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Compartmentalisation of cytokines and cytokine inhibitors in ventilator-associated pneumonia

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Abstract *Objective:* To examine whether cytokine concentrations change in the pulmonary compartment during the development of ventilator-associated pneumonia (VAP). *Design:* Non-directed bronchial lavage (NBL) was performed every 48 h in critically ill mechanically ventilated patients. Serial measurements of the cytokines tumor necrosis factor (TNF) α, interleukin (IL)-1α, IL-1β, IL-6, and IL-10 and the cytokine inhibitors soluble TNFα receptor type I (sTNFαRI), IL-1 receptor antagonist (IL-1Ra) and soluble IL-1 receptor II (sIL-1RII) were performed on the NBL fluid and matching plasma samples by ELISA. *Setting:* An adult medical and surgical university hospital intensive care unit. *Patients:* Nine patients who developed VAP and nineteen patients who did not develop VAP served as controls. *Interventions:* None. *Results:* Plasma concentrations of the measured cytokines and cytokine inhibitors did not change significantly in any patients. In control patients, NBL fluid concentrations of sIL-1RII decreased significantly over time (P=0.01). In patients who developed VAP, NBL fluid concentrations of TNFα, sTNFαRI, IL-1α, and IL-1β increased significantly (P=0.002, P=0.03, P=0.04 and P=0.02, respectively). Furthermore, NBL fluid/plasma concentration ratios for TNFα, sTNFαRI, IL-1α, IL-1Ra and IL-6 increased significantly as VAP developed (P=0.001, P=0.001, P=0.04, P=0.03, and P=0.04, respectively). *Conclusion:* Our results suggest that the production of important cytokines and cytokine inhibitors is compartmentalised within the lung in critically ill mechanically ventilated patients who develop VAP.

Keywords Ventilator-associated pneumonia · TNFα · IL-1α · IL-1β · IL-6 · IL-10 · sTNFαRI · IL-1Ra · sIL-1RII

Introduction

Critically ill patients requiring mechanical ventilation are at risk of ventilator-associated pneumonia (VAP) [1]. This common and serious complication affects between 8% and 28% of critically ill mechanically ventilated patients and is estimated to increase the risk of death by 20–30% [2]. Cytokines are key factors in the pathogenesis of pneumonia [3, 4] and compartmentalisation of the host response has been clearly demonstrated in animal models [5, 6, 7].

In clinical studies, compartmentalised cytokine production has been demonstrated in uncomplicated community-acquired pneumonia (CAP) [8, 9]. In patients with CAP and a wide range of initial APACHE II scores (3 – 24), increased severity of illness was associated with higher levels of interleukin (IL)-6 and IL-10 in the systemic circulation [10]. In another study, circulating tumor necrosis factor (TNF) α was higher in patients with pneumonia leading to acute respiratory distress syndrome (ARDS) than in less severely ill patients [11]. Taken together, these data suggest that with increasing severity

of pneumonia, circulating cytokine concentrations are likely to be elevated. However, critically ill mechanically ventilated patients may have elevated systemic cytokine levels for a number of reasons [12], and VAP is not associated with an increase in circulating levels of IL-6 or IL-8 [13]. Thus, it is possible that VAP may be associated with changes in cytokine concentrations in the lung that are not apparent in the systemic circulation.

We therefore compared the effect of VAP on lung and systemic cytokine concentrations over time. We measured concentrations of important primary cytokines and their inhibitors: TNFα and soluble TNFα receptor type I (sTNFαRI); IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) and soluble IL-1 receptor II (sIL-1RII); IL-6; and IL-10. Samples of non-directed bronchial lavage (NBL) fluid and plasma were obtained simultaneously every 48 h. Serial changes in lung and systemic cytokine concentrations and NBL fluid to plasma concentration ratios were compared in patients who did or did not develop VAP.

Methods

Subjects

The John Radcliffe ICU is a general adult medical and surgical unit based in a university hospital. Mechanically ventilated patients were enrolled into the study within 72 h of admission to the ICU if it was anticipated that ventilation would be required for at least a further 5 days. Patients were not considered for inclusion if they were immunosuppressed.

