invasion of the pleural space was associated with large increases in PLF leukocytosis, predominantly neutrophils and monocytes (Figure 4).

Progress of Pleural Infection after Intravenous and Intrapleural Injection

The above data suggested bacterial migration into the pleural cavity through the visceral pleura from the infected lung parenchyma. However, hematogenous spread from the bloodstream to the pleura remained another potential path of entry. In addition, little is known about the clearance of bacteria reaching the pleural cavity. We therefore inoculated CD1 mice with 5×10^6 D39 CFU in 0.1 ml by intravenous ($n = 12$) or intrapleural injection ($n = 12$). After intravenous inoculation, the concentration of bacteria in the blood remained static over time, and only a small number of bacteria ($< 10^4$ CFU) were recovered from PLF or lung tissue (Figure 5 and data not shown), demonstrating that in this model pleural infection is unlikely to occur as a consequence of septicemia. All the animals survived

Figure 2. Histological appearances of the pleura at 4, 12, and 24 hours after intranasal inoculation of D39 *S. pneumoniae*. (A–C) Developing morphological changes of mesothelial cells (arrowheads) with largely normal appearances at 4 hours and increased rounding associated with an influx of inflammatory cells into the subpleural space but preservation of the mesothelial layer integrity at 12 and 24 hours. (D–F) Relationship of *S. pneumoniae* stained with anticapsular antibody (brown, indicated by arrows) to the mesothelium, with an increase in visible bacteria in close proximity to the submesothelial layer with time. (G) Quantitative analysis of *S. pneumoniae* bacterial numbers within and adjacent to the pleural over time. Each dot represents number of bacteria visible within and adjacent to the pleura for a single lung section from one of five mice for each time point.

TABLE 1. CYTOKINE LEVELS IN PLEURAL FLUID FROM HUMAN SUBJECTS (N = 6 TO 7) WITH EMPYEMA AND AT 48 HOURS IN THE MOUSE MODEL OF EMPYEMA (N = 3 TO 4) COMPARED WITH HEART FAILURE AND PHOSPHATE-BUFFERED SALINE-TREATED CONTROLS, RESPECTIVELY

Source of Pleural Fluid	Cytokine			
	TNF- α (pg/ml)	MCP-1 (CCL-2) (ng/ml)	IL-8/MIP-2 (CXCL-2) (pg/ml)	VEGF (pg/ml)
Murine samples				
Murine empyema	833 $\pm$ 153 [†]	39.9 $\pm$ 6.94	2,565 $\pm$ 558	2,394 $\pm$ 543
Murine PBS controls	147 $\pm$ 108	0.02 $\pm$ 0.06	65.9 $\pm$ 33.8	295 $\pm$ 182
P values [‡]	0.030	0.001	0.004	0.050
Human samples				
Human empyema	63.7 $\pm$ 39	3,468 $\pm$ 863	11,556 $\pm$ 2136	3,883 $\pm$ 619
Human CHF [§]	105.7 $\pm$ 54	470 $\pm$ 179	645 $\pm$ 607	1,079 $\pm$ 519
P values [‡]	0.35	0.093	<0.001	0.006

Definition of abbreviations: CHF = congestive heart failure.

[†] Values are mean $\pm$ SEM.

[‡] Unpaired *t* tests for comparisons between empyema samples versus controls.

[§] Effusions due to heart failure.

and showed no clinical signs of septicemia at 24 hours. In contrast, direct intrapleural delivery of bacteria resulted in persistently high levels of bacterial CFU in the PLF over 24 hours and the rapid spread of bacteria into the blood and the lung (Figure 5), increasing by 4 log₁₀ CFU after 24 hours and accompanied by signs of infection in the mice (reduced mobility and piloerection). The data suggest that clearance of *S. pneumoniae* from the pleural space is poor, even in healthy animals without concurrent pneumonia, whereas passage of bacteria from the pleura into the blood (and lung) is rapid. Thus, the pleural cavity can act as a reservoir of bacteria for continuing infection.

In Vitro Bacterial Migration across the Mesothelial Cell Layer

The mouse model data suggested that *S. pneumoniae* can rapidly cross the pleural layer. To investigate this in more detail, we used D39 infection of a mesothelial cell line, Met5A, and isolated primary mesothelial cells in a Transwell cell culture model. After growth on Transwell filter inserts for 21 or 35 days, for primary and Met5A cells, respectively, a confluent cellular layer formed (Figures E2A, E3A, and E3B) and developed

