Research Article

Bronchoalveolar lavage fluid cytokine bead array profile for prognostication of ventilated trauma patients

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Abstract

Aim of Study: Ventilator-associated pneumonia (VAP) is a common cause of mortality in trauma patients admitted to Intensive Care Units. The outcome of such patients may be dependent on local host immune response, which may be best reflected in studies using bronchoalveolar lavage (BAL) fluid. The present study was conducted to ascertain the cytokine profile of BAL using the cytometric bead array (CBA) in a flow cytometer and to correlate the levels of Th-1/Th-2 cytokines in BAL with the clinical outcome of ventilated trauma patients. **Patients and Methods:** BAL was collected from the patients with suspected VAP. CBA was performed to assess the levels of interleukin-4 (IL-4), IL-6, IL-8, IL-1 β, interferon gamma (IFN-γ), and tumor necrosis factor-alpha in the BAL samples. After acquiring the BAL samples on the flow cytometer, the results were generated using FCAP Array™ software. The cytokine profile was correlated to clinical outcomes. **Results:** A total of forty patients were enrolled during the study period. Of these, 12 patients (30%) had confirmed VAP and 8 (20%) patients had a fatal outcome. The levels of IL-8 and IFN-γ correlated significantly with the development of VAP and elevated IL-6 in BAL was associated with a poor outcome. **Conclusion:** A proinflammatory response in the form of elevated IL-6 and IL-8 correlated poorly with the clinical outcome. Th-1 response was significantly reduced in patients with VAP. A proinflammatory response in the form of elevated IL-6 and IL-8 correlated poorly with the clinical outcome.

Keywords: Bronchoalveolar lavage, cytokine bead array, prognosis, trauma, ventilator-associated pneumonia

Introduction

Ventilator-associated pneumonia (VAP) is the specific type of hospital-acquired pneumonia that occurs after the first 48 h of initiating mechanical ventilation.[1,2] The Institute of Healthcare Improvement has recognized VAP as one of the most important preventable causes of morbidity and mortality in critically ill patients.[3,4] The direct cost due to excess stay in patients suffering from VAP has been estimated to be approximately $40,000/per patient.[5,6] The immense cost and adverse outcomes of VAP is a growing medical challenge.

Bronchoalveolar lavage (BAL) has become a standard diagnostic procedure for cytological, microbiological, or

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immunological analysis for the majority of patients with pulmonary disease. The technique is safe, minimally invasive, reveals specific information, providing useful insight into the underlying pathophysiology of lung diseases. If a BAL finding is compatible with the clinico-radiological diagnosis, this can be sufficient for disease confirmation.[7,8]

Trauma usually afflicts middle-aged males, without underlying risk factors for the development of pneumonia. Ventilated trauma patients have a high risk of development of VAP.[9] The outcome of such patients is highly variable, raising the possibility that the host immunological response may also be governing the patient's outcome. The paradigm of pulmonary inflammatory response in ventilated trauma patients is largely unknown. In the face of increasing antimicrobial resistance, immune modulation may have to be looked into as a treatment avenue for VAP. Thus, we conducted the present study to ascertain the cytokine profile of BAL using the cytometric bead array (CBA) in a flow cytometer. The CBA is a multiplexing platform, which provides a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using a flow cytometry. The objective of this study was to correlate the levels of Th-1/Th-2 cytokines in BAL with the clinical outcome of ventilated trauma patients.

Patients and Methods

A total of forty patients of either gender, aged between 19 and 65 years, admitted to the surgical Intensive Care Unit (ICU) of our hospital setting with severe injuries were included in the study. The further inclusion criteria were primary admission to our hospital, within 24 h of trauma and survival for more than 48 h in the ICU. In all cases, BAL was performed for a diagnostic purpose, when the treating physicians had a clinical suspicion of VAP. The collection of BAL was done as per the standard recommendations. The diagnosis of VAP was done based on the CDCs NHSN definition.[10]

Processing of bronchoalveolar lavage samples

The samples were processed for microbiological culture and susceptibility testing as per the standard methods.[11,12] For the CBA study, the BAL specimens were transferred from the collection vial to a 5 ml tube (Becton Dickinson, Franklin Lakes, NJ, USA). During this transfer process, the BAL samples were filtered through a two-layer sterile gauze into sterile plastic vials (Falcon, Oxnard, CA, USA) to reduce the amount of mucus and large, noncellular debris that

might be present. An aliquot of the same was stored at −80°C immediately for further processing by CBA.