Protocol

Clinical data, specimens of non-directed bronchial lavage (NBL) fluid, and plasma were collected every 48 h. If sepsis was suspected, the ICU staff would obtain blood, NBL, and other appropriate specimens for culture at any time.

Non-directed bronchial lavage technique

The NBL technique was performed as described previously [14, 15]. Briefly, a sterile 50-cm, 14-Fr tracheal suction catheter was attached to a 20-ml syringe filled with 0.9% saline. The distal end was introduced via the endotracheal tube and advanced until significant resistance was encountered. The saline was instilled over 10–15 s, and immediately aspirated before withdrawal of the catheter. Usually 5–10 ml of fluid was recovered.

Non-directed bronchial lavage sample processing

The NBL samples were kept at 4 °C and processed within 1 h. An aliquot of the NBL sample was sent to the microbiology laboratory. The remainder of the NBL sample was centrifuged at 1,500 × *g* for 15 min at 4 °C. Supernatant (NBL fluid) was stored at −80 °C for subsequent cytokine measurements. In the microbiology laboratory, semi-quantitative culture was performed, as described previously [14]. Briefly, 1 ml of the NBL sample was diluted in 19 ml of sterile 0.9% saline and 50 μl aliquots were spread onto blood agar to be incubated both anaerobically and in 5% CO_2, onto chocolate agar to be incubated in 5% CO_2, and onto MacConkey agar to be incubated aerobically. After overnight incubation, colonies were counted and converted to bacterial concentrations as follows: 2–24 colonies represent 10^3 colony forming units (cfu).ml^{-1}, 25–249 colonies represent 10^4–10^5 cfu.ml^{-1}, and >250 colonies represent >10^5 cfu.ml^{-1}. Organisms present at $\geq 10^4$ cfu.ml^{-1} were identified to genus level using routine laboratory methods.

Blood samples

Immediately prior to the lavage procedure, blood samples were drawn into sterile vacutainer tubes containing heparin. Blood samples were centrifuged at 1,500 × *g* for 15 min at 4 °C. The plasma was stored at −80 °C until cytokine assays were performed.

Diagnosis of VAP

Ventilator-associated pneumonia was diagnosed by intensive care unit clinicians independently from the study. To corroborate the diagnosis, the study investigators recorded temperature, peripheral white cell count, quantity and colour of respiratory secretions, and P_aO_2/F_iO_2 (kPa) on each occasion that samples were obtained. Serial chest X-rays were assessed by a specialist chest physician (CM). Microbiologically, a diagnosis of VAP required either a significant growth ($\geq 10^4$ cfu.ml^{-1}) on semi-quantitative culture of the NBL or growth of a lower colony count (10^3 cfu.ml^{-1}) associated with isolation of the pathogen from culture of blood. If the study investigators agreed that the clinical, radiological, and microbiological data were consistent with a diagnosis of VAP, then the diagnosis was considered to be confirmed. The day of onset of VAP was then taken to be the day that the attending clinicians started antibiotic treatment.

Other clinical data

APACHE II score [16]and the Multiple Organ Dysfunction Score (MODS) [17] were determined on ICU admission. Occurrence of acute respiratory distress syndrome (ARDS) [18] at any point during the ICU admission was noted.

Assays

TNFα, IL-1α, IL-1β, IL-6, IL-10, sTNFαRI, IL-1Ra, and sIL-1RII concentrations were measured using commercially available ELISAs according to the manufacturer's instructions. The limits of detection were 1.4 pg.ml^{-1} for TNFα, 0.6 pg.ml^{-1} for IL-6, and 1.2 pg.ml^{-1} for IL-10 (assay kits from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and 23.4 pg.ml^{-1} for IL-1α, 0.4 pg.ml^{-1} for IL-1β, 6.2 pg.ml^{-1} for sTNFαRI, 13.7 pg.ml^{-1} for IL-1Ra, and 58.5 pg.ml^{-1} for sIL-1RII (assay kits from R&D Systems, Minneapolis, Minn., USA).

Consent

The microbiological surveillance policy of obtaining NBL on alternate days from all ventilated patients was established locally and has been described previously elsewhere [14]. In view of the established nature of this policy, the observational nature of the study, the relatively small volumes of blood sampled (15 ml on alternate days), and the impossibility of obtaining informed consent prospectively, the investigators requested that neither retrospective

Table 1 Demographic, clinical, and outcome data for the study patients. Data presented as mean±SD unless stated otherwise. (*VAP* ventilator-associated pneumonia, *APACHE II score* Acute Physiology and Chronic Health Evaluation II score, *MODS* Multiple Organ Dysfunction Score, *ARDS* Acute Respiratory Distress Syndrome)

	All patients (*n*=28)	Non-VAP patients (*n*=19)	VAP patients (*n*=9)	*p*
Age, years	57±16	56±15	59± 18	NS
Male, *n* (%)	19 (68%)	11 (58%)	8 (89%)	NS
APACHE II score	22±9	21±10	25±6	NS
MODS score	9±4	8±4	10±3	NS
ARDS, *n* (%)	9 (32%)	4 (21%)	5 (56%)	NS
Length of ICU stay, days	21±13	16±11	30±11	0.04
Mortality, *n* (%)	3 (11%)	2 (11%)	1 (11%)	NS

patient consent nor formal assent from relatives be required. The study protocol was approved by the Central Oxford Regional Ethics Committee.

Statistical analysis

Student's *t*-test, the Chi-squared test, and the Wilcoxon rank sum test were used to compare data as appropriate. For comparisons within individual patients developing VAP, cytokine concentrations on the day of diagnosis of VAP were compared with cytokine concentrations 5 days previously. For comparisons between patients developing VAP and controls, cytokine concentrations on the day of diagnosis of VAP were compared to cytokine concentrations on the day of enrolment into the study. Analysis of repeated measures was performed using linear regression on logarithmically transformed data using the MIXED procedure of the SAS statistical package. To calculate ratios, where cytokine concentrations were at or below the lower limit of detection for a given assay, the cytokine concentration was assumed to be the value of the lower limit of detection. Values for *P* of less than 0.05 were considered statistically significant.

Results

Study population

Specimen collection was initiated in 60 patients where it was anticipated that mechanical ventilation would be required for 5 or more days. However, in 32 cases, the duration of mechanical ventilation was less than 5 days and therefore only one or two sets of matching plasma and NBL fluid were obtained. As our intention was to examine trends, we elected not to measure cytokine concentrations in these cases. All of these patients were successfully extubated and none of these patients developed VAP.

In the other 28 cases, three or more serial sets of specimens (median 5, range 3–12) were obtained and these patients constitute the study population. VAP was diagnosed in nine of these patients. The other 19 patients were considered to be controls. The median (range) time from ICU admission to onset of VAP was 9 (8–18) days. The minimum time from enrolment into the study to diagnosis of VAP was 5 days. Demographic, clinical and outcome data for the study patients are given in Table 1. Primary ICU admission diagnoses for the study patients are given in Table 2. The clinical and microbiological data confirming the diagnosis of VAP are given in Table 3.

Table 2 Primary diagnosis on ICU admission

	Non-VAP patients (*n*=19)	VAP patients (*n*=9)
Abdominal sepsis	4	1
Cardiothoracic surgery	1	3
Pelvic fracture	1	1
Trauma (other)	2	2
Infective endocarditis	1	–
Pancreatitis	3	1
Necrotising fasciitis	2	–
Liver failure	1	1
Drug overdose	1	–
Intracerebral haemorrhage	3	–

Pro-inflammatory cytokines in plasma and NBL fluid

Serial measurements of the concentration of the pro-inflammatory cytokines TNFα, IL-1α, IL-1β, and IL-6 in plasma and NBL fluid are shown in Fig. 1. In control patients, neither plasma nor NBL fluid concentrations of TNFα, IL-1α, IL-1β, and IL-6 changed significantly. In patients who developed VAP, plasma concentrations of TNFα, IL-1α, IL-1β, and IL-6 again did not change significantly. However, NBL fluid concentrations of TNFα, IL-1α, and IL-1β increased significantly as patients developed VAP (P=0.002, P=0.04, and P=0.02, respectively). NBL fluid concentrations of IL-6 increased, but this was not statistically significant.