TEER values of 300 to 500 Ω /cm², indicative of intact tight junction formation. Immunocytochemistry confirmed that the cells had mesothelial cell characteristics, with positive staining for mesothelin, von Willebrand factor, and calretinin and negative staining for smooth muscle actin (22, 23) (Figures E3 and E4). The Met5A cells displayed positive staining for HBME-1, a marker for mesothelioma, whereas the primary untransformed mesothelial cells stained negatively for HBME-1. Addition of D39 to the Met5A transwell layers at a MOI of 10 or 50 caused a fall in the TEER (Figure 6A). Scanning electron microscopy demonstrated Met5A cytoskeletal rearrangement, with cell extrusion and rounding with atrophy of the microvilli (Figure E2). Despite this, there was no leakage of a fluorescently labeled macromolecule (FITC-dextran) across infected Met5A and primary cell monolayers (Figures 6B and 6F). Supernatant LDH levels and the TUNEL assay showed no evidence of significant cell death in infected transwells even with a MOI of 50 (Figure E5). These data demonstrate that, after infection with *S. pneumoniae*, the integrity of the mesothelial cell monolayers was not affected. However, *S. pneumoniae* D39 bacterial CFU were readily detectable in the lower well medium within 2 hours of addition of bacteria at a MOI of 10 to the upper well of Met5A transwells or 1 hour after the addition of bacteria at

Figure 3. *S. pneumoniae* colony-forming units (CFU) (D39) within target sites of infection after intranasal inoculation over time. (A) CFU within PLF. (B) CFU within the lung homogenates. (C) CFU within the blood. Data in panels A, B, and C are presented as box plots (median and interquartile range) with whiskers showing minimum and maximum values, and *P* values represent results for Kruskal-Wallis tests comparing CFUs over time. (D–E) Correlation between the CFU within the PLF and lung and PLF and blood, respectively, with *P* values and *R*² calculated using Pearson's correlation coefficients.

Figure 4. Differential cell counts within pleural lavage fluid (PLF) after intranasal inoculation with D39 over time. (A) Total white blood cell (WBC) counts. (B) Total polymorphonuclear cell counts (PMNs). (C) Total monocytes/macrophages counts. (D) Total lymphocyte counts. Data are presented as box plots (median and IQRs) with whiskers showing minimum and maximum values, and P values represent results for Kruskal Wallis tests comparing CFU over time.

a MOI of 50 to Met5A or primary mesothelial cell transwells (Figure 6C). The bacterial effects on TEER and bacterial CFU accumulating in the bottom well were markedly reduced by treatment of the Met5A or primary cells with the microtubule inhibitors nocodazole and colchicine and with the inhibitor of actin polymerization cytochalasin D (Figures 6D, 6E, 6G, and 6H), whereas dextran-FITC leakage (Figure 6F data for Met5A not shown), supernatant LDH levels (Figures E5B and E5C), and TUNEL staining were unaffected (data not shown). Overall, the data demonstrate that *S. pneumoniae* crosses Met5A and primary mesothelial cell layers by an intracellular route that is dependent on microtubules and actin polymerization.

In Vitro Evidence for *S. pneumoniae* Invasion of Mesothelial Cells

The above data demonstrate that D39 crosses mesothelial layers by invasion of mesothelial cells. Therefore, we confirmed internalization of *S. pneumoniae* by Met5A cells using infection of cultured Met5A cells (MOI 50:1) and the antibiotic protection assay, and by flow cytometry and confocal microscopy using FAM5E-labeled *S. pneumoniae*. Increasing numbers of *S. pneumoniae* were associated with Met5A cells over time, reaching a maximum of approximately 10 per mesothelial cell at 4 hours. Selective killing of extracellular bacteria by the addition of antibiotics at different time points showed that a proportion of *S. pneumoniae* had been internalized (Figures 7A and 7B). Flow cytometry demonstrated bacterial association with approximately 4% of Met5A cells. Quenching of extracellular fluorescence with trypan blue also showed that, although *S. pneumoniae* association with Met5A cells was mainly due to extracellular bacteria, there was a small but significant population of internalized bacteria (Figure 7C). This was confirmed by confocal

Figure 5. Log_{10} D39 CFU in blood (A) or pleural cavity (B) after intrapleural (IPI) or intravenous (IV) injections of D39. The data are presented as box plots (median and IQRs) with whiskers showing minimum and maximum values, and P values represent results for Mann-Whitney U tests comparing CFU at 1 and 24 hours. (C and D) Correlation between bacterial CFU (log_{10}) in the PLF and blood after intrapleural and intravenous inoculation, respectively, with P values and R^2 calculated using Pearson's correlation coefficients.

microscopy and Z-stacking, which demonstrated that 11% of *S. pneumoniae* bacteria associated with Met5A cells had been internalized (Figure 7D).

