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The impact of time on the systemic inflammatory response in pneumococcal pneumonia

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ABSTRACT: The aim of our study was to analyse the impact of time from onset of symptoms on the systemic cytokine concentrations in patients with pneumococcal pneumonia.

Adults with severe pneumococcal pneumonia were prospectively included. At admission, vital signs, time from onset of pneumonia symptoms and circulating levels of C-reactive protein (CRP), serum amyloid A (SAA), tumour necrosis factor (TNF)-α, and interleukin (IL)-1β, IL-6, IL-8, IL-10 and IL-1ra were recorded.

32 patients were included; 13 patients had <48 h of evolution and 19 patients had been sick for >48 h. The group with a longer time of evolution presented higher plasmatic levels of TNF-α (19.1±8.5 *versus* 35.5±26 pg·mL^{-1}), fibrinogen (6±1.8 *versus* 9±2); CRP (130±85 *versus* 327±131) and SAA (678±509 *versus* 984±391). Concentrations of TNF-α were associated with the presence of bacteraemia, initial blood pressure <90 mmHg and with a lower oxygen saturation at admission. Likewise, TNF-α levels were correlated with concentrations of IL-1β (r=0.49), IL-6 (r=0.41) and IL-8 (r=0.40).

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.

KEYWORDS: Cytokines, immunology (infection), innate immune response, pneumonia, *Streptococcus pneumoniae*

The most frequently isolated organism in patients with community-acquired pneumonia (CAP) is *Streptococcus pneumoniae*. Pneumococcal pneumonia is characterised by an intense inflammatory response induced mainly by cell wall components and orchestrated by cytokines [1]. Tumour necrosis factor (TNF)-α is one of the earliest mediators of the inflammatory response and induces a second wave of pro- and anti-inflammatory cytokines that 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 of pneumococcal pneumonia 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 CAP 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 could also explain why a delay in antimicrobial therapy could influence the prognosis of 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, production of acute phase reactants, severity of disease and outcomes in patients with severe pneumococcal pneumonia.

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We also analysed the relationship between TNF-α 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 now been conducted. The local ethics and research committee (Hospital Universitari Mútua de Terrassa, Barcelona, Spain) 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 score by CHARLSON *et al*. [10], prognosis measured by the PSI [8] and Acute Physiology and Chronic Health Evaluation (APACHE) II scores [11], consumption of statins, use of nonsteroidal 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 indicated by either the patient or his/her relatives was obtained by one of the investigators (E. Calbo). Either the patient or his/her relatives were questioned about the moment at 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 $1,500 \times g$ for 15 min at 4°C, distributed in four aliquots of 2 mL and stored at -80°C. Circulating levels of C-reactive protein (CRP), serum amyloid A (SAA) and cytokines TNF-α, interleukin (IL)-1β, -6, -8, -10 and -1ra 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 BacT-Alert® system (bioMérieux Inc., Durham, NC, USA). When available, sputum samples were processed for Gram staining and culture. Only sputum samples with <10 squamous epithelial cells per low-power field and >25 polymorphonuclear cells per low-power field were accepted for Gram staining 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, Scarborough, ME, USA). Urine samples were boiled for 5 min and centrifuged at $1,000 \times g$ for 15 min. To optimise time to diagnosis the first nonconcentrated urine was used; if the test result was 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. The Kolmogorov–Smirnov test was used to assess normality.

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

In a similar fashion, we analysed the relationships between TNF-α serum levels at admission and clinical presentation, inflammatory response, presence of bacteraemia and severity. To assess these relationships, we applied the Spearman test (r) for quantitative variables and the Mann–Whitney U-test for qualitative variables.

Data analysis was performed with the use of SPSS software, version 13 (SPSS Inc., Chicago, IL, USA). Statistical significance was taken as a p-value $\leqslant 0.05$.

RESULTS

32 patients with pneumococcal pneumonia were included and pneumococcal aetiology was confirmed in all patients. 26 (81%) had a positive antigen in urine, 14 (44%) were bacteraemic; in 14 (44%), diplococci were present in sputum Gram stain, and in eight (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 h, with a median of 48 h and a mean±SD of 58±48 h. 13 patients were included within a time frame of <48 h (early-comers group) and the other 19 patients at ⩾48 h from onset of symptoms (late-comers group). Both groups were homogenous and without significant differences in terms of age (the mean age in the early-comers group was 70.3 yrs *versus* 65.7 yrs in the late-comers group), presence of comorbidities, previous statin therapy (15.4% of early comers *versus* 11.1% of late comers), smoking habit (early comers 31% *versus* latecomers 47%), alcohol consumption (early comers 38.5% *versus* latecomers 15.8%) and lieu of residence.