IL-10 and cytokine inhibitors in plasma and NBL fluid

Serial measurements of the concentration of IL-10, sTNFαRI, IL-1Ra, and sIL-1RII in plasma and NBL fluid are shown in Fig. 2. In control patients, plasma concentrations of IL-10, sTNFαRI, IL-1Ra, and sIL-1RII did not change significantly. NBL fluid concentrations of IL-10, sTNFαRI, and IL-1Ra did not change significantly,

Table 3 Clinical and microbiological data from the day of diagnosis of ventilator-associated pneumonia (*Temp* temperature, *WCC* peripheral white cell count, *Sec* secretions graded as 0, + or ++, *CXR* number of quadrants of consolidation on the chest radiograph, *P/F* P_aO_2/F_IO_2, *ARDS* acute respiratory distress syndrome, *NBL* non-directed bronchial lavage, *BC* blood culture)

Patient	Age	Sex	Temp (°C)	WCC ($\times 10^9.l^{-1}$)	Sec	CXR	P/F (kPa)	ARDS	Semi-quantitative culture of NBL	Other microbiology	Antibiotic(s) initiated
1	79	M	36.7	22.4	++	1Q	19	N	*E. coli* $>10^5$ cfu.ml^{-1}		Piperacillin-tazobactam
2	77	M	38.5	7.8	+	4Q	22	Y	Coliforms $<10^4$ cfu.ml^{-1}	BC - *Klebsiella*	Piperacillin-tazobactam
3	50	M	37.2	18.6	++	4Q	20	Y	Polymicrobial (aspiration)		Meropenem
4	67	M	37.8	21.0	++	4Q	17	Y	*Stenotrophomonas maltophilia* $>10^5$ cfu.ml^{-1}		Ceftriaxone
5	59	F	39.0	50.0	++	2Q	8	Y	*Pseudomonas aeruginosa* $10^{4–5}$ cfu.ml^{-1}	Abdominal sepsis	Piperacillin-tazobactam
6	27	M	37.3	21.0	++	4Q	19	Y	Coliforms $10^{4–5}$ cfu.ml^{-1}		Meropenem
7	71	M	37.3	7.8	+	1Q	20	N	Coliforms $<10^4$ cfu.ml^{-1}	BC - *Citrobacter koseri*	Meropenem
8	35	M	39.0	13.4	++	1Q	25	N	*Strep. pneumoniae* $>10^5$ cfu.ml^{-1}		Benzyl penicillin
9	69	M	37.6	9.7	++	1Q	26	N	*P. aeruginosa* $>10^5$ cfu.ml^{-1}		Meropenem

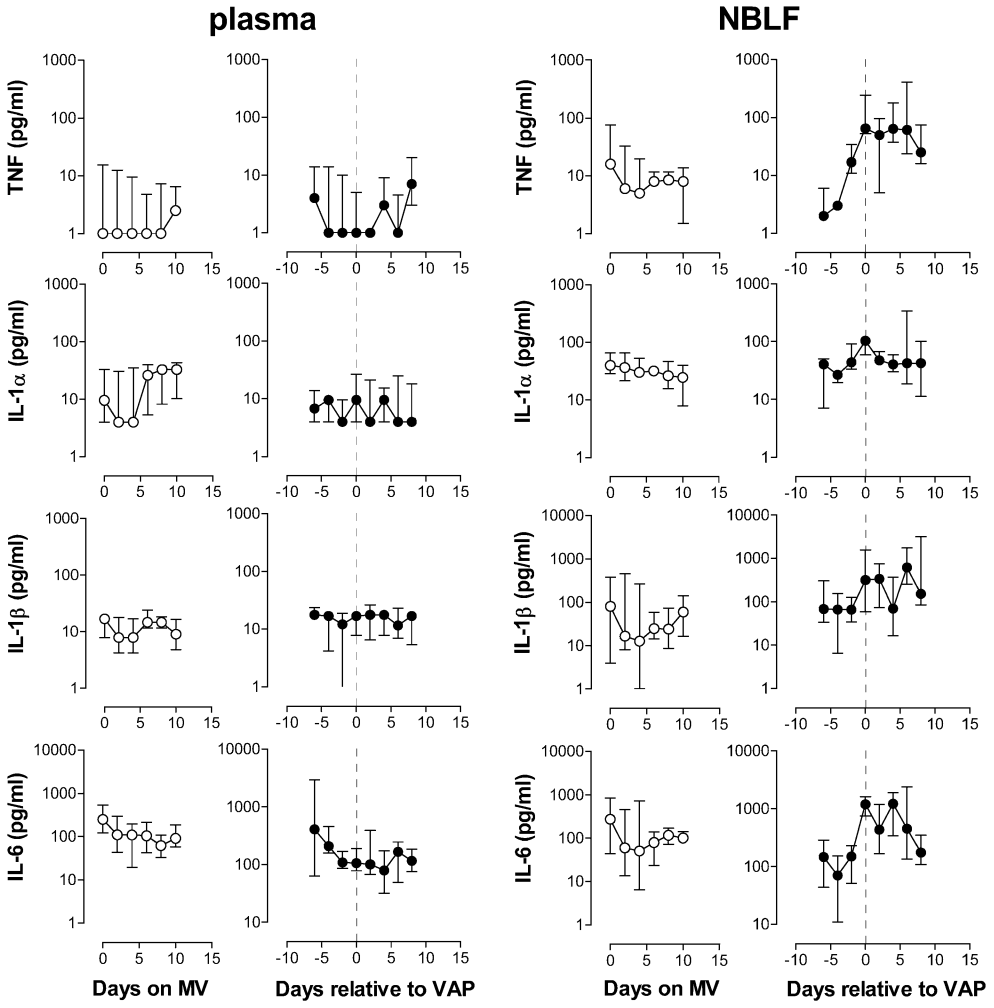

Fig. 1 Pro-inflammatory cytokine concentrations in plasma and non-directed bronchial lavage fluid against time. Median±IQR of TNFα, IL-1α, IL-1β, and IL-6 concentrations for control patients (-◯-, n=19) and patients developing VAP (-●-, n=9). Time (days) is relative to the day of enrolment into the study for control patients and relative to the day of diagnosis for patients developing VAP (vertical broken line)