DISCUSSION

Despite the growing incidence of empyema worldwide (2–5), therapeutic advances have been limited, and major questions remain about the pathogenesis of pleural infection. One of the main hurdles in empyema research has been the lack of an animal model that resembles human disease. Existing published *in vivo* models using intrapleural delivery of animal-specific pathogens in rabbits or other large animals (6, 8, 9) preclude the study of the natural evolution of empyema and offer little insight into the biology of bacteria relevant to human disease. This study describes a new murine model of pneumonia-associated empyema due to *S. pneumoniae*, a common cause of empyema (13). The model mimics human disease phenotypically, immunologically, and histologically and is characterized by bacterial invasion of the pleural space from the lung, intrapleural influx of inflammatory cells with raised levels of cytokines that are elevated in patients with empyema, and the development of fibrinous pleural adhesions. The histological appearances with fibrinous pleural adhesions were similar to early-stage empyema in humans. This empyema model has the following advantages over existing models: it uses a human relevant pathogen; it arises from preceding pneumonia and therefore resembles the common clinical course of community-acquired human empyema; it uses an intranasal

CFU in the presence of 1 μ M colchicine, 1 μ M nocodazole, and 1 μ M cytochalasin compared with untreated Met5A and primary cells, whereas dextran-FITC leakage was unaffected. Data shown are the mean of at least three independent experiments (+ SD). Statistical significance was evaluated using an unpaired *t* test and denoted as reaching statistical significance (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$) to PBS-treated controls.

route of delivery, which is technically easier than intrapleural injection or tube thoracostomy (9); it uses mice, which are easier to handle and cheaper than larger animals and allow experiments in transgenic animals; and it has a natural history similar to disease in humans (resulting in fatality if untreated).

This model has limitations. In clinical practice, some patients present with a more chronic form of pleural infection, often without concomitant pneumonia, but this model resembles early phases of human empyema and needs adaptation to represent more chronic forms. Murine susceptibility to different bacterial species and strains vary, so although the model presents an opportunity to study different bacteria, optimization is necessary for each species. Furthermore, the model has a steep dose-response with bacterial inocula of less than 5×10^6 CFU frequently failing to establish significant infection.

Using this model, we were able to describe, for the first time, the sequence of events during the development of pneumonia-associated empyema. The data show that *S. pneumoniae* can invade the pleural space as early as 4 hours after inoculation into the lungs, before there are major inflammatory changes affecting the underlying lung parenchyma and pleura. Pleural infection developed rapidly, with the majority of mice having very high numbers of bacteria in the pleural space by 24 hours

Figure 6. *S. pneumoniae* D39 migration across mesothelial Transwells layers. (A) The effect of D39 on Met5A monolayer transepithelial electrical resistance (TEER) over time. (B) Effect of D39 on migration of FITC-dextran across Met5A Transwell layer. The concentration of FITC-dextran was 0.45 mg/ml in the upper chamber as denoted by the dotted line. Only a low concentration of FITC-dextran migrated across the mesothelial layer (< 0.1 mg/ml), and there were no significant increases between infected (multiplicity of infection [MOI] 50, white columns; MOI 10, black columns) and noninfected cells (grey shaded columns). (C) Bacterial CFU (10^6 /ml) found in the lower chamber after addition of 1×10^7 or 5×10^7 *S. pneumoniae* D39 CFU to the upper chamber (MOI of 10 or 50 to 1) over a 4-hour period. (D–H) Effects at 2 hours of 1 μ M colchicine, nocodazole, or cytochalasin on monolayer TEER (D and G), migration of bacteria across Transwells layers (E and H), and leak of FITC-dextran into the bottom well (F and data not shown) for Met5A (D and E and data not shown) or primary mesothelial cells (F, G, and H). There were significant differences in changes in TEER and bacterial

and with formation of adhesions by 48 hours. Hence, *S. pneumoniae* can readily establish pleural infection in the naive host that has not previously been exposed to *S. pneumoniae*. Direct inoculation of *S. pneumoniae* into the pleural space confirmed that the pleural space is permissive for bacterial replication and that *S. pneumoniae* can overwhelm the local immune defense and leak from there into the blood. In contrast, *S. pneumoniae* injected intravenously struggled to maintain septicemia, and only low numbers of bacteria reached the pleural space. These data demonstrating the ease with which at least this strain of *S. pneumoniae* can infect the pleural space contrast with human infection because only a minority of patients with pneumonia develop empyema. This important observation suggests that humans have efficient mechanisms that prevent pleural infection, which, if characterized, may allow the prevention of empyema. The most likely mechanisms are preexisting immunity in humans due to previous colonization with *S. pneumoniae* (24) and the early use of antibiotics, but further investigation is required. In addition, mice only have approximately five branching generations of airways, whereas humans have approximately 21. Therefore, intranasal administration of *S. pneumoniae* in a mouse could migrate to the pleural space more rapidly and more easily than in a human, where it would have to travel