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

TABLE 1 Time from onset of symptoms and inflammatory response

	Early-comers group <48 h	Late-comers group ⩾48 h	p-value
Subjects n	13	19	
TNF-α pg·mL^{-1}	19.1±8.5	35.5±26	0.03
IL-1β pg·mL^{-1}	6.7±9	3.4±4	0.2
IL-8 pg·mL^{-1}	79.5±112	175±573	0.5
IL-6 pg·mL^{-1}	3569±6646	2122±3149	0.4
IL-10 pg·mL^{-1}	44±40	23±28	0.09
IL-1ra pg·mL^{-1}	8754±7734	7379±7182	0.6
CRP pg·mL^{-1}	130±85	327±131	<0.001
SAA pg·mL^{-1}	678±509	984±391	0.025
Fibrinogen mg·dL^{-1}	6±1.8	9±2	0.001
Albumin mg·dL^{-1}	40±5	36±5	0.043

Data are presented as mean±SD, unless otherwise indicated. TNF: tumour necrosis factor; IL: interleukin; CRP: C-reactive protein; SAA: serum amyloid A.

Severity of disease at presentation (measured by PSI and APACHE scores), presence of bacteraemia 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 bacteraemia (p=0.008), an initial blood pressure <90 mmHg (p=0.050) and a lower oxygen saturation (p=0.047) at admission. Likewise, TNF-α levels were correlated with concentrations of IL-1β (r=0.49; p=0.008); IL-6 (r=0.41; p=0.03) and IL-8 (r=0.40; p=0.03). No differences were found with the other measured cytokines.

TABLE 2 Time from onset of symptoms, severity of disease and outcome

	Early-comers group <48 h	Late-comers group >48 h	p-value
Subjects n	13	19	
Bilobar Rx involvement	3 (23)	8 (42)	0.1
Empyema	1 (8)	2 (10.5)	0.6
PSI class IV	7 (54)	13 (68)	0.4
PSI class V	2 (15)	5 (26)	0.6
APACHE score	13.6±4	15.2±4.5	0.3
Shock	2 (15)	1 (5)	0.3
Bacteraemia	4 (31)	10 (53)	0.1
Mechanical ventilation	0	2 (10)	0.3
Length of stay days	10.6±5.1	12.16±15.2	0.7
30-day mortality	0	1 (5)	0.5

Data are presented as n (%) or mean±SD, unless otherwise indicated. Rx: chest radiograph; PSI: Pneumonia Severity Index; APACHE: Acute Physiology and Chronic Health Evaluation.

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 with 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. It is noteworthy that none of the patients had previously received any antibiotics, thus the release of cell wall components and other pro-inflammatory 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 mouse model [12], bacterial growth was shown to reach a plateau of 10^7 cfu·g^{-1} in lung tissue 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 to be due to lysis of dying pneumococci and release of large amounts of toxins rather than by living pneumococci in a well-established infection. Conversely, 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 84 h). In addition, higher levels of inflammatory mediators were observed as bacteria reached the bloodstream.

To our knowledge, in human pneumonia, the cytokine profile has not been previously described with respect to its relationship with time from onset of symptoms. Intuitively, we associate a higher inflammatory response with a longer period of infection evolution and a more severe clinical presentation. This has been indirectly demonstrated by the relationship 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 aged >65 yrs and showed that mortality was lower among those who received antibiotics within 8 h and 4 h of arrival at hospital. No timing outcome association could be demonstrated among patients aged <65 yrs. 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 closely parallels 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] analysed cytokine kinetics in mice with pneumococcal pneumonia. TNF-α was either absent or detected in very low levels in serum until 48 h and 72 h, and then increased rapidly. This increase paralleled the migration of bacteria into 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 a reduced capacity to produce TNF-α or following systemic neutralisation of TNF-α during pneumococcal pneumonia [17]. In our study, TNF-α levels also correlated with the presence of bacteraemia. 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 upregulation of receptors implicated in tissue invasion of pneumococci, such as the platelet-activating factor receptor, favouring the development of bacteraemia [19]. Conceivably, TNF-α level could be used as a predictor of the risk of bacteraemia in pneumococcal pneumonia.

Our data show a correlation between IL-1β, -6 and -8 concentrations and TNF-α levels. All these ILs are secreted by macrophages as a part of the innate inmunitary response. TNF-α also plays a major role in the clinical manifestations of septic shock [20, 21]. This could explain the fact that higher TNF-α levels were associated with blood pressure $<$90 mmHg 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 pro-inflammatory 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 defence in the early stages of infection and hence continue its multiplication without being eliminated [24, 25]. In clinical practice, patients arrive at hospital in a wide range of stages of their infection and the key to improving the management of the inflammatory response could be to select those severely ill patients in the very early phases of the infection who could benefit from the use of an inflammatory modulator.

Our study has some limitations. First, the time from onset of symptoms is not an exact measure; 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. Secondly, the small sample size has not allowed us to find other important differences in the patterns of inflammatory response. We were unable to 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 with 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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STATEMENT OF INTEREST

None declared.

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