Cytometric bead array

CBA was performed to assess the levels of interleukin-4 (IL-4), IL-6, IL-8, IL-1 β, interferon gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) in the BAL samples. Each capture bead CBA Human Soluble Protein Flex Set System (BD Biosciences, Sans Jose, CA, USA) had a distinct fluorescence and was coated with a capture antibody specific for each soluble protein. The detection reagent was a mixture of phycoerythrin (PE)-conjugated antibodies, which provided a fluorescent signal in proportion to the amount of bound analytes, i.e., the cytokines present in the BAL fluid. When the capture beads and detection bead were incubated with the standards or the unknown samples containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) were formed. These complexes were then measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector.

The bead population was resolved in two fluorescence channels of a flow cytometer. Each bead population was given an alphanumeric position designation indicating its position relative to other beads in the CBA Human Soluble Protein Flex Set System. Beads with different positions were combined in the assay to create a six-plex assay. The bead positions of the cytokines assessed in this study-IL-4, IL-6, IL-8, IL-1 β, IFN-γ, and TNF-α were A5, A7, A9, B4, B8, and D9, respectively. The intensity of PE fluorescence of each sandwich complex revealed the concentration of each particular cytokine [Figure 1].

Flow cytometer setup

BD FACS Aria™ III flow cytometer equipped with FACS Diva™ software, version 6.1 (BD Biosciences, San Jose, CA, USA) [Table 1] was used for data acquisition and FCAP Array™ software (v3.0.1), Soft Flow Hungary, Ltd., Pecs, Hungary for data analysis. To verify cytometer performance, establish the appropriate compensation adjustments and instrument settings, FACS Diva™ software, version 6.1 (BD Biosciences, San Jose, CA, USA) was run in lyse/no-wash mode using CaliBRITE beads (BD Biosciences, San Jose, CA, USA). The BD™ CBA Flex set the template for flow cytometer was designed as per the procedure recommended for BD FACS Aria™ III flow cytometer using the BD CBA Human Soluble Protein Master Buffer Kit (BD Biosciences, Sans Jose, CA, USA) (Catalog #558264). PE was used as the reporter channel while APC and APC-Cy™ 7 were used as the bead channels. Table 1 shows the flow cytometer setup.

Human Soluble Protein Flex Set Assay

The lyophilized human flex set standards were reconstituted in assay diluent provided in the BD CBA Human Soluble Protein Master Buffer Kit and serially diluted to obtain the following concentrations: Top standard (2500 pg/mL), 1:2 (1250 pg/mL), 1:4 (625 pg/mL), 1:8 (312.5 pg/mL), 1:16 (156 pg/mL), 1:32 (80 pg/mL), 1:64 (40 pg/mL), 1:128 (20 pg/mL), 1:256 (10 pg/mL), 1:512 (5 pg/mL), and 1:1024 (2.5 pg/mL). In addition to this, a negative control was also prepared having 0 pg/mL of protein. Then, the Human Soluble Protein Flex Capture Beads were mixed according to the manufacturers' instructions. The BAL samples were diluted with the assay diluent to a concentration of 1:4 (which was standardized based on a previous set of experiments).

Acquisition strategy

An acquisition dot plot of forward scatter versus side scatter was displayed on a log scale. An anchor gate was drawn to create a region that included a singlet population of beads. An additional acquisition dot plot of APC-Cy7 versus APC was created to further characterize each of the six beads clusters (IL-4, IL-6, IL-8, IL-1 β, IFN-γ, and TNF-α) at their specific designated bead positions.

Table 1: Flow Cytometer Set-Up

Laser lines	633nm	
Emission filters	660/20	780/60
Fluorochrome	APC	APC.Cy7

All the 12 standard tubes were run, followed by the BAL samples. After acquiring the BAL samples on the flow cytometer, the results were generated in graphical and tabular formats using FCAP Array™ software.

Clinical follow-up

All patients were clinically followed up daily based on the receipt of the first BAL sample. The development of VAP and the clinical course was evaluated by the critical care specialists in-charge of the patients as per the standard protocols.[10]

Statistical analysis

Group comparison of data was done by Kruskal–Wallis test and Pearson Chi-squared test, respectively. A pair-wise comparison was done with Mann–Whitney U-test if a significant difference was found with Kruskal–Wallis test. A $P < 0.05$ was considered as significant. The study was approved by the Institute's Ethical Committee.