Figure 7. *S. pneumoniae* D39 association and translocation across Met5A cells. (A) Total (adherent and intracellular) bacterial CFU obtained by plating cell lysates at different time points as marked without prior addition of antibiotics. (B) CFU for intracellular D39 found within Met5A cells when assessed by an antibiotic protection assay involving the addition of antibiotics to kill extracellular bacteria before plating cell lysates at different time points as marked. Error bars represent SEMs. (C) Association of fluorescent D39 with Met5A cells when assessed by flow cytometry with (open columns) and without (black columns) addition of trypan blue to quench the fluorescence of extracellular bacteria. Data are presented as a fluorescence index (sum of the proportion of Met5A cells associated with fluorescent bacteria and the intensity of their fluorescence). (D) Confocal microscopy of MET5A cells incubated with fluorescent D39, with intracellular bacteria identified by the arrow. Cell cytoplasm is stained with Texas Red, and the nuclei were stained with DAPI. Z stacking demonstrated 11% of visible bacteria was found within Met5A cells.

much further and cope with greater exposure to host defense mechanisms, such as alveolar macrophages. Another relevant observation is that aspiration of bacteria into the lungs resulted in bacterial translocation into the pleural space before histological evidence of pneumonia develops. This could explain why a surprisingly high proportion of cases of empyema have no radiological evidence of pneumonia (25).

One important question is how the bacteria reach the pleural space during the development of empyema. Potential mechanisms include disruption of mesothelial integrity by inflammation or toxins allowing bacteria access to the pleural space via a paracellular route, translocation through mesothelial cells, or indirectly via the blood. In our model, pleural infection preceded septicemia, and intravenous inoculation of *S. pneumoniae* resulted in only small numbers of bacteria in PFL, whereas intrapleural inoculation resulted in high-level septicemia. Hence, empyema was unlikely to be due to indirect spread of bacteria into the pleura from the blood; instead, the data suggest that pleural infection could contribute to septicemia. The rapid development of pleural infection in some animals before macroscopic evidence of mesothelial inflammation suggest that translocation of bacteria through mesothelial cells could be an important route by which bacteria reach the pleural space. This was confirmed *in vitro* using a mesothelial cell line as well as primary *ex vivo* mesothelial cells. To ensure that even a high density of infection does not cause barrier breakdown and translocation of bacteria via a paracellular route, for these experiments we chose relatively high MOIs. Even with an MOI of 50, there was no leakage of macromolecules across mesothelial transwell layers despite translocation of significant numbers of *S. pneumoniae*. MOIs of 10 and 50 resulted in similar numbers of bacteria translocating across mesothelial layers, suggesting that the mechanism of translocation can be saturated. During actual infection, the MOIs are unlikely to be as high as 50 but could approach 10 locally, and microscopy did show *S. pneumoniae* in close proximity to the pleural layer during pneumonia. Translocation of even small numbers of *S. pneumoniae* to the pleural space could readily lead to infection because our data suggest that the bacteria are relatively

protected against immune clearance at this site. Translocation across mesothelial cells was dependent on mesothelial cell actin polymerization and microtubule function and was associated with a fall in TEER but not with cell death or disruption of the mesothelial barrier function. Antibiotic protection assays, confocal microscopy, and flow cytometry demonstrated that small numbers of bacteria were internalized by Met5A cells. However, confirming that the bacteria invade mesothelial cells during infection in mice would require electron or confocal microscopy of recovered mesothelium and would be technically very challenging due to the low number of events. Several mechanisms of cell invasion dependent on different bacterial protein/host ligand interactions have been described for *S. pneumoniae* and can result in translocation across epithelial and endothelial layers (21, 26–29). Further research is required to clarify whether similar mechanisms are relevant for translocation across mesothelial cells, characterization of which could offer potential targets for therapies that prevent empyema. Differences in the ability to translocate across mesothelial cells may explain why certain bacterial pathogens and strains are particularly associated with pleural infection (e.g., the *S. milleri* group and capsular serotype 1 *S. pneumoniae*) (30–32). During later stages of infection, the pleural layer showed evidence of severe disruption, which could allow significant bacterial movement into the pleural space from the lung and *vice versa* via a paracellular route.

In summary, we have developed a novel murine model of empyema with significant advantages over published models. Using this model, we have described the development of *S. pneumoniae* empyema during pneumonia and have shown that the pleural space is permissive for bacterial infection. We have identified that pleural invasion occurs surprisingly early during infection, probably via bacterial translocation across mesothelium. This model will allow investigation of the pathogenesis of empyema, the pleural immunological responses to infection, and the efficacy of antibiotic or other therapies and could be adapted to assess why there are major differences between and within species of bacterial pathogens in their ability to cause pleural infection.

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