

Why does pneumococcus kill?

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Acute lower respiratory tract infections remain the most common cause of death due to infection worldwide, and *Streptococcus pneumoniae* is responsible for approximately 30% of all cases of community-acquired pneumonia. While many virulence factors have been associated with fatal pneumococcal pneumonia, there is growing evidence that some components of the immune response contribute significantly to the high mortality rate. This paper reviews the major bacterial virulence factors and pathogenesis steps that characterize fatal pneumococcal pneumonia, with a focus on the inflammation that was observed from the initial infection to death in an experimental murine pneumonia model. These steps involve the successive recruitment of polymorphonuclear neutrophils (PMNs), monocytes and lymphocytes; the pulmonary and/or systemic release of inflammatory mediators that characterize the prebacteremic and bacteremic phases of infection; and the participation of parenchymal cells in the host response. Although the kinetics of cytokines differ considerably from blood to lung tissue to alveoli, and blood levels do not correlate to tissue levels, the kinetics of tumour necrosis factor (TNF) and interleukin-6 in blood, as well as TNF and nitric oxide in bronchoalveolar lavage (BAL) fluid are good indicators of the evolution of the disease. Nitric oxide release is biphasic and corresponds mostly to monocyte recruitment in BAL fluid and concomitant serious tissue injury. Pneumococci activate leukotriene B₄ (LTB₄) release, but PMN recruitment is not primarily mediated by LTB₄. Bacteremia, leukopenia, thrombocytopenia and lipid peroxidation closely precede death. Knowledge of the chronology of microbiological and inflammatory events that occur during pneumonia may help to design appropriate diagnostic tests that could be used to monitor the evolution of this deadly infection. There has been an explosive growth in the use of biological response modifiers that may be given to treat pneumonia. The proper use of these agents requires prior identification of biological markers in humans with pneumonia.

Key Words: *Cytokines; Pathogenesis; Pneumococcal pneumonia*

Pourquoi le pneumocoque tue-t-il ?

RÉSUMÉ : Les infections aiguës des voies respiratoires inférieures demeurent, à travers le monde, la cause la plus courante de décès attribuable à une infection, et *Streptococcus pneumoniae* est responsable d'environ 30 % de tous les cas de pneumonie extra-hospitalière. Alors que de nombreux facteurs de virulence sont associés à la pneumonie à pneumocoques mortelle, de plus en plus de preuves semblent indiquer que certaines composantes de la réponse immunitaire contribuent de façon significative au taux de mortalité élevé. Le présent article passe en revue les principaux facteurs de virulence bactérienne et les étapes de la pathogénie qui caractérisent la pneumonie à pneumocoques fatale, en insistant sur l'inflammation qui a été observée à partir de l'infection initiale jusqu'à la mort dans un modèle expérimental de pneumonie murine. Ces étapes impliquent la mobilisation successive de neutrophiles polymorphonucléaires (NPM), de monocytes et de lymphocytes, la libération systémique ou pulmonaire de médiateurs de l'inflammation qui caractérise les phases prébactériémique et bactériémique de l'infection, et la participation des cellules du parenchyme à la réponse de l'hôte. Bien que la cinétique des cytokines diffère considérablement à partir du sang jusqu'aux tissus pulmonaires, et jusqu'aux alvéoles, et que les taux sanguins ne soient pas en corrélation avec ceux mesurés dans les tissus, la cinétique de la cachectine (TNF) et de l'interleukine-6 dans le sang, de même que la TNF et l'oxyde nitrique dans le liquide du lavage broncho-alvéolaire (LBA) sont de bons indicateurs de l'évolution de la maladie. La libération de l'oxyde nitrique est biphasique et correspond en grande partie à la mobilisation des monocytes dans le LBA et à une lésion tissulaire concomitante grave. Les pneumocoques activent la libération des leucotriènes B₄ (LTB₄), mais la mobilisation des NPM n'est pas principalement médiée par les LTB₄. La bactériémie, la leucopénie, la thrombocytopénie et la peroxydation des lipides précèdent de près la mort. La connaissance de la chronologie des événements inflammatoires et microbiologiques

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qui surviennent pendant une pneumonie pourrait favoriser la mise au point de tests diagnostiques appropriés pouvant être utilisés pour surveiller l'évolution de cette infection mortelle. On assiste à une croissance explosive de l'utilisation des modificateurs de la réponse biologique qui peuvent être administrés pour traiter la pneumonie. L'utilisation adéquate de ces agents exige au préalable une identification des marqueurs biologiques chez les humains atteints de pneumonie.

The World Health Organization reported 52 million deaths worldwide in 1997; 17 million of these resulted from infectious diseases and a high percentage from acute lower respiratory tract infections (four million). *Streptococcus pneumoniae* is responsible for approximately 30% of all community-acquired pneumonia cases (1-3). Mortality rates vary from 5% to 20% (4), or even 40% in patients with bacteremia (1) and 60% in patients requiring intensive care (5). In fact, the use of potent antibiotics and aggressive intensive care support has not reduced fatality rates during the first five days of bacteremic infections over the past 40 years (1,6). Death still occurs days after antibiotic therapy has started, when tissues are sterile and the pneumonia is clearing.

There are major gaps in our understanding of interactions between the host and pneumococci. In fact, the persistence of this deadly infection in both immunosuppressed and immunocompetent hosts, the profound problem posed by childhood pneumonia in developing countries (7), and the widespread emergence of penicillin-resistant strains throughout the world (8) emphasize the importance of acquiring a better understanding of the mechanisms by which this formidable pathogen causes disease. Many bacterial virulence factors and host immune responses together contribute to the outcome of pneumonia. The colonization of airways, development of pneumonia and bacteremia, cellular and humoral responses, and the release of inflammatory mediators need to be investigated from the initial infection to death through extensive pathogenesis studies to properly elaborate preventive and therapeutic strategies with more effective vaccines, antibiotics and immunomodulator drugs that will control the bacteria and its toxins, and optimize host response.

There is growing evidence that some components of the immune response contribute significantly to the high mortality rate: while immunosuppressed patients die as a consequence of poor host response, immunocompetent hosts face overwhelming inflammatory reactions that contribute to tissue injury, shock and death (9-12). This paper reviews microbiological and inflammatory aspects of fatal pneumococcal pneumonia, with particular focus on five major pathogenesis steps that were observed in a murine model established at our laboratory.