Results and Discussion

A total of forty patients (35 males; 5 females) admitted to the trauma surgical ICU, who were on the ventilator, were enrolled in the study. The age of these patients ranged from 19 to 65 years (median age 40.8 years). The length of their stay varied from 7 to 90 days (average 36.3 days). The duration of ventilation ranged from 5 to 90 days (average 18.7 days). The primary site of injury was the chest in 12 (30%), abdomen in 8 (20%), pelvis,
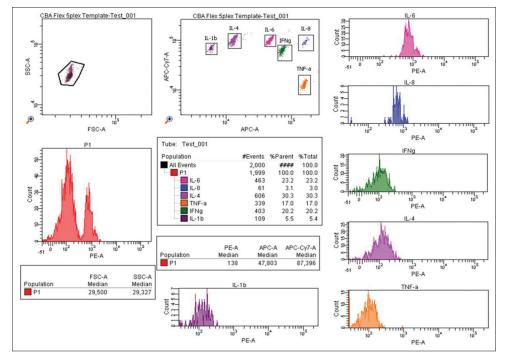

Figure 1: Cytometric bead arrayCBA profile picture on flow cytometer

femur and spine in 4 each (10% each), and head in 1 patient. Seven patients were admitted with polytrauma.

A total of 12 patients (30%) had confirmed VAP. The duration of ventilator days in these patients ranged from 8 to 75 days (average 26 days). In 9 of these 12 patients (75%), a single microorganism was isolated from BAL culture in significant counts (*Acinetobacter baumannii* in six, *Klebsiella pneumoniae* in two, and *P. aeruginosa* in one). Three patients had two organisms isolated from BAL culture (one patient each with *A. baumannii + P. aeruginosa*; *Burkholderia cepaciae + Serratia marcescens*; *A. baumannii + K. pneumoniae*). The blood samples from 5 of the 12 patients confirmed to have VAP grew the same organism (identification and susceptibility profile similar) as the BAL samples. Thus, three patients had *A. baumannii*, one had *P. aeruginosa*, and one had *A. baumannii + P. aeruginosa* in both blood and BAL samples.

Of the forty patients, eight (20%) patients had a fatal outcome. The cause of death was septicemia in five patients. One each of the remaining three fatal cases had disseminated intravascular coagulation, shock, and acute renal failure as the causes of death.

The cytokine profile of BAL in the various categories of ventilated trauma patients is shown in Table 2.

When the difference in cytokine levels was compared between the culture positive sample which further survived or had a fatal outcome due to septicemia, multiple-organ dysfunction syndrome, VAP, and acute respiratory distress syndrome (ARDS), it was found that only IL-6 was significantly raised in patients who had a fatal outcome. The difference in other cytokines was not significant. Similarly, when the difference was compared with respect to patients who had VAP as compared to patients without VAP, the levels of IL-8 were significantly elevated in patients with VAP and the levels of IFN-γ were significantly low in patients with VAP. For all other cytokines, the difference in levels between the patients' groups was not significant.

Thus, in our present study, the levels of IL-8 and IFN-γ correlated significantly with the development of VAP and elevated IL-6 in BAL was associated with a poor outcome. Th-1 response was therefore significantly reduced in patients with VAP. A proinflammatory response in the form of elevated IL-6 and IL-8 correlated poorly with the clinical outcome.

A. baumannii predominated as the cause of VAP in our study. A high prevalence of multidrug resistance is seen at our center in all genera of Gram-negative bacteria.[13] Although not reported in the present study, we have observed that not all patients suffering from VAP with the same clone of *A. baumannii* have a fatal outcome.[14] Thus, there must be some host immune response-driven factors which propel a patient toward a poor outcome.

CBA-based tests are economical and time efficient, enabling the analysis of a wide range of cytokines. A disadvantage of such assays is that they give the overall picture of extracellular cytokines in a body fluid, without identifying the cellular source of those cytokines. Most studies on cytokine profiling of BAL have been done on chronic inflammatory conditions such as asthma and sarcoidosis.[15-17] To our knowledge, this is the first such study on ventilated trauma patients. Given that many such patients ultimately succumb to ARDS or systemic inflammatory response syndrome, immune monitoring or immunomodulating interventions need to be looked into by conducting larger studies.

Table 2: CBA profile of cytokines in BAL fluid of ventilated trauma patients

Cytokines	IL-4	IL-6	IL-8	IL-1β	IFNϒ	TNFα
All Patients N=40						
Range	0-17.14	0-40629	173.6-69753.8	0-74373	0-41.9	0-4574
Average	2.4	4921.4	22821.0	4712.7	9.1	263.9
Patients who survived N=32						
Range	0-17.14	0-34519.6	173.62-64795.48	0-74373.03	0-42489.01	0-4574.3
Average	2.76	3702.5	19755.3	5004.47	1337.29	286.39
Patients who died N=8						
Range	0-8.83	358-40629	5527.03-69753.2	417-14373.2	0-41.9	4.38-1028.4
Average	1.3	9472	35084.1	3545.9	7.9	179.5
Patients with VAP N=12						
Range	0-8.83	96.2-40629	6735.5-69753.2	428-9726.4	0-41.9	1.56-1651.2
Average	1.56	7499.2	35678.3	3717	9.9	267.9
Patients without VAP N=28						
Range	0-14.14	0-34519	173.6-61392.8	0-16141.2	0-41.9	0-4574.3
Average	3.1	3516.49	14426.98	2346.3	1642.7	261.62

