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Cytokine profiles in bronchoalveolar lavage fluid and blood in HIV-seronegative patients with *Pneumocystis carinii* pneumonia

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Abstract. Concentrations and *ex vivo* production of interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF), interleukin-6 (IL-6), interleukin-1 receptor antagonist (IL-1RA) and TNF soluble receptors were followed in bronchoalveolar lavage (BAL) fluid and blood from 10 HIV-seronegative patients with *Pneumocystis carinii* pneumonia (PCP) and compared with values found in healthy volunteers. During the acute phase of PCP, TNF but not IL-6 or IL-1 β was detectable in BAL fluid. At that time, plasma concentrations of the proinflammatory cytokines were low, whereas plasma concentrations of the anti-inflammatory cytokines were high. The *ex vivo* production capacity of proinflammatory cytokines was suppressed in the acute phase, in the blood as well as at the site of infection. During convalescence the production capacity of the blood cells normalized. The IL-1RA production capacity of the alveolar cells was also suppressed in the acute phase, but preserved in blood cells.

Keywords. Interleukin-1, interleukin-1 receptor antagonist, interleukin-6, *Pneumocystis carinii* pneumonia, sTNF receptors, TNF- α .

Introduction

Pneumocystis carinii is an intriguing pathogen, mainly causing pulmonary infections in immunocompromised hosts. Although deficient cellular immunity is considered a major predisposing factor for the development of *Pneumocystis carinii* pneumonia (PCP), the underlying mechanisms are still unclear. Clinical and experimental data suggest that CD4⁺ T-lymphocytes are critical in protection against PCP [1–3]. However, CD4⁺ lymphocytes are unlikely to function directly as effector cells in killing the organisms. Which cells act as effector cells is not clear, although macrophages have been shown to interact with the organisms [4–5]. Cytokines, the

intercellular messengers, mainly derived from macrophages or T-helper lymphocyte subsets, are involved in many inflammatory processes and are likely to be involved in the host defence against *Pneumocystis carinii* (*P. carinii*), but their exact role in PCP is not yet clear.

In vitro studies have shown that *P. carinii* possesses binding sites for tumour necrosis factor- α (TNF) and that this cytokine is capable of killing *P. carinii* [6–8]. In mice TNF and interleukin-1 β (IL-1 β) are critical in the clearance of *Pneumocystis*, and interleukin-6 (IL-6) regulates pulmonary inflammation and antibody response during resolution of PCP [9–11]. In addition, several authors have reported the ability of *Pneumocystis* to stimulate *in vivo* and *in vitro* production of TNF by alveolar macrophages [12–17]. Thus, as in many bacterial and protozoal infections, IL-1, IL-6 and TNF seem to play a role in the pathogenesis of PCP.

We therefore studied the profile of these cytokines and their inhibitors in blood as well as at the site of infection in patients with PCP. Although at present most PCP episodes are seen in patients with HIV infection, it is still an important opportunistic infection in other immunocompromised patients, notably organ transplant patients and patients with lymphatic malignancies.

A series of cases of PCP in such HIV-seronegative patients, mainly renal transplant recipients, recently diagnosed at our hospital enabled us to follow cytokine concentrations and production capacity in bronchoalveolar lavage (BAL) fluid and in the blood.

Materials and methods

Patients

All HIV-seronegative adult patients with PCP, diagnosed by BAL, between October 1992 and March 1993 were included in the study. Diagnosis of PCP was based on demonstration of *P. carinii* organisms

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Table 1. Cell differential (median and range) in BAL and peripheral blood

	BAL			Peripheral blood		
	Granulocytes (%)	Lymphocytes (%)	Macrophages (%)	Granulocytes (%)	Lymphocytes (%)	Monocytes (%)
Healthy control subjects	2 (1-5)	3 (1-8)	95 (92-99)	57 (18-65)	35 (25-61)	4 (3-6)
PCP patients	7 (2-45)	7 (1-25)	75 (45-97)	82 (4-99)	10 (1-77)	4 (0-16)

on Churukian-Schenk amoniactal silver-stained and Giemsa-stained BAL specimen [18]. At the time of BAL, blood samples from all patients were taken for measurement of circulating and *ex vivo*-produced cytokines and inhibitors; another sample was obtained after full recovery. Nine patients were non-smokers. All patients were proved to be seronegative for HIV. None of the patients received non-steroidal anti-inflammatory drugs or pentoxifylline.