SEQUENTIAL PATHOGENESIS

Various virulence factors of pneumococci and elements of the host response to this bacteria have already been characterized. They include the polysaccharide capsule, cell wall components, intracellular proteins, immune and nonimmune cells, pro- and anti-inflammatory cytokines, the chemotactic lipid metabolite leukotriene (LT) B₄, oxygen radicals that are released on activation of phagocytes, and many additional mi-

crobial and immunological elements. Some pathogenesis studies have focused on aspects of colonization or inflammation in relation to edema and histological lesions. However, a thorough study of the inflammatory response to pneumococci in the lung as a single time course evaluation of the infection is difficult to locate in the literature. Although cytokines have been found in bronchoalveolar lavage (BAL) fluid or plasma of animals or patients, ours is the first laboratory to make correlations between cytokine levels within lung tissue (BAL fluid and serum simultaneously), time course of the disease and outcome of pneumonia, and to evaluate at the same time the chronology of LT release and inflammatory cell recruitment in association with kinetics of cytokines, and to assess the relationship between nitric oxide release and histopathology during pneumococcal pneumonia.

To characterize the chronology of events associated with fatal pneumococcal pneumonia, we developed a murine model that allowed us to follow the pathogenesis steps from the initial infection to death in immunocompetent mice. In this model, which was previously described (13), four-week-old anesthetized CD1 mice received an intranasal suspension of 10⁷ log-phase colony forming units (CFUs) of bacteria in phosphate-buffered saline (PBS), which they inhaled involuntarily. The parameters that were followed were physiological, microbiological, hematological, immunological and biochemical aspects of infection and host response. All assays were performed on samples obtained from BAL fluid (alveoli), lung tissue homogenates (tissue interstitium) and blood (or serum). All samples were obtained and processed as previously described (13). Body weight was recorded daily. Animal prostration and tachypnea were noted. Bacterial growth in lungs and blood was quantified on blood agar after twofold dilutions in PBS. Hematology was recorded using a Coulter counter #T890 (Beckman Coulter, Florida), including white blood cell counts, hematocrit and thrombocyte counts. White blood cells in BAL fluid were differentiated using a cytopsin preparation stained with Diff-Quick reagent #B4132-1 (Baxter). Polymorphonuclear cells (PMNs) in lung tissues were quantified through the dosage of myeloperoxidase (MPO) (13). Lung weight was noted as an indicator of edema. The cytokines were assayed using ELISAs (tumour necrosis factor [TNF], interleukin [IL]-1 and IL-6: Genzyme # 80-2802-00, 1900-01, 80-3748-01, respectively; Genzyme, Massachusetts). LTB₄ was measured with a radioimmunoassay kit # 8-6020 (Cedarlane). Malondialdehyde release was followed as an indicator of lipid peroxidation by a colorimetric method using thiobarbituric acid, as already described (13). Light and electron microscopy were performed according to standard procedures (13), with particular attention paid to tissue injury, type II pneumocyte proliferation and surfactant secretion.

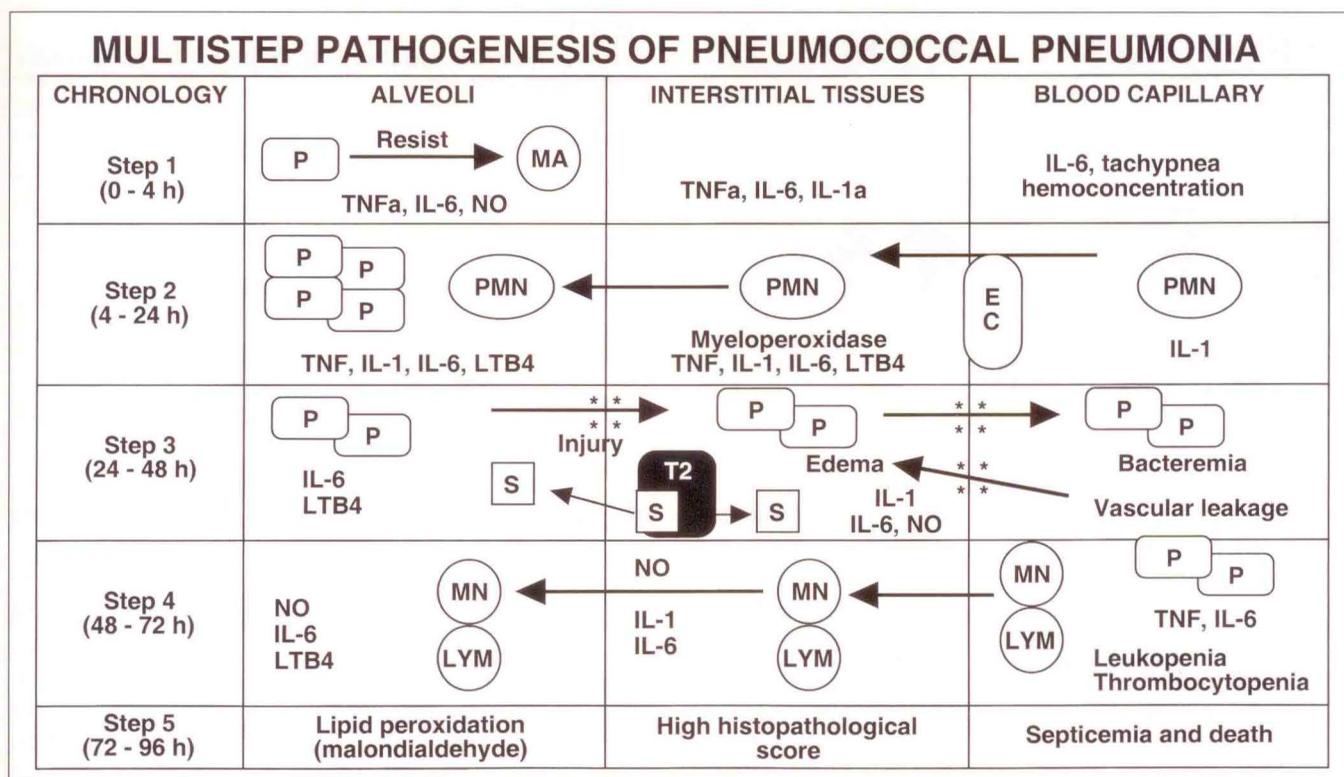


Figure 1) Multistep pathogenesis of pneumococcal pneumonia after intranasal inoculation of CD1 mice with 10⁷ colony forming units per mouse. The chronology from 0 to 96 h postinfection highlights the major changes that characterize each step in every lung compartment, in the alveolar spaces (bronchoalveolar lavage fluid analysis), the tissue interstitium (lung homogenate supernatant analysis) or the blood capillaries. **Tissue injury. EC Endothelial cell; IL-6 Interleukin-6; LTB4 Leukotriene B4; LYM Lymphocyte; MA Alveolar macrophage; MN Monocyte; NO Nitric oxide; P Pneumococcus; PMN Polymorphonuclear cell (neutrophil); S surfactant; T2 Type II pneumocyte; TNFα Tumour necrosis factor alpha

Figure 1 is a schematic representation highlighting the particular events that characterize the five pathogenesis steps of pneumococcal pneumonia that were observed from the initial infection to death. The first two steps correspond to pulmonary infection in the absence of bacteremia, the third step coincides with transition from pulmonary to systemic infection, and the last two steps are characterized by widespread overwhelming inflammatory reactions that contribute to severe tissue injury, hematological and biochemical disorders, and death.