Conclusions

A proinflammatory response in the form of elevated IL-6 and IL-8 correlated poorly with the clinical outcome. Th-1 response was significantly reduced in patients with VAP. A proinflammatory response in the form of elevated IL-6 and IL-8 correlated poorly with the clinical outcome.

Conflicts of interest

There are no conflicts of interest.

References

1. Lynch JP 3rd. Hospital-acquired pneumonia: Risk factors, microbiology, and treatment. Chest 2001;119 2 Suppl: 373S-84S.
2. Tablan OC, Anderson LJ, Besser R, Bridges C, Hajjeh R; CDC; Healthcare Infection Control Practices Advisory Committee. Guidelines for preventing health-care – Associated pneumonia, 2003: Recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee. MMWR Recomm Rep 2004;53:1-36.
3. Chastre J, Fagon JY. Ventilator-associated pneumonia. Am J Respir Crit Care Med 2002;165:867-903.
4. Baker AM, Meredith JW, Haponik EF. Pneumonia in intubated trauma patients. Microbiology and outcomes. Am J Respir Crit Care Med 1996;153:343-9.
5. Fagon JY, Chastre J, Hance AJ, Montravers P, Novara A, Gibert C. Nosocomial pneumonia in ventilated patients: A cohort study evaluating attributable mortality and hospital stay. Am J Med 1993;94:281-8.
6. Rello J, Ollendorf DA, Oster G, Vera-Llonch M, Bellm L, Redman R, et al. Epidemiology and outcomes of ventilator-associated pneumonia in a large US database. Chest 2002;122:2115-21.
7. Costabel U, Guzman J. Bronchoalveolar lavage in interstitial lung disease. Curr Opin Pulm Med 2001;7:255-61.
8. Tøndell A, Moen T, Børset M, Salvesen O, Rø AD 2nd, Sue-Chu M. Bronchoalveolar lavage fluid IFN-γ+ Th17 cells and regulatory T cells in pulmonary sarcoidosis. Mediators Inflamm 2014;2014:1-9.
9. Batra P, Mathur P, John NV, Nair SA, Aggarwal R, Soni KD, et al. Impact of multifaceted preventive measures on ventilator-associated pneumonia at a single surgical centre. Intensive Care Med 2015;41:2231-2.
10. Horan TC, Andrus M, Dudeck MA. CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. Am J Infect Control 2008;36:309-32.
11. Collee JG, Diguid JP, Fraser AG, editors. Mackie and McCartney Practical Medical Microbiology. 14th ed. Edinburgh: Churchill Livingstone; 1996.
12. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 16th Informational Supplements, M100-S16. CLSI 2006. Wayne, PA, USA: Clinical and Laboratory Standards Institute; 2006.
13. Lalwani S, Mathur P, Tak V, Janani S, Kumar SI, Bagla R, et al. Diagnosis of ventilator-associated pneumonia: Comparison between ante-mortem and post-mortem cultures in trauma patients. Indian J Med Microbiol 2014;32:294-300.
14. Khurana S, Mathur P, Batra P, Nair SA, Varghese P, John NV, et al. Does Infection with Multidrug Resistant Bacteria Necessarily Lead to Adverse Patient Outcome? A Prospective Study. Abstract Presented in 15th Congress of International Federation of Infection Control, XIII National Conference of Hospital Infection Society, 21-24 March, 2015, New Delhi, India; 2015.
15. Urbankowski T, Hoser G, Domagala-Kulawik J. Th1/Th2/Th17-related cytokines in the bronchoalveolar lavage fluid of patients with sarcoidosis: Association with smoking. Pol Arch Med Wewn 2012;122:320-5.
16. Mortaz E, Gudarzi H, Tabarsi P, M Adcock I, Masjedi MR, Jamaati HR, et al. Flow cytometry applications in the study of immunological lung disorders. Iran J Allergy Asthma Immunol 2015;14:12-8.
17. Boomer JS, Parulekar AD, Patterson BM, Yin-Declue H, Deppong CM, Crockford S, et al. A detailed phenotypic analysis of immune cell populations in the bronchoalveolar lavage fluid of atopic asthmatics after segmental allergen challenge. Allergy Asthma Clin Immunol 2013;9:37.