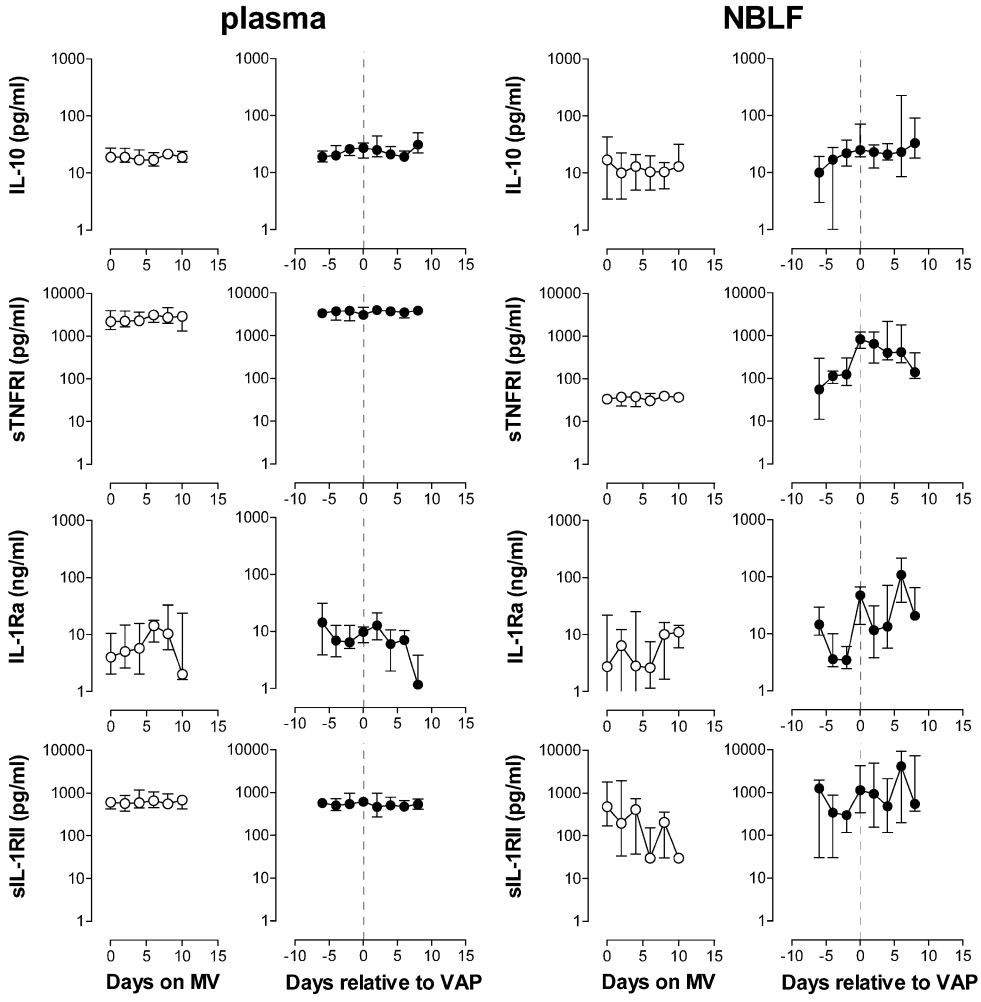

Fig. 2 IL-10 and cytokine inhibitor concentrations in plasma and non-directed bronchial lavage fluid against time. Median±IQR of IL-10, sTNFαRI, IL-1Ra, and sIL-1RII concentrations for control patients (-◯-, n=19) and patients developing VAP (-●-, n=9). Time (days) is relative to the day of enrolment into the study for control patients and relative to the day of diagnosis for patients developing VAP (*vertical broken line*)

but NBL fluid concentrations of sIL-1RII decreased significantly (P=0.01). In patients who developed VAP, plasma concentrations of IL-10, sTNFαRI, IL-1Ra, and sIL-1RII again did not change significantly. However, NBL fluid concentrations of sTNFαRI increased significantly as patients developed VAP (P=0.03).

NBL fluid/plasma ratios

To demonstrate whether cytokine and cytokine inhibitor production is compartmentalised during VAP, ratios of NBL fluid concentration to plasma concentration were calculated. In control patients, there were no statistically significant changes with time in NBL fluid/plasma concentration ratios (data not shown). In patients developing VAP, NBL fluid/plasma concentration ratios are shown in Fig. 3. NBL fluid/plasma concentration ratios for TNFα, sTNFαRI, IL-1α, IL-1Ra, and IL-6 increased significantly as VAP developed (P=0.001, P=0.001, P=0.04, P=0.03, and P=0.04, respectively).

Discussion

In these critically ill mechanically ventilated patients, as VAP developed we observed a significant increase in the concentrations of TNFα, sTNFαRI, IL-1α, and IL-1β in NBL fluid. This suggests that the local production of cytokines and cytokine inhibitors is an early feature of VAP. We also found that as patients developed VAP, the NBL fluid/plasma concentration ratios for TNFα, sTNFαRI, IL-1α, IL-1Ra, and IL-6 increased significantly, indicating that the host response during VAP is compartmentalised, i.e., restricted to the site of infection.

The patients studied were representative of a general medical and surgical adult intensive care unit population. The admission APACHE II scores were high, confirming that these patients were critically ill. A proportion of the

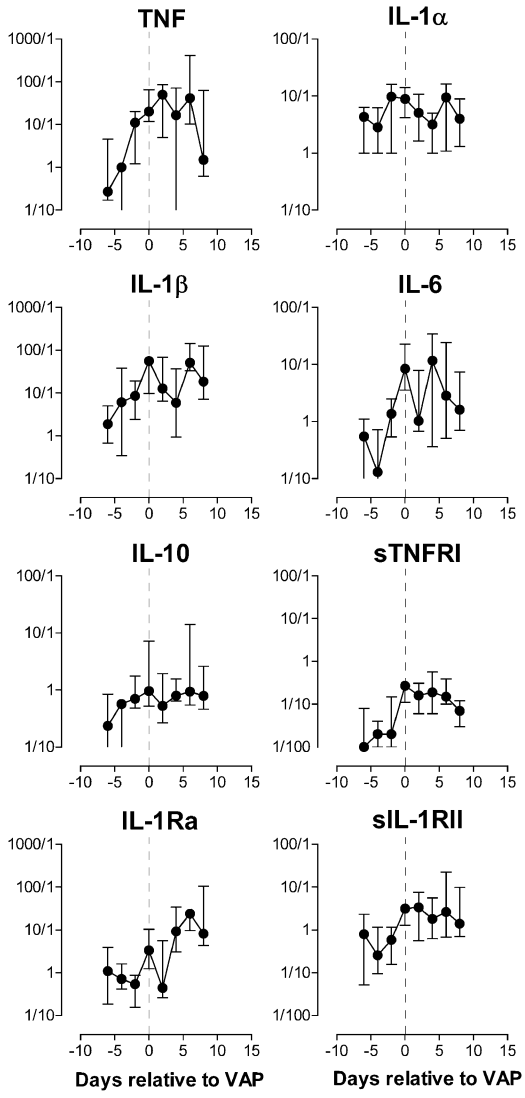

Fig. 3 NBL fluid to plasma concentration ratios of cytokines and cytokine inhibitors in patients developing VAP (n=9) against time. Median±IQR of the ratios for each point in time (days) relative to the day of diagnosis of VAP (*vertical broken line*)

patients developed VAP. The demographic and clinical characteristics of patients developing VAP and control patients, who did not develop VAP, were similar except that length of ICU stay was significantly longer for the VAP group as would be expected.

Where only one or two sets of matching plasma and NBLF specimens were obtained, we elected not to measure cytokine concentrations because we wanted to examine trends. As no patient developed VAP before his or her fifth day in the study, we were able to compare concentrations on the day of diagnosis with concentrations 5 days previously.

A potential limitation of our study is that the amount of fluid recovered by NBL is not constant, which may cause variable dilution of cytokines. However, cytokines are not evenly distributed throughout a homogenous layer of fluid lining the airway. Respiratory secretions are a complex mixture of aqueous, mucus, and cellular components and cytokines and their inhibitors are variably membrane-associated. Therefore we did not feel that it was appropriate to correct our results by, for example, urea dilution. Importantly, although the variation in lavage fluid recovered was twofold, the changes in cytokine concentration we observed were log-fold and would therefore render error due to dilutional effects negligible.

Different time courses have been observed for the changes in concentration of the functionally related but distinct IL-1 inhibitors IL-1Ra and sIL-1RII in meningococcal sepsis [19]. Moreover, different patterns of change over time were observed in different compartments (systemic and subarachnoid). In our study, the concentration of IL-1Ra in NBL fluid increased significantly but sIL-1RII did not. Our results reflect the complexity of cytokine signalling pathways. The compartmentalisation we have demonstrated suggests that in making a presumptive diagnosis of VAP, systemic cytokine levels are of little value.

The use of alternate-day NBL has been advocated for early recognition of VAP [15]. However, delay caused by the time needed for culture weakens this approach. The magnitude of the increase in cytokine concentrations that we have observed in NBL fluid suggest that measurement of cytokine concentration in NBL fluid may be a useful test to support a diagnosis of VAP. This hypothesis warrants further investigation in a larger number of patients.

In conclusion, high concentrations of cytokines and cytokine inhibitors are present in the lung in VAP, demonstrating the complexity of cytokine signalling pathways during critical illness in general, and VAP in particular. Furthermore, although our results involved a relatively small number of patients and should be interpreted with caution, our data suggest that cytokine measurements in NBL fluid may be a useful method to support a diagnosis of VAP.

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