Step 1: Step 1 occurs from 0 to 4 h after intranasal inoculation with 10⁷ CFU of *S pneumoniae* serotype 3 (transparent in colonial morphology, isolated clinically from blood culture, and penicillin susceptible). Step 1 is characterized by ineffective bacterial clearance by resident alveolar macrophages, release of TNFα, IL-6 and nitric oxide in alveoli, TNF, IL-6 and IL-1α in lung tissues, and IL-6 in serum. Concomitant but transient physiological and hematological anomalies that accompany the microbiological challenge and immunological response include tachypnea and hemoconcentration.

Step 2: Step 2 covers the 4 to 24 h period after infection. It is characterized by significant bacterial growth in alveoli, high release of TNF, IL-6 and IL-1 and LTB₄ in alveoli and lung tissues, and recruitment of PMNs from the bloodstream to lung tissue (through endothelial cells) to alveoli, as detected by high MPO levels in lung homogenates and cell counts in BAL

fluid and on tissue sections. This is associated with transient 'spillover' of IL-1 in serum.

Step 3: Step 3 occurs between 24 and 48 h. It is characterized by down-regulation of the proinflammatory cytokines TNF and IL-1 in the BAL fluid and lungs. However, injuries to the alveolar ultrastructure become visible and they are associated with edema in the interstitium as a result of vascular leakage. Lung weight starts to increase. Regeneration processes are detected through the proliferation of type II pneumocytes, which is associated with an increased secretion of surfactant. A marked progression of bacteria from alveoli to tissue to the bloodstream is seen, as well as a noticeable loss in body weight of the animals.

Step 4: Step 4 occurs from 48 to 72 h postinfection. It is characterized mainly by strong monocyte recruitment from blood to the alveoli, which is associated with high nitric oxide release in tissues and BAL fluid. Noticeable lymphocyte migration also occurs, so that leukopenia is observed. The overall infection and inflammation are characterized by an increase in tissue damage and an increase in systemic anomalies, including high TNF and IL-6 levels and thrombocytopenia.

Step 5: Step 5 occurs from 72 to 96 h and closely precedes death. Pulmonary histopathological features include severe airspace disorganization with no remaining alveolar architecture and diffuse tissue damage. This is evaluated through histopathological scores that take into consideration the per-

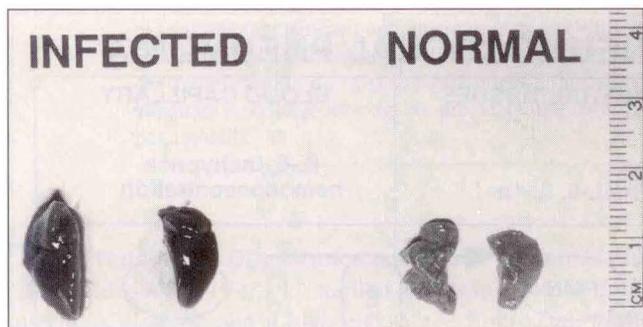


Figure 2) Gross pathology of the lungs of CD1 mice infected with 10^7 *Streptococcus pneumoniae* and killed 72 h later

centage of lung that is inflamed, the number of inflammatory cells recruited to the site of infection, hemorrhage in the alveoli, edema, injuries to organelles and alveolar architecture, and regeneration of parenchymal cells. A high histopathological score in step 5 coincides with high nitric oxide levels detectable in lung tissue and BAL fluid, and lipid peroxidation of membranes detectable through malondialdehyde release. There is also unrestrained bacterial growth, further loss in body weight and a high mortality rate. It should be noted that in fatal pneumococcal pneumonia in mice, body weight falls from 20 g to 18 g to 15.2 g at 0 h, 24 h and 72 h, respectively, indicating a loss of appetite, while lung weight increases from 183 mg to 258 mg to 326 mg over the same period, indicating strong edema that affects pulmonary function and the gross morphology of the lung (Figure 2).

VIRULENCE FACTORS, COLONIZATION AND BACTEREMIA

Fatal pneumococcal pneumonia is, thus, characterized by progressive spreading of bacteria from lung alveoli to the bloodstream, successive participation of various immune and nonimmune cell populations, and pulmonary or systemic release of inflammatory mediators that together with virulence factors contribute, in immunocompetent hosts, to induce tissue injury, shock and death. Bacterial virulence factors that contribute to the colonization of the respiratory tract and pathogenesis of pneumonia include the polysaccharide capsule, the peptidoglycan/teichoic acid complex, pneumolysin, autolysin, pneumococcal surface protein A, pneumococcal surface adhesin A (also called the 37 kDa protein), neuraminidase, hyaluronidase and immunoglobulin (Ig) A1 protease (Table 1). In fact, for a long time, the polysaccharide capsule was thought to determine virulence because it was always present in freshly isolated clinical strains (14), but immunization with pneumococcal proteins or mutations altering these proteins significantly reduced virulence of inoculated pneumococci, thus confirming a role for both polysaccharides and proteins in the virulence of pneumococci.

The antigenic determinants of the polysaccharide capsule have been used to identify 90 different serotypes of *S pneumoniae* (15). However, only 23 of these induce more than 90% pneumonia; therefore, a 23-valent vaccine is administered to

TABLE 1
Virulence factors associated with *Streptococcus pneumoniae*

Polysaccharide capsule
Peptidoglycan/teichoic acid complex
Pneumolysin
Autolysin
Pneumococcal surface protein A
Pneumococcal surface adhesin A (37 kDa protein)
Neuraminidase
Hyaluronidase
Immunoglobulin A1 protease

the population at risk (16). More than 60% of individuals are healthy carriers of one or many serotypes at a specific time, and most people will be colonized throughout a lifetime. In fact, pneumococci are part of the normal flora of humans and sometimes of animals (17). The same serotypes do not necessarily affect children and adults (18) or express the same virulence (19,20). Serotypes also vary from Asia to Europe to the Americas (16). Types 3, 4, 14 and 19 induce bacteremia most frequently (21). Colonization takes about six weeks in human beings but may last up to one year (21). Most of the time, illness prevails when contamination by a new serotype progresses rapidly (17,22) because slow and prolonged colonization allows protective antibodies to develop (14). During colonization of the nasopharynx, pneumococci face mechanical and humoral barriers (eg, mucociliary clearance, cough, IgA) at the surface of the trachea (23). Different serotypes vary in their capacity to adhere to these cells. Serotypes whose colonial morphology appears transparent on agar plates more successfully and reproducibly colonize the nasopharynx than opaque strains after intranasal infection in experimental animal models (24-26), which suggests that the former have a better capacity to bind to receptors on epithelial cells. Such receptors include the disaccharide GlcNAcB1-3Gal and GlcNAcB1-4Gal present in glycolipids and glycoproteins of the respiratory tract (27,28), and the platelet activating factor (PAF) receptor (29). In fact, these receptors are also present in type II pneumocytes and endothelial cells, which apparently contributes to bacterial dissemination to the bloodstream (29). Additional binding sites may appear when coinfection with influenza virus induces tracheal epithelial cell injury (30). The corresponding ligands on pneumococci include phosphorylcholine in teichoic acid of the cell wall, which recognizes the PAF receptor on epithelial cells (29), and the pneumococcal surface adhesin A, a 37 kDa protein specific to pneumococci and antigenically highly conserved among serotypes (31). The latter has been shown to protect mice when used for immunization (14,32). Thus, adherence is mainly mediated by protein components of the outer surface rather than by capsular polysaccharides.

When bacteria overcome mechanical and humoral barriers at the surface of the trachea (23), they progress, sometimes through aerosols, to the lower respiratory tract where they

reach the alveoli and face phagocytosis by alveolar macrophages. Encapsulated strains are 10^5 more virulent than strains devoid of the capsule (33). Both the thickness and chemical nature of the capsule influence the virulence of a particular serotype (3,34). In fact, virulence may be increased or reduced by the transformation of bacteria with a gene responsible for a particular serotype (35). Serotypes appear to respond differently to the complement (36,37), antibodies (38) and phagocytes, resulting in variable phagocytic efficacy and extent of bacterial proliferation. This issue is further discussed in the next section.

Other bacterial components contribute to the virulence of strains (39), mostly by triggering inflammatory reactions. Although the capsule may induce slight activation of cytokines, cell wall components are 1000 times more potent at inducing inflammation (2,40). The cell wall peptidoglycan/teichoic acid complex stimulates TNF, IL-1 and nitric oxide release, and contributes to lung edema (3,41). Pneumolysin, an intracellular protein that is conserved among pneumococcal serotypes and that is released on bacterial lysis, can create transmembrane pores in lipid bilayers of virtually every type of cell in the lungs (3,17,42-44). Pneumolysin stimulates TNF and IL-1 (45) and can reproduce all the histological features of pneumococcal pneumonia when injected into animal lungs (46). Mutant strains that lack pneumolysin become less virulent than wild-type strains (47,48), and immunization of mice with purified pneumolysin shows significant protection against infection (49). The same observations were made when immunizing animals with the autolysin or other virulence factors (47-51). The pneumococcal surface protein A, a 60 to 200 kDa protein that is expressed at the surface of most clinically isolated pneumococci, is also associated with virulence and high immunogenicity (52-54). Moreover, antigenicity is quite variable among serotypes due to variations in the N-terminal portion of this protein (55). Neuraminidase and hyaluronidase also contribute to colonization and bacteremia by altering, respectively, sialic and hyaluronic acids in host cells (16,56).

Bacterial virulence factors, together with proinflammatory mediators, contribute most likely to membrane injury, thus facilitating bacterial invasion. Although early TNF and IL-1 secretion has been associated with protective immunity in a number of pulmonary disorders (57-61), their combination also mediates cell toxicity (9,62). Considering that PMNs also induce tissue injury through the release of oxygen radicals and degradative enzymes (11,63-66), the concomitant activity of inflammatory cells and mediators, as well as bacterial virulence factors in the first two steps of the pathogenesis process, may initiate membrane injuries that become visible in step 3, thus contributing to bacterial growth inside pneumocytes (67) and bacteremia. In immunocompetent humans, the coincidence of pneumonia and bacteremia ranges from 30% to 50% in the absence of antibiotic therapy, varying with serotype and the population studied (17). Bacteremia is associated with a two- to threefold increase in mortality rates in humans (68). Our own results with mice show that death is directly correlated with bacteremia because 100% of mice that develop bac-

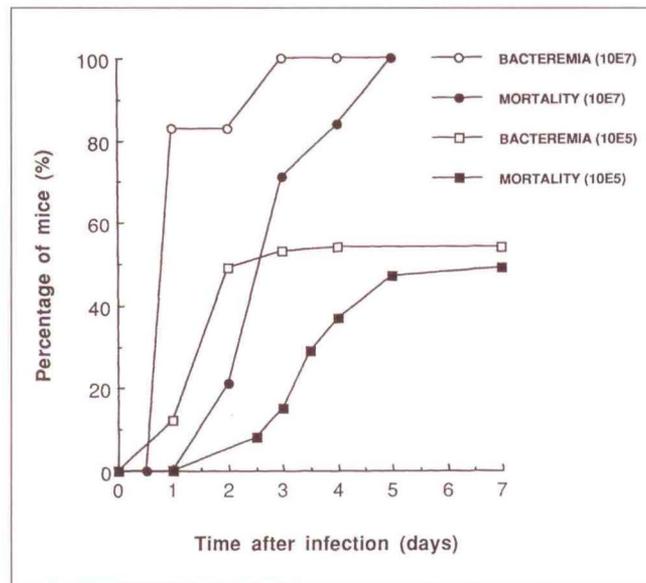


Figure 3) Relationship between bacteremia and mortality in pneumococcal pneumonia in CD1 mice in a maximal lethal dose model of infection with 10^7 bacteria per mouse and in a median lethal dose model of infection with 10^5 bacteria per mouse

teremia in a maximal lethal dose model of infection (10^7 CFU inoculum) die, while 50% of mice die when bacteremia develops in 50% of them after a median lethal dose inoculum (10^5 CFU per mouse) (Figure 3). Thrombocytopenia and leukopenia coincide with bacteremia in both humans and mice; they are considered to be negative prognostic factors in community-acquired pneumonia and sepsis (21,69,70). Although thrombocytopenia may result from interactions of platelets with bacterial toxins, endogenous cytokines also contribute to coagulation anomalies (70,71). During bacteremia, both the liver and spleen participate in bacterial clearance; in particular, the C-reactive protein binds to the C-polysaccharide of the cell wall and acts as an opsonin, facilitating phagocytosis and the killing of pneumococci (72).

INFLAMMATORY CELLS

Once pneumococci reach the lower respiratory tract, immunity is mainly mediated through phagocytosis by alveolar macrophages after opsonization by the complement and antibodies (40,65,73,74) (although direct binding of pneumococci to macrophages through lipoteichoic acid receptors has already been reported) (63). While IgA predominate in the upper airways, IgG and IgM but mostly IgG2 prevail against pneumococci in the lower respiratory tract (73,75). People who have had a splenectomy or cirrhosis, infants, the elderly, alcoholics and immunosuppressed patients who have low antibody reservoirs or reduced phagocytic capacity resist pneumococci poorly (7,12,76-79). Alveolar macrophages may rapidly be overcome by virulent pneumococci, even in immunocompetent hosts because it takes five to seven days for the body to produce effective antibodies against a new invading strain, while antibodies are available after a few hours in BAL fluid through plasma exudation in immunized people (65).

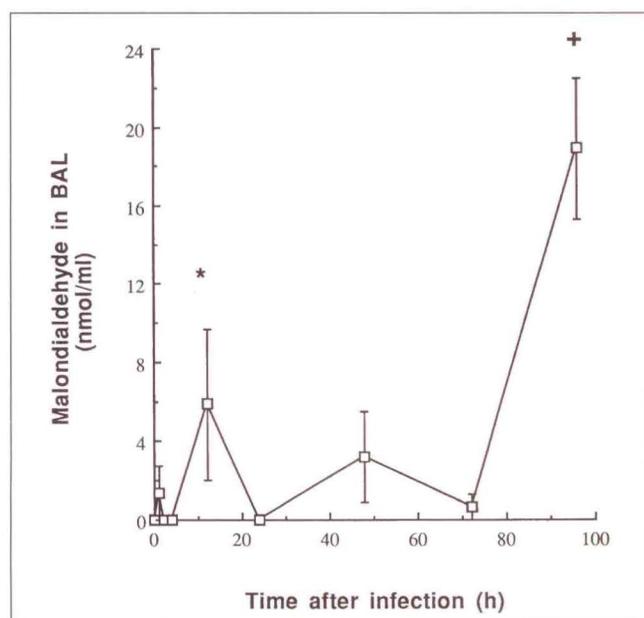


Figure 4 Malondialdehyde levels in bronchoalveolar lavage (BAL) fluid of CD1 mice challenged with intranasal inoculation of 10^7 *Streptococcus pneumoniae* serotype 3. * $P < 0.05$; + $P < 0.01$ compared with preinfection value

The secretion of TNF and chemotactic mediators by alveolar macrophages contributes to the expression of adhesion molecules on endothelial cells and PMN recruitment to the site of infection (80,81). It is of interest that the successive recruitment of polymorphonuclear cells and mononuclear cells in pneumococcal pneumonia closely parallels the successive waves of PMNs, monocytes and lymphocytes that was reported after intratracheal administration of endotoxin, IL-1 or TNF in rats (82).

PMNs are effective bacterial killers through oxidative-dependent and -independent mechanisms. Oxidation of membranes is mediated through the release of oxygen radicals, such as superoxide, hydroxyl radical and hypochloric acid. The respiratory burst that is associated with PMN activation involves the participation of a constitutive enzyme, the MPO, whose detection in tissue homogenates allows quantification of PMN recruitment to the infected site. Enzyme release from PMNs includes elastase and collagenase, which participate in bacterial killing. Unfortunately, both oxidative and nonoxidative mechanisms also contribute to host cell injury because peroxidation of lipid bilayers reduces membrane fluidity, and degradation of lipids in tissues may be followed by malondialdehyde release in fluids, as was observed in step 5 of the pathogenesis process (Figure 4).

The recruitment of PMNs is thought to be mediated by various chemotactic substances, including the C5a fraction of the complement, PAF, LTB_4 and chemokines (75,83-85). LTs are lipid mediators that are synthesized from arachidonic acid from membrane phospholipids under the influence of lipoxygenases that are activated by bacterial toxins. While LTC_4 , LTD_4 and LTE_4 have been associated mostly with asthma and

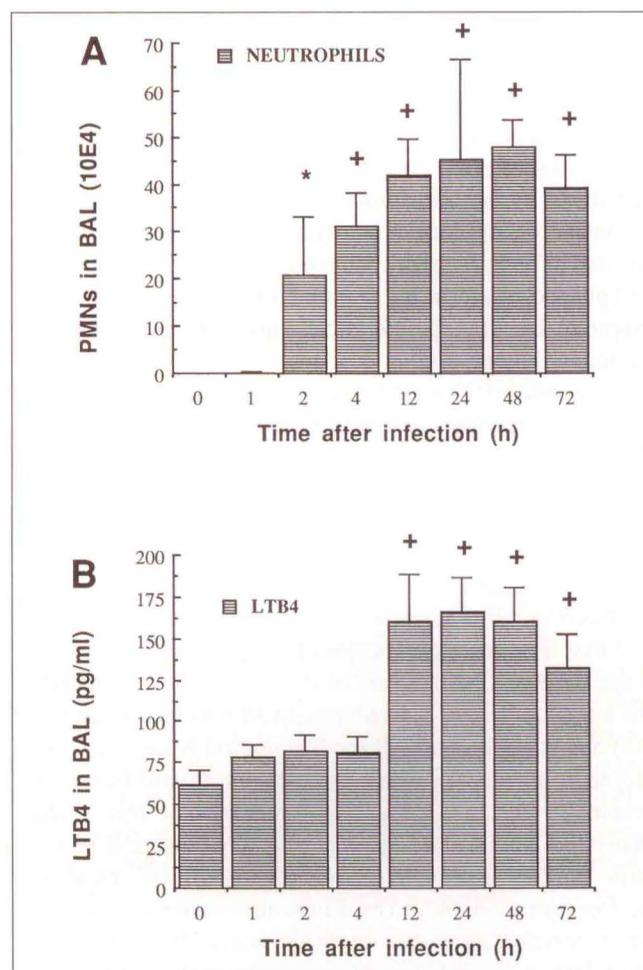


Figure 5 A Polymorphonuclear neutrophil (PMN) counts and B leukotriene B₄ (LTB_4) levels in bronchoalveolar lavage (BAL) fluid of CD1 mice after intranasal challenge with 10^7 *Streptococcus pneumoniae*. * $P < 0.05$; + $P < 0.01$ compared with preinfection value. Reproduced with permission from reference 13

allergy, LTB_4 exerts chemotaxis on PMNs. Although mouse and human PMNs show high reactivity to LTB_4 (86), and levels of this lipid are elevated in various pulmonary infections in animals and humans (65,85,87), LTB_4 does not appear to be the primary chemotactic substance for PMNs in pneumococcal pneumonia, because its synthesis parallels and follows, rather than precedes, PMN influx to the alveoli, as can be seen in step 2 of the pathogenesis process (Figure 5).

Chemokines most likely participate in the early recruitment of PMNs and late monocyte recruitment. IL-8 has been detected in patients with acute pulmonary infection (88,89), and elevated IL-8 levels in BAL fluid correlate with fatal outcome (90). Macrophage inflammatory protein (MIP)-2 is most likely the functional murine homologue of IL-8 because it performs the functions of human IL-8 in mice (83,91,92). It has been associated with lung PMN influx in *Klebsiella pneumoniae* pulmonary infection (89), and most likely plays an important role in pneumococcal pneumonia. Other chemotactic mediators, including granulocyte-colony stimulating factor (G-CSF), also

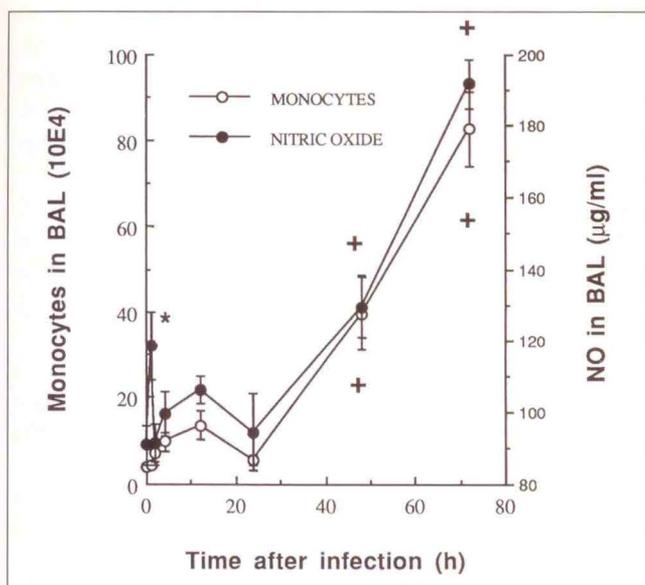


Figure 6 Correlation between monocyte recruitment and nitric oxide (NO) release in bronchoalveolar lavage (BAL) fluid of CD1 mice after intranasal challenge with 10^7 *Streptococcus pneumoniae*. * $P < 0.05$; + $P < 0.01$ compared with preinfection value

stimulate PMN maturation and release from the bone marrow. G-CSF levels are increased in the lungs and blood during pneumococcal pneumonia (93). G-CSF increases the PMN recruitment and survival rate of animals when given as a prophylactic treatment (94). However, the efficacy of late administration when treatment is initiated at different pathogenesis steps of acute pneumonia remains to be confirmed. Overall, the beneficial versus detrimental effects of PMN influx in immunocompetent hosts, which may occur through CD18-dependent and -independent mechanisms (9,95-98), still need to be clarified.

The recruitment of monocytes to the lungs for clearance of debris usually occurs once PMN activity declines and infection subsides (99). Many chemokines increase chemotaxis and phagocytic activity of monocytes/macrophages, including MIP-1 alpha and monocyte chemoattractant protein-1 (89,91). However, in fatal pneumonia, it is not clear what role these chemokines play and to what extent monocytes contribute to bacterial clearance or to increased tissue damage because their recruitment in step 4 of the pathogenesis process is associated with recrudescence of inflammatory mediator release, including nitric oxide, increased tissue injury and death (Figure 6).

Cell-mediated immunity through T lymphocyte activation plays a major role against chronic infections induced mostly by intracellular micro-organisms. On recognition of microbial antigens expressed by antigen-presenting cells, such as monocytes/macrophages, T helper (Th)1 cells stimulate phagocytosis and the killing of micro-organisms through the release of interferon and other activating cytokines, while Th2 cells stimulate B cell proliferation for antibody production. Capsular polysaccharides may also directly stimulate B cell proliferation (16,17). In healthy carriers of pneumococcal

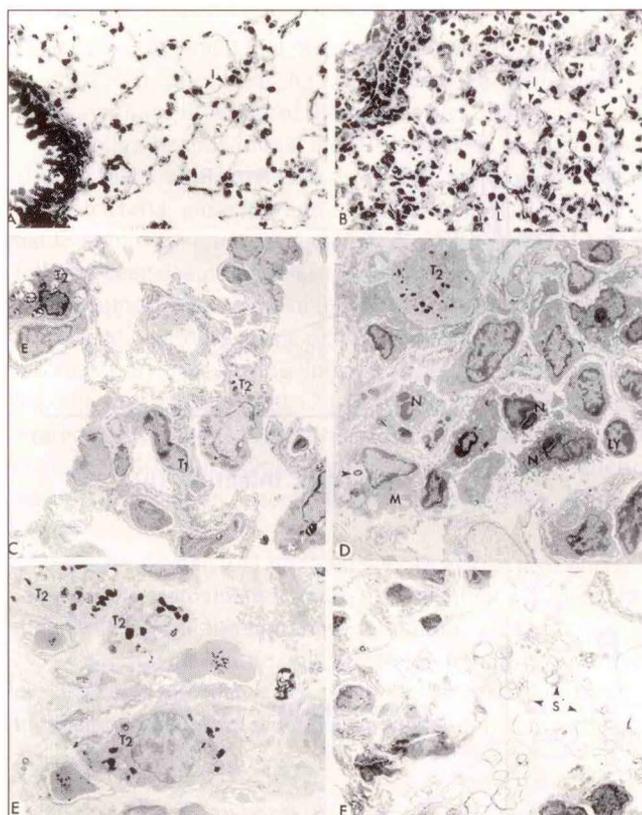


Figure 7 Lung histology of normal mice (A,C) and mice infected with 10^7 *Streptococcus pneumoniae* cells and killed 72 h later (B,D,E,F). Interstitial tissues (I) in alveolar areas (A,B) were enlarged in infected mice (B), and leukocytes (L) can be seen in alveoli. Tissue injury characterized the infectious and inflammatory processes (D). Macrophages (M) containing ingested bacteria (arrowhead) were seen, as well as recruited neutrophils (N) and lymphocytes (LY). Type II pneumocytes (T2) proliferated after infection (E) and secreted abnormal amounts of surfactant (S) in alveoli (F). E Endothelial cell; T1 Type I pneumocyte. A,B magnification $\times 400$; C,D $\times 5000$; E $\times 9600$; F $\times 6000$. Reproduced with permission from reference 13

strains, T and B lymphocytes most likely participate in host defence by elaborating protective antibodies, but in the case of acute pneumococcal pneumonia, death may occur before antibodies to the infecting serotype are produced. The high incidence of pneumococcal pneumonia in people with human immunodeficiency virus and elderly patients provides support for the important roles played by T lymphocytes in host response to pneumococci (100-102). However, lymphocyte recruitment to the lungs occurs late in experimental pneumonia (step 4), and their participation may reflect anti-inflammatory cytokine secretion, such as IL-10. IL-10 has been shown to downmodulate proinflammatory cytokine release, including TNF, IL-1, G-CSF and interferon, as well as nitric oxide and oxygen radicals (89,103,104).

Lung epithelial cells also participate in host response to infection. Type II pneumocytes proliferate from step 3 of the pathogenesis process as a repair mechanism for regeneration of both type II and type I epithelial cells once tissue injury has started (10) (Figure 7). Indeed, edema to tissue interstitium gradually develops (Figure 7A,B) as leukocytes are recruited (Figure 7C,D) and inflammatory mediators are released. While

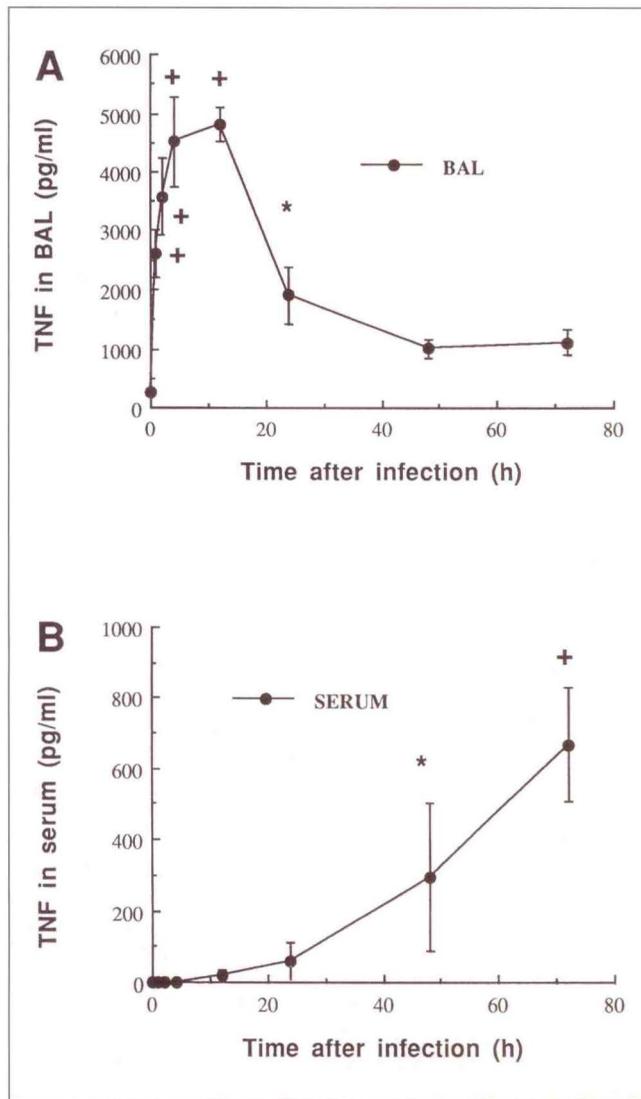


Figure 8 A Tumour necrosis factor (TNF)-alpha levels in bronchoalveolar lavage (BAL) fluid and B serum of CD1 mice after intranasal challenge with 10^7 Streptococcus pneumoniae. * $P < 0.05$; + $P < 0.01$ compared with preinfection value

type I pneumocytes are very sensitive to PMN-induced cytotoxicity (10), type II pneumocytes stimulated by IL-1 express surface receptors for pneumococci, which contribute to infection (3,105). Increased type II to type I ratio (Figure 7E) may play a role in the outcome of pneumonia (106). Also, the abundant surfactant secretion by type II cells (Figure 7F) possibly dampens inflammatory reactions, mostly by reducing pro-inflammatory cytokine levels in the alveoli (10).

INFLAMMATORY MEDIATORS

The pre-septicemic and septicemic phases of pneumonia clearly manifest a confinement of TNF to the pulmonary or systemic site of infection (Figure 8). This phenomenon was also reported in rats after intratracheal or intravenous injection of endotoxin (107) and in patients diagnosed with unilateral pneumonia (108). The down-regulation of TNF in BAL

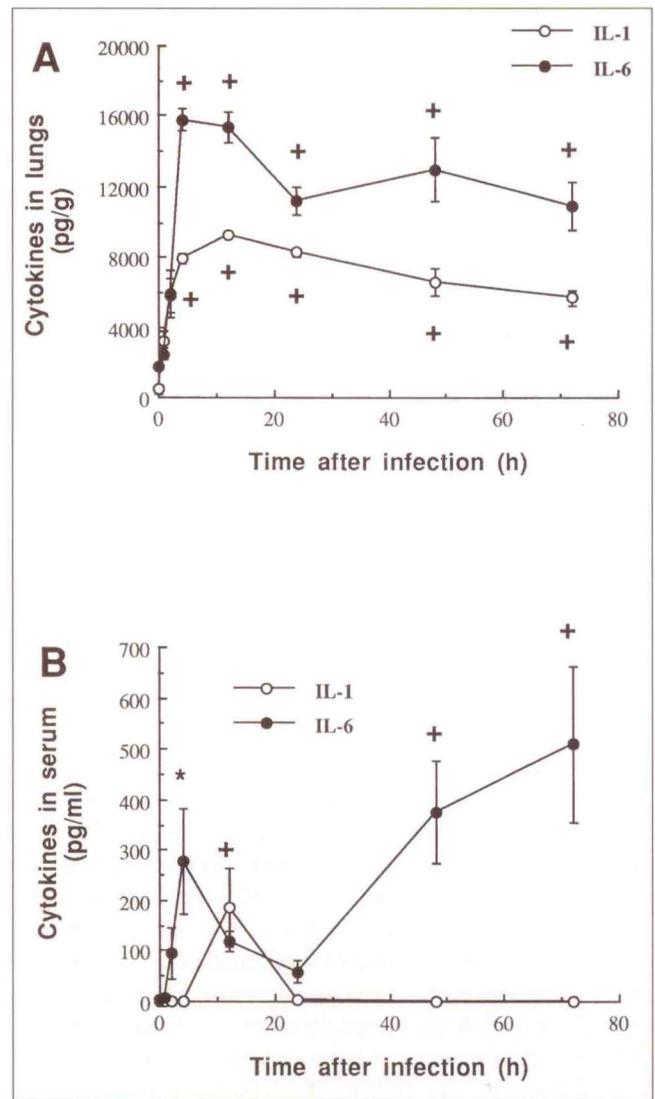


Figure 9 A Interleukin (IL)-1 alpha and IL-6 levels in lung homogenates and B serum of CD1 mice after intranasal challenge with 10^7 Streptococcus pneumoniae. * $P < 0.05$; + $P < 0.01$ compared with preinfection value

fluid after step 2, despite sustained stimulation by bacterial components, also corroborates other data from septicemic animals (109). Thus, in the context of pneumococcal pneumonia, the detection of TNF appears to reflect appropriately the pre-septicemic and septicemic phases of infection.

Our findings in animal pneumococcal pneumonia models indicate that blood levels of cytokines do not reflect tissue levels because the latter remain extremely high from infection to death, while the former show transient appearance only (Figures 8,9). The release of TNF, IL-1 or IL-6 in blood has already been detected in other experimental models, as well as in patients (69,108,110,111), but our observations may explain the discrepancies in the literature concerning the attempts to correlate the outcome of pneumonia with cytokine levels. Blood analysis should be interpreted in terms of overall chronological events that mediate pathogenesis of pneumonia. For example, the presence of high levels of TNF and IL-6 in blood at

the same time in our model indicates fully developed infection with bacteremia; by contrast, increased IL-6 level in blood in the absence of TNF indicates early disease with limited tissue injury and no bacteremia. According to Puren et al (69), IL-6 probably reflects severity of stress rather than severity of infection during various pathological states; thus, early high blood IL-6 levels reflect stress associated with exposure of airways to antigens, and they are associated with physiological derangements.

The monitoring of inflammatory mediators in BAL fluid may also generate markers of the evolution of disease, including cytokines, LTB₄ and nitric oxide. While IL-6 should not be considered in this fluid as a good indicator of the progression of pneumonia, the secretion of TNF and IL-1, by contrast, correlates with early pulmonary inflammatory response. IL-1 α exerts its biological activity mostly in a membrane-associated form and it appears transiently in BAL fluid when peak tissue concentrations are very high, suggesting a spillover from cells to fluids. The detection of IL-1 in serum or BAL fluid may, thus, indicate very active inflammatory processes in tissues. Studying the chronology of cytokine release also reveals that low TNF and IL-1 levels in BAL fluid do not necessarily indicate good health status, especially when nitric oxide is detected, but rather signify transition from steps 1 and 2 to steps 4 and 5 in the pathogenesis process and, thus, an evolution toward a more profound state of illness. Nitric oxide succeeds to TNF in BAL fluid as infection and inflammation progress. In fact, the combination of the profiles of TNF in BAL fluid and blood, and nitric oxide in BAL fluid, provide an accurate estimation of the disease state that chronologically corresponds to a worsening of the pathological score. The profiles could, therefore, be viewed as good biological markers for pneumonia, and antagonists should be investigated at appropriate stages of infection from the perspective of immunotherapy.

Nitric oxide is also released into the lungs during pneumococcal pneumonia, and its secretion correlates with monocyte recruitment and tissue injury (steps 4 to 5) (Figure 6). This provides support for monocytes/macrophages as the main sources of nitric oxide during pneumococcal pneumonia, and for a potential cytotoxic role for this molecule and the detrimental effects of massive monocyte recruitment. Nitric oxide is secreted by alveolar and interstitial macrophages during endotoxemia and *Pneumocystis carinii* pneumonia (87,112), but type II pneumocytes, endothelial cells, fibroblasts and lymphocytes can also release nitric oxide (113-117). In fact, nitric oxide production appears to be biphasic during pneumococcal pneumonia: an early but transient release (step 1) that possibly results from the activation of constitutive nitric oxide synthases by resident alveolar macrophages under the influence of bacterial virulence factors or TNF

(112,118-120), and a late massive and sustained release that most likely results from the activation of an inducible nitric oxide synthase in recruited monocytes and other cells.

Although several animal models have been used to demonstrate a role for nitric oxide as an effector molecule for the killing of bacteria, parasites and fungi (119,121,122), and nitric oxide enhances immunity against *K pneumoniae* (121), no link has been demonstrated so far between nitric oxide release and pneumococcal counts in tissue. In addition to its potential microbicidal activity, nitric oxide possibly has a multifaceted inflammatory role during infection, ranging from capillary leakage and edema (119,123,124), through modulation of leukocyte activity (119,120,125,126), to tissue cytotoxicity (117,127-130). Nitric oxide may affect cell activity by altering signal transduction, energy production and DNA synthesis (114,127).

Reactive oxygen and nitrogen species also act in concert to induce lipid peroxidation, defective membrane permeability and cell injury through the formation of peroxynitrites (131,132). It is not surprising, therefore, that nitric oxide overproduction in pneumococcal pneumonia is associated with a high histopathological score, lipid peroxidation and death (step 5).

CONCLUSION

Five major pathogenesis steps can be identified from the initial infection to death in pneumococcal pneumonia. They involve the successive recruitment of PMNs, monocytes and lymphocytes; the pulmonary and/or systemic release of inflammatory mediators that characterize the prebacteremic and bacteremic phases of infection; and the participation of parenchymal cells in the host response. Although the kinetics of cytokines differ considerably from blood to lung tissue to alveoli, and blood levels do not correlate to tissue levels, the kinetics of TNF and IL-6 in blood, as well as TNF and nitric oxide in BAL fluid, are good indicators of the evolution of the disease. Nitric oxide release is biphasic and corresponds mostly to monocyte recruitment in BAL fluid and concomitant serious tissue injury. Monocyte counts in sputum, a very simple test, should be further investigated. Pneumococci activate LT release, but PMN recruitment is not primarily mediated by LTB₄. Bacteremia, leukopenia, thrombocytopenia and lipid peroxidation closely precede death. Knowledge of the chronology of microbiological and inflammatory events that occur during pneumonia may help to design appropriate diagnostic tests that could be used to monitor the evolution of this deadly infection. There has been an explosive growth in the use of biological response modifiers that may be given to treat pneumonia. The proper use of these agents requires prior identification of biological markers in people with pneumonia.

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