The study was approved by the hospital ethical committee and informed consent was obtained from all patients and healthy control subjects.

Control subjects

Eight non-smoking subjects who volunteered to undergo BAL were used as controls. They were all in good health and none had risk factors for HIV infection. HIV testing was not done for ethical reasons. None of them used medication or had evidence of lung disease or a history of recent respiratory tract infections. Before the BAL, a blood sample was taken for measurement of cytokines and white blood cell count.

Bronchoalveolar lavage

After topical lidocaine anaesthesia of the oropharynx and bronchial tree, a flexible fiberoptic bronchoscope was introduced into the bronchial tree and, after inspection, wedged in a subsegmented bronchus serving the area of greatest radiological abnormality. In the controls the lavage was done in the right middle lobe. Bronchoalveolar-lavage was performed by injecting and aspirating 6-8 aliquots of 20 mL of sterile saline. The BAL fluid was collected via a closed system in a polypropylene flask. In patients most of the BAL fluid was used for diagnosis, the remaining fluid (in general 10-20 mL) for cytokine assessment.

Processing BAL fluid

Immediately after BAL, the flask was put on ice and transported to the laboratory. A 30 mL sample was taken for a cell count and cell viability was assessed by trypan blue dye exclusion. The remaining fluid was centrifuged at 500 *g* for 15 min. The supernatant was removed and 1% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) was added. The fluid was then

Table 2. Clinical characteristics of patients with PCP

Patient number	1	2	3	4	5	6	7	8	9	10
Underlying condition	Renal TX	Renal TX	NHL	Renal TX	Renal TX	Renal TX	Renal TX	Renal TX	Auto-immune disease	Renal TX
Duration of symptoms (weeks)	4	3	< 1	< 1	< 1	3	1	2	6	1
Concomitant infections	PTB	<i>Staphylococcus aureus</i> sepsis	-	-	CMV	-	-	-	CMV	-
<i>Pao</i> ₂ (kPa)	5.0	6.5	10.2	8.3	6.0	8.5	8.5	9.4	6.9	8.0
On respirator	Yes	Yes	No	No	Yes	No	No	No	Yes	No
Body temperature (°C) at time of first sample	38.5	37.8	38.3	37.8	39.5	39.2	39.0	40.4	39.6	38.0
Prednisone dose (mg) at time of BAL	80	15	0	80	40	80	80	80	80	80
at recovery or before death	20	10	0	10	50	20	20	10	100	25
Other immuno suppressive drugs	AZA	CsA	-	AZA	CsA	CsA	CsA	CsA	-	CsA
Outcome	Cure	Cure	Cure	Cure	Death	Cure	Cure	Cure	Death	Death
Cure/death at week	5	7	2	2	3	1	2	1	2	3

TX, Transplantation; NHL, non-Hodgkin lymphoma; PTB, pulmonary tuberculosis; CMV, Cytomegalovirus early antigen detected in BAL fluid and therapy with gancyclovir; *Pao*₂, arterial oxygen pressure; AZA, azathioprine; CsA, cyclosporin A.

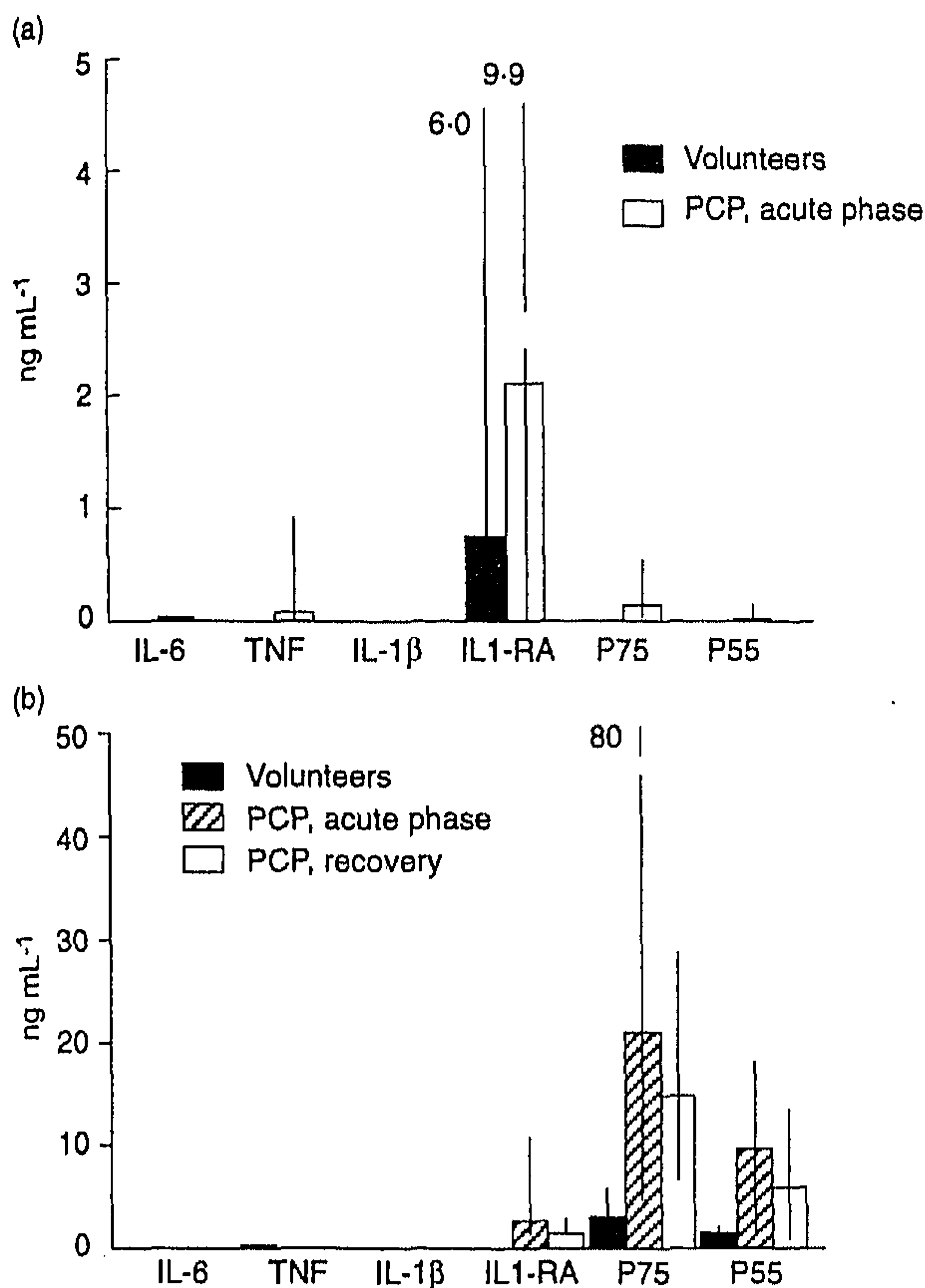


Figure 1. Concentration (median and range, in ng mL⁻¹) of cytokines in BAL fluid (a) and plasma (b) in volunteers and in HIV-seronegative patients with PCP during the acute phase and in recovery (for plasma only). Bars = ranges.

filter sterilized, aliquoted and frozen at -80°C until assayed.

The pellet was resuspended in Dulbecco's modified Eagle medium (DMEM) at a concentration of 0.5×10^6 viable macrophages per mL; $100 \mu\text{L}$ was taken for preparing cytocentrifuged Giemsa-stained preparations and half of the remaining fluid was then incubated in 4-mL closed polystyrene tubes (Greiner, Alphen aan de Rijn, The Netherlands) without any addition (unstimulated culture) and the other half was incubated with lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5, Sigma) at a final concentration of $10 \mu\text{g mL}^{-1}$. All incubations were performed at 37°C for 24 h. After incubation the tubes were centrifuged at 1200 G for 10 min and the supernatant and cell pellets separately frozen at -80°C until cytokine analysis.

Processing whole blood

Venous blood was drawn from patients and volunteers for cytokine assessment in whole blood as described previously [19]. In brief, venous blood was collected aseptically into three 5-mL-draw sterile vacuum tubes, containing 7.2 mg of lyophilized EDTA and $250 \mu\text{L}$ of aprotinin (Trasylol 2500 KIU, Bayer, Leverkusen,

Germany; final concentration 625 KIE mL^{-1} . One tube was immediately centrifuged at $1250 g$ for 10 min and then plasma was transferred to a polypropylene tube (Eppendorf, Sarstedt, Nümbrecht, Germany) and centrifuged at $15\,000 g$ for 1 min to remove the platelets. This platelet-poor plasma was pipetted into another sterile polypropylene tube and frozen at -80°C until cytokine analysis. In one of the two remaining tubes $50 \mu\text{L}$ of LPS was added (final concentration $10 \mu\text{g mL}^{-1}$) to stimulate cytokine production. This tube and the third tube were then incubated at 30°C for 24 h. After incubation the tubes were centrifuged and the plasma frozen at -80°C as described above.

Cytokine assays

Plasma and BAL samples were analysed by means of specific radioimmunoassays for IL-1β, IL-1 receptor antagonist (IL-1RA) and TNF [20,21]. An enzyme-linked binding assay (ELIBA; Hoffman la Roche, Basel, Switzerland) was used for soluble receptors of TNF (sTNF-R; p55, p75) and a bioassay (B9 hybridoma cell line) for IL-6. The detection limits of the various assays were: IL-1β, 20 pg mL^{-1} ; IL-1RA, 800 pg mL^{-1} ; TNF, 20 pg mL^{-1} ; sTNF-R:p55, 80 pg mL^{-1} ; p75, 300 pg mL^{-1} ; and IL-6, 20 U mL^{-1} . Since sTNF-R levels are reported to correlate with serum creatinine levels and with the glomerular filtration rate, a corrected value for sTNF-R concentration was calculated as follows [22]:

$$\text{Actual sTNF concentration} \times \text{creatinine clearance} \times 1/100$$

Statistical analysis

Values are reported as medians and range. Differences between medians were analysed using the Wilcoxon signed-rank test for paired and unpaired samples. $P < 0.05$ was considered significant. One-way analysis of variance (ANOVA) was used to assess differences in the observed cytokine patterns between patients with and patients without concomitant infections.

Results

Clinical characteristics of patients and control subjects

All 10 patients (median age 45 years; range 29–69, four males, six females) had fever, dry cough and/or dyspnoea. Nine patients had interstitial or alveolar infiltrates on the chest radiograph; in one patient (number 4) the chest radiograph was normal.

Of the 10 patients, eight were renal transplant recipients with a median interval between transplantation and PCP of 3 months (range 2–30). One patient

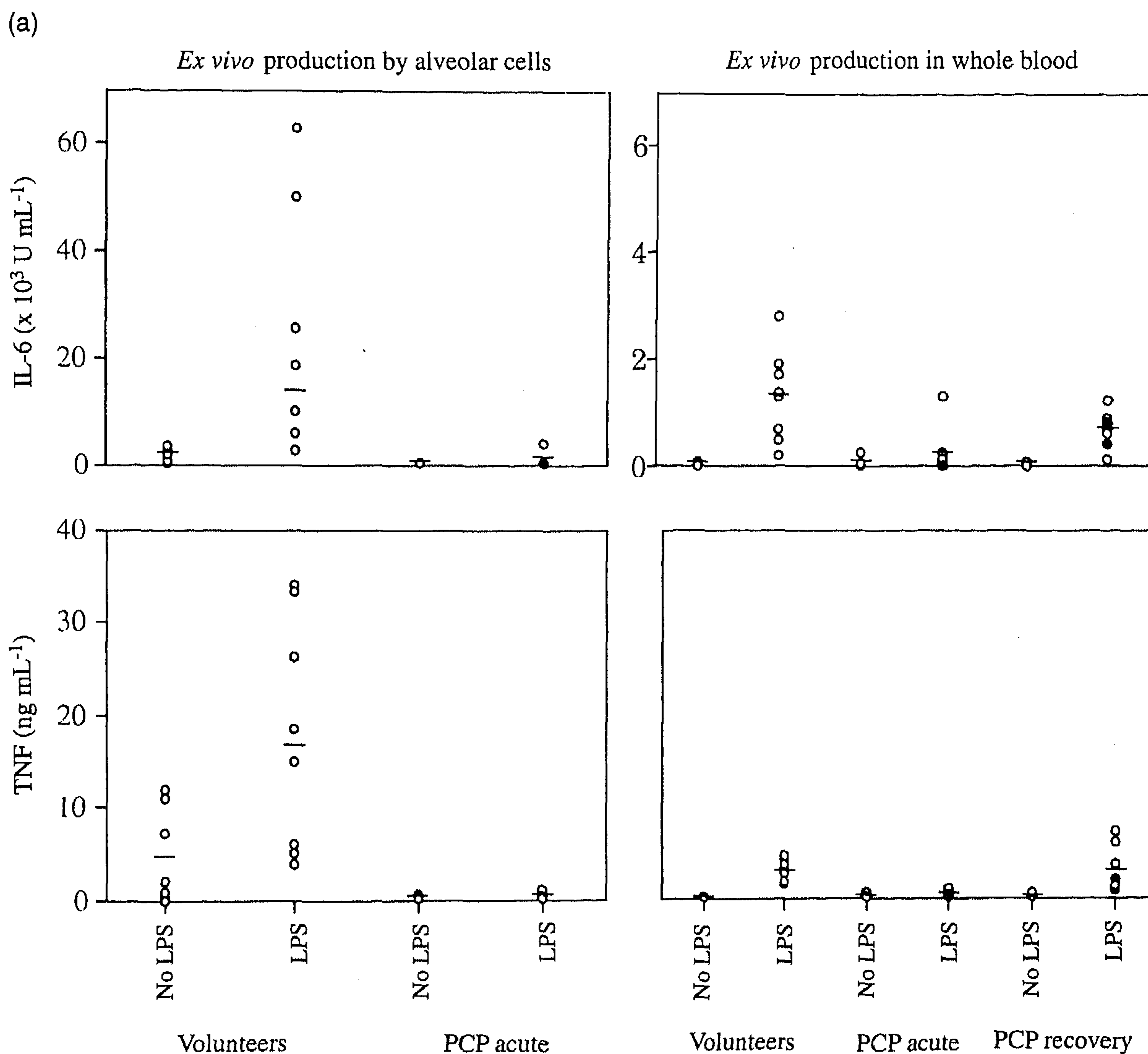


Figure 2. Unstimulated and LPS-stimulated ex-vivo production of cytokines, (IL-6 and TNF; IL-1 and IL-1RA) by alveolar cells and in whole-blood cultures from volunteers and HIV-seronegative patients with PCP in the acute phase and at recovery -, Median; ●, patients without concomitant infections; ○, patients with concomitant infections.

developed PCP while using prednisone for an undetermined auto-immune disease, and one patient was treated with chemotherapy (including prednisone) for non-Hodgkin's lymphoma. None of the patients used PCP chemoprophylaxis. The clinical and laboratory characteristics are summarized in Tables 1 and 2.

The median age of the eight healthy volunteers, two males and six females, was 24 (20–25) years.

Cytokines in BAL and blood

A measurable concentration of IL-6 (446 U mL^{-1}) was present in BAL fluid from only 1 of the 10 PCP patients (Fig. 1a). The concentration of TNF in the BAL fluid was high (6.1 ng mL^{-1}) in one patient (number 7), detectable ($0.1\text{--}0.6 \text{ ng mL}^{-1}$) in five patients and below the detection limit in four patients.

Interleukin- 1β concentrations were detectable in three patients ($0.16\text{--}0.24 \text{ ng mL}^{-1}$) and below the detection limit in seven. The concentration of IL-1RA in the BAL fluid from the PCP patients was 2.1 ($0.4\text{--}9.9$) ng mL^{-1} (Fig. 1a). In 4 of the 10 patients, the IL-1RA concentration in BAL fluid was above the upper normal limit of healthy volunteers ($\geq 95\%$ percentiles). Soluble TNF receptors could not be detected in BAL fluid, except in one patient (number 10), in whom values just above the detection limit were found (p55, 1.2 ng mL^{-1} ; p75, 1.3 ng mL^{-1}). The IL-1RA concentration in BAL fluid from healthy volunteers was 0.75 ($< 0.4\text{--}6.6$) ng mL^{-1} , which was significantly lower than in patients ($P = 0.03$, Fig. 1a). The concentrations of IL-6, TNF, IL- 1β and sTNF-Rs in the BAL fluid from volunteers were below the detection limit.

In the patients, the plasma concentrations of the proinflammatory cytokines IL-6, TNF and IL- 1β

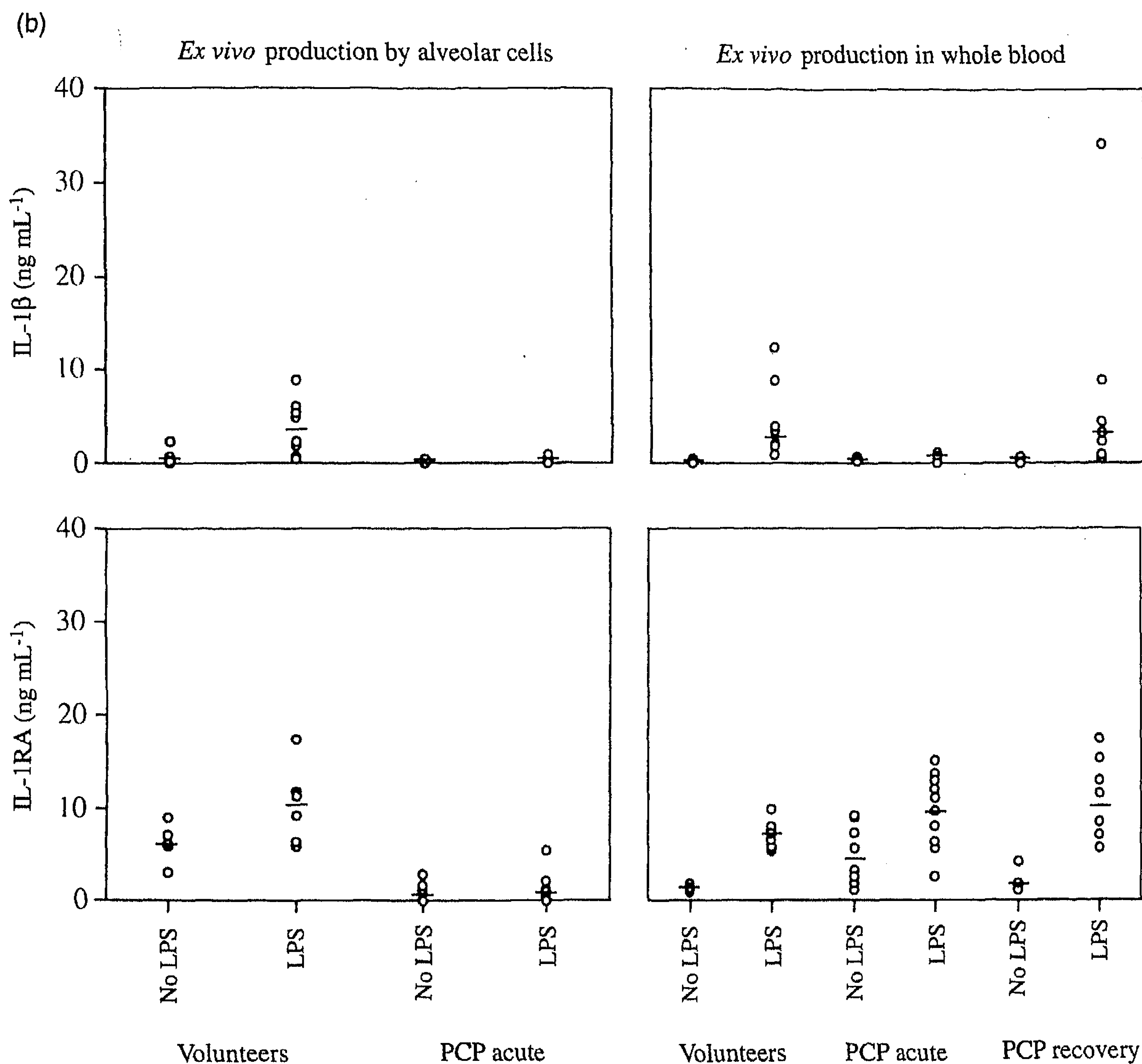


Figure 2. Continued.

were low in the acute phase as well as at recovery (Fig. 1a). In contrast, the plasma concentration of the cytokine inhibitors were elevated in the acute phase. For IL-1RA we found 2.4 (< 0.8–10.3) ng mL⁻¹, for sTNF-R-p55 10.8 (6.6–17.7) ng mL⁻¹ and for sTNF-R-p75 23 (14–36) ng mL⁻¹ (Fig. 1b). At recovery, while using a low, maintenance dose (10–20 mg) of prednisone, the concentration of these inhibitors decreased in the patients but remained significantly higher than in healthy volunteers ($P \leq 0.04$). Even after correction for renal function, sTNF-R concentrations remained significantly higher than in healthy volunteers, both in the acute phase [p55, 3.5 (1.5–7.4) ng mL⁻¹; p75, 9.1 (3.7–19.5) ng mL⁻¹] and during recovery [p55, 2.7 (0.8–8.7) ng mL⁻¹, p75, 3.9 (2.6–15.2) ng mL⁻¹]. Corrected for renal function, p75 concentration was higher in the acute phase ($P = 0.04$) than during recovery, whereas for p55 concentration the difference between the acute

phase and the recovery period did not reach statistical difference.

Cytokine concentrations in blood and BAL were not correlated with severity of infection.

Ex vivo production of cytokines by alveolar cells and blood cells

In six patients (patients 1, 2, 5, 7, 8 and 10) adequate BAL material was obtained for assessment of *ex vivo* production by alveolar cells. The LPS-stimulated *ex vivo* production of the proinflammatory cytokines by the alveolar cells was suppressed in the acute phase of PCP (Fig. 2a and b). We also found a significant suppression of unstimulated and LPS-stimulated *ex vivo* production of IL-1RA by alveolar cells, compared with the production in healthy volunteers ($P < 0.005$, Fig. 2b). Bronchoalveolar lavage was not repeated during follow-up.

The same pattern as with the alveolar cells was seen for the *ex vivo* production of the proinflammatory cytokines in the whole-blood cultures, i.e. suppression of the LPS-stimulated *ex vivo* production in the acute phase of PCP. At recovery the *ex vivo* production returned to the values of the volunteers. In contrast, the *ex vivo* production of IL-1RA did not appear to be affected by disease. After correction for circulating concentrations present in the cultured blood, both unstimulated and LPS-stimulated IL-1RA production was virtually identical in the acute phase and at recovery and similar to the production in volunteers (Fig. 2b). The *ex vivo* production of sTNF-R was not measured since previous experiments demonstrated that TNF receptors were hardly released during LPS-stimulation in the alveolar cell and whole-blood culture system (data not shown).

In two patients with PCP who were investigated recently, blood was drawn just before and 24 h after the start of high-dose (80 mg) prednisone; in both samples the proinflammatory cytokine production was strongly suppressed (240 pg mL⁻¹ and 260 pg mL⁻¹ for IL-1, 300 pg mL⁻¹ and 580 pg mL⁻¹ for TNF before the high-dose prednisone vs. 100 pg mL⁻¹ and 320 pg mL⁻¹ for IL-1, 220 pg mL⁻¹ and 320 pg mL⁻¹ for TNF after the high dose). Analysis of variance failed to detect differences in cytokine patterns between patients with and patients without concomitant infections (for all cytokines $P > 0.13$).

Discussion

In this study, we followed, in 10 HIV-seronegative immunocompromised patients with PCP, circulating and *ex vivo* production of cytokines in whole-blood cultures during the course of infection and measured in the acute phase cytokine concentrations and *ex vivo* production at the site of infection (BAL fluid and alveolar cells).

Although *in vitro* and animal studies suggest an important role of the proinflammatory cytokines in the pathogenesis of PCP early in the course of the infection, we found low concentrations of these cytokines in the blood as well as at the site of infection (i.e. in the BAL fluid) [9–11]. One explanation could be that at first examination of the patient the infection has passed its initial acute stage, as most patients had symptoms for at least a couple of days.

We could not detect a relationship between body temperature and plasma concentrations of the pