THE IMPACT OF TIME ON THE SYTEMIC INFLAMMATORY RESPONSE IN PNEUMOCOCCAL PNEUMONIA

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RUNNING TITTLE: TIME, CYTOKINES AND PNEUMOCOCCAL PNEUMONIA
ABSTRACT

OBJECTIVE: To analyze the impact of time from onset of symptoms on the systemic cytokine concentrations in patients with pneumococcal pneumonia.

METHODS: Adults with severe pneumococcal pneumonia were prospectively included. At admission, vital signs, time from onset of pneumonia symptoms and circulating levels of CRP, serum amyloid A (SAA), TNFα, IL1β, IL6, IL8, IL10, and IL1ra were recorded.

RESULTS: 32 patients were included; 13 patients had <48h of evolution and 19 patients had been sick for >48h. The group with a longer time of evolution presented higher plasmatic levels of TNF-α (19.1(SD8.5) vs. 35.5(SD26) pg/mL; p=0.035), fibrinogen (6(SD1.8) vs. 9(SD2); p=0.001); CRP (130 (SD 85) vs. 327(SD 131; p=0.000) and SAA (678 (SD 509) vs. 984 (SD 391); p=0.025). Concentrations of TNFα were associated with the presence of bacteraemia (p=0.008), initial blood pressure < 90 mm Hg (p=0.050), and with a lower oxygen saturation at admission (p=0.047). Likewise, TNFα levels were correlated with concentrations of IL1β (r=0.49, p=0.008), IL6 (r=0.41, p=0.03), and IL8 (r=0.40, p=0.03)

CONCLUSIONS: In pneumococcal pneumonia, patients with a longer time of evolution presented with higher levels of pro-inflammatory cytokines and a higher expression of acute phase proteins, suggesting a sustained release of pneumococcal antigens over time.

KEY WORDS: Cytokines; Immunology (infection); Innate immune response; Pneumonia; Streptococcus pneumoniae.
INTRODUCTION:

*S. pneumoniae* is the most frequent isolated organism in patients with community-acquired pneumonia. Pneumococcal pneumonia is characterized by an intense inflammatory response induced mainly by cell wall components and orchestrated by cytokines. [1] Tumor necrosis factor α (TNFα) is one of the earliest mediators of the inflammatory response and induces a second wave of pro and anti-inflammatory cytokines which are mediators of the inflammatory process. Its activity has been shown to be a critical factor in the protective response in pneumococcal pneumonia. [2,3] Although a great deal of information on pneumococcal pneumonia is now available, the chronology of infection and the inflammatory response in humans remain to be determined.

One of the most unexplored factors is time from onset of symptoms to hospital admission. Infection is a dynamic process and time represents one of the main contributors to bacterial growth. Data suggest that time can be a critical factor in the evolution of sepsis. In a recent study, time to initiation of effective antimicrobial therapy was the single strongest predictor of outcome in patients with septic shock. [4] In the same vein, a large and well designed prospective study in patients with community acquired pneumonia has shown that at the time of presentation to hospital systemic cytokine concentrations had already peaked indicating that the cytokine cascade was already activated by the time patients sought hospital care. [5] This also could explain why a delay in antimicrobial therapy could influence the prognosis of community acquired pneumonia (CAP). [6,7]

The magnitude of the inflammatory response in patients with severe pneumococcal pneumonia could be due, at least in part, to the time that had elapsed from onset of symptoms to the initial determination of cytokines at hospital admission. To test this
hypothesis, we analysed the impact of time from onset of symptoms to hospital admission on pro and anti-inflammatory cytokines concentrations, acute phase reactants production, severity of disease and outcomes in patients with severe pneumococcal pneumonia. We also analysed the relationship between TNFα serum levels at admission and clinical presentation, severity of disease, inflammatory response, and presence of bacteraemia.

MATERIAL AND METHODS

SETTING AND STUDY DESIGN: Consecutive adults with pneumonia classes III, IV and V of the Pneumonia Severity Index (PSI) [8] and with a confirmed pneumococcal aetiology were included. This cohort of patients has been previously described in a study reported elsewhere. [9] A new analysis with no data duplication has been conducted. The local ethics and research committee approved the study and informed consent was obtained from all patients. Inclusion and exclusion criteria have been described elsewhere. [9]

On admission, data were prospectively collected and included demographics, smoking and alcohol habits, comorbidities based on the Charlson [10] score, prognosis measured by the PSI[8] and APACHE II scores, [11] consumption of statins, use of non-steroidal or steroidal anti-inflammatory drugs during the process, length-of-stay, and 30-day mortality.

Accurate information of time between pneumonia onset and inclusion as referred by the patient or his/her relatives was obtained by one of the investigators (EC). The patient or his/her relatives were questioned about the moment in which an abrupt worsening in
his/her general state took place, with or without the simultaneous presence of chills and fever.

Data on vital signs (heart rate, blood pressure, axillary temperature, respiratory rate) and oxygen saturation while patients were breathing room air were obtained at entry.

**COLLECTION OF BLOOD SAMPLES AND LABORATORY PROCESSING:**

Blood samples were collected at inclusion, immediately prior to initiation of antibiotic therapy. Samples were centrifuged at 1500 x g for 15 min at 4 ° C, distributed in 4 aliquots of 2 mL and stored at – 80°C. Circulating levels of CRP, serum amiloyd A (SAA) and cytokines TNFα, IL1β, IL6, IL8, IL10 and IL1ra were measured. The procedure is described elsewhere. [9]

**MICROBIOLOGICAL STUDIES.** In all patients, two sets of blood cultures were obtained prior to commencing antibiotic therapy. Blood cultures were processed with the system BacT-Alert® (bioMérieux, Durham, EE.UU.) When available, sputum samples were processed for Gram stain and culture. Only sputum samples with less than 10 squamous epithelial cells/low power field and more than 25 polymorphonuclear cells/LPF were accepted for Gram stain and culture.

The pneumococcal aetiology was also pursued by the detection of *S pneumoniae* antigen in urine (Binax NOW® *S pneumoniae Urinary Antigen Test;* Binax). Urine samples were boiled for 5 minutes and centrifuged at 1000xg for 15 min; to optimise time to diagnosis the first unconcentrated urine was used; if the test resulted negative the urine was then concentrated 25-fold by selective ultra filtration (Urifil-10 Concentrator; Millipore Corporation, Bedford, MA, USA).

**STATISTICAL ANALYSIS:**

Evolution of the inflammatory response was assessed. Two groups were considered: Patients that came to hospital within a time frame below the median of the whole cohort
were considered the Early comers group. Those coming to hospital after this median time were considered the Late comers group.

Normally distributed data were compared using unpaired t-tests. To assess normality Kolmogorov-Smirnov test was used.

Nonparametric tests were used to compare not normally distributed variables: Mann-Whitney U-test was used to compare quantitative variables between groups, F Fisher to compare qualitative variables.

In a similar fashion, we analyzed the relation among TNFα serum levels at admission and clinical presentation, inflammatory response, presence of bacteraemia and severity. To assess these relations we applied r Spearman test for quantitative variables and Mann Whitney U test for qualitative variables.

Data analysis was performed with the use of SPSS software, version 13. Statistical significance was taken as a p-value ≤ 0.05.

RESULTS:
Thirty two patients with pneumococcal pneumonia were included. The pneumococcal aetiology was confirmed in all patients. Twenty six (81%) had a positive antigen in urine, 14 (43%) were bacteraemic, in 14 (43%) diplococci were present in sputum Gram stain, and in 8 (25%) S pneumoniae was isolated in sputum cultures. In fact, most patients had several positive tests. Only four patients had a pneumococcal aetiology sustained exclusively on the sputum results; all had diplococci in sputum Gram stain and positive sputum cultures for pneumococci.

Time from onset of symptoms to hospital admission ranged from 3 to 168 hours, with a median of 48h, and a mean of 58 h (SD 48h). Thirteen patients were included within a
time frame below 48h (Early comers group) and the other 19 patients at 48 h or after from onset of symptoms (Late comers group). Both groups were homogenous and without significant differences in terms of age (mean age in the Early comers group was 70.3 years vs. 65.7 years in the Late comers group), presence of co morbidities, previous statin therapy (15.4% of the Early comers vs. 11.1% of the Late comers), smoking habit (31% vs. 47%), alcohol consumption (38.5% vs. 15.8%) and lieu of residence.

The Late comers group, with a longer time of evolution at entry, presented higher plasmatic levels of fibrinogen, serum amyloid A and lower albumin concentrations. Among all cytokines studied only TNF-α showed higher concentrations in the patients who sought hospital care later. (Table 1)

Severity of disease at presentation (measured by PSI and APACHE scores), presence of bacteremia and radiological involvement were also similar among groups (table 2). Concentrations of TNF-α irrespective of time from onset to admission, were associated with the presence of bacteremia (p=0.008); initial blood pressure < 90 mm Hg, (p=0.050) and with a lower oxygen saturation (p=0.047) at admission. Likewise, TNFα levels were correlated with concentrations of IL1β (r = 0.49; p=0.008); IL6 (r = 0.41; p=0.03); and (IL 8 r = 0.40; p=0.03). No differences were found with the other measured cytokines.

**DISCUSSION**

We studied a homogenous group of patients with severe and well documented pneumococcal pneumonia. In a previous analysis [9] we described the evolution of the inflammatory response upon the initiation of antimicrobial therapy. In the present study
we have analysed the impact of time that had elapsed from onset of symptoms to inclusion in the initial immune response. In this group of patients, those with a longer time of evolution presented at inclusion higher levels of pro-inflammatory cytokines and a higher expression of acute phase proteins, and tended to be more severely ill. These data suggest that a sustained release of pneumococcal antigens took place over time which led to a higher pro-inflammatory pattern. Of note, none of the patients had received any antibiotic previously, thus the release of cell wall components and other proinflammatory antigens could only be attributable to the spontaneous lysis of bacterial cells in the phase of an increasing inoculum.

Bacterial growth in the alveolar space is a time dependent process. In a pneumococcal pneumonia mice model, [12] bacterial growth was shown to reach a plateau of $10^7$ cfu/gram in lung tissue after 36 h post inoculation. However, the inflammatory response further amplified after 36 h and peak levels of most mediators were observed at 84 h post infection. This late burst of inflammation was most likely due to lysis of dying pneumococci and release of large amounts of toxins rather than by living pneumococci in a well established infection. On the other hand, in the group of mice with higher bacterial growth more animals became bacteraemic as the infection developed in the lungs (25% at 36 h, 60% at 60 h and 100% at 84h). Higher levels of inflammatory mediators were observed as bacteria reached the bloodstream, as well.

To our knowledge, the cytokine profile in relation to time from onset of symptoms in human pneumonia has not been previously described. Intuitively, we associate a higher inflammatory response with a longer time of infection evolution and a more severe clinical presentation. This has been indirectly demonstrated by the relation between timeliness of antibiotic administration with a better outcome in sepsis [13] and in CAP.(6,7) In the latter, two large retrospective studies focused on antibiotic timing in
patients older than 65 years and showed that mortality was lower among those who received antibiotic within 8 h and 4 h from hospital arrival, respectively. No timing outcome association could be demonstrated among patients less than 65 years old. Although these two retrospective studies have some limitations (they did not evaluate time from onset of symptoms before patients sought medical care) they reflect how the time elapsed from hospital admission to the initiation of antimicrobial therapy has a close parallel with the evolving dynamics of the inflammatory response, and how this process is critical in determining pneumonia severity and outcome.

Among all cytokines studied only TNF-α showed higher concentrations in the Late comers group. This is in agreement with the mice pneumococcal pneumonia model. Bergeron et al. [14] analyzed cytokine kinetics in mice with pneumococcal pneumonia. TNF-α was absent or detected in very low levels in serum until 48 and 72 h and then rapidly increased. This increase paralleled the migration of bacteria to the bloodstream. In the same model, IL 1β showed a transient appearance in serum, and IL 6 levels remained elevated during the whole experiment.

TNF-α is known to play a key role in the immune response against *S pneumoniae*. Pneumococcal cell wall components [15] and pneumolysin [16] are potent stimulators of production of TNF-α by human monocytes in vitro. Increased susceptibility to infection and higher bacterial loads have been found in mice strains with reduced capacity to produce TNFα or following systemic neutralization of TNF-α during pneumococcal pneumonia. [17] In our study, TNF-α level correlated with the presence of bacteraemia as well. Bacteraemia has been repeatedly associated with higher levels of TNF-α in the murine model. [14,18] This association could be explained by a higher offer of cell wall components in the bloodstream that stimulates the release in blood of TNF-α. In addition, TNF-α expression can cause up regulation of receptors implicated
in tissue invasion of pneumococci such as the platelet-activating factor receptor, favoring the development of bacteraemia. [19] Conceivably, TNF-α level could be used as predictors of the risk of bacteraemia in pneumococcal pneumonia.

Our data show a correlation between IL1β, IL6 and IL8 concentrations and TNF-α level. All these ILs are secreted by macrophages as a part of the innate immune response. TNFα also plays a major role in the clinical manifestations of septic shock. [20,21] This could explain that higher TNF-α levels were associated with blood pressure < 90 mm Hg and with lower oxygen saturation.

Many anti-inflammatory strategies have failed to improve survival in pneumonia. [22] It seems that timing is crucial to achieve an optimal modulation of the inflammatory response. In the mice model, increased survival has been observed when proinflammatory compounds were injected in concomitance with the inoculum suggesting that a stronger inflammatory response in the early hours of infection can be beneficial. [12] By the same token, when anti TNF-α was administered together with antibiotic 25 h after the induction of pneumococcal pneumonia, it led to decreased survival, and was associated with enhanced bacterial outgrowth. [23] This could be due to the pneumococcus ability to inhibit some components of the host defense in the early stages of infection and hence continue its multiplication without being eliminated. [24,25] In clinical practice, patients arrive to hospital in a wide range of stages of their infection and the key to improve the management of the inflammatory response could be to select those severe ill patients in the very early phases of the infection that could benefit from the use of an inflammatory modulator.

Our study has some limitations. First, time from onset of symptoms is not an exact measure and it has a strong subjective component, and could be influenced by several circumstances such as patient tolerance. Even so, a correlation between this subjective
impression and the inflammatory pattern was found. The small sample size has not allowed us to find other important differences in the patterns of inflammatory response. We could not establish a direct relationship between the intensity of the inflammatory response and the severity of the episode probably because only patients with severe pneumonia were included in our study. Finally, only one death occurred during the study and no firm statement can be made about the clinical relevance of the changes we have observed.

In summary, we found that patients with severe pneumococcal pneumonia with a longer time of evolution at inclusion presented higher levels of pro-inflammatory cytokines and a higher expression of acute phase reactant proteins. To our knowledge, no previous studies have evaluated the impact of time from onset of symptoms on the inflammatory response. Bacterial growth and the inflammatory response that it generates are dynamic processes, and this time variable must be taken into account in the analysis of the cytokine production pattern.

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REFERENCES


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<th>EARLY COMERS GROUP</th>
<th>LATE COMERS GROUP</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>&lt; 48 H N=13</td>
<td>&gt; 48 H N=19</td>
<td></td>
</tr>
<tr>
<td>TNFα pg/mL; (SD)</td>
<td>19.1 (8.5)</td>
<td>35.5 (26)</td>
<td>0.03</td>
</tr>
<tr>
<td>IL 1β pg/mL; (SD)</td>
<td>6.7 (9)</td>
<td>3.4 (4)</td>
<td>0.2</td>
</tr>
<tr>
<td>IL 8 pg/mL; (SD)</td>
<td>79.5 (112)</td>
<td>175 (573)</td>
<td>0.5</td>
</tr>
<tr>
<td>IL 6 pg/mL; (SD)</td>
<td>3569 (6646)</td>
<td>2122 (3149)</td>
<td>0.4</td>
</tr>
<tr>
<td>IL 10 pg/mL; (SD)</td>
<td>44 (40)</td>
<td>23 (28)</td>
<td>0.09</td>
</tr>
<tr>
<td>IL 1 ra pg/mL; (SD)</td>
<td>8754 (7734)</td>
<td>7379 (7182)</td>
<td>0.6</td>
</tr>
<tr>
<td>CRP pg/mL; (SD)</td>
<td>130 (85)</td>
<td>327 (131)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>SAA pg/mL; (SD)</td>
<td>678 (509)</td>
<td>984 (391)</td>
<td>0.025</td>
</tr>
<tr>
<td>FIBRINOGEN mg/dL</td>
<td>6 (1.8)</td>
<td>9 (2)</td>
<td>0.001</td>
</tr>
<tr>
<td>ALBUMIN mg/dL</td>
<td>40 (5)</td>
<td>36 (5)</td>
<td>0.043</td>
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TABLE 2. TIME FROM ONSET OF SYMPTOMS, SEVERITY OF DISEASE AND OUTCOME.

<table>
<thead>
<tr>
<th></th>
<th>EARLY COMERS GROUP ≤ 48 H</th>
<th>LATE COMERS GROUP &gt; 48 H</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>Bilobar Rx involvement</td>
<td>3 (23%)</td>
<td>8 (42%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Empyema</td>
<td>1 (8%)</td>
<td>2 (10.5%)</td>
<td>0.6</td>
</tr>
<tr>
<td>PSI Class IV</td>
<td>7 (54%)</td>
<td>13 (68%)</td>
<td>0.4</td>
</tr>
<tr>
<td>PSI Class V</td>
<td>2 (15%)</td>
<td>5 (26%)</td>
<td>0.6</td>
</tr>
<tr>
<td>APACHE; mean; (SD)</td>
<td>13.6 (4)</td>
<td>15.2 (4.5)</td>
<td>0.3</td>
</tr>
<tr>
<td>Shock</td>
<td>2 (15%)</td>
<td>1 (5%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>4 (31%)</td>
<td>10 (53%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>0</td>
<td>2 (10%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Length of stay, days; (SD)</td>
<td>10.6 (5.1)</td>
<td>12.16 (15.2)</td>
<td>0.7</td>
</tr>
<tr>
<td>30-day-mortality</td>
<td>0</td>
<td>1 (5%)</td>
<td>0.5</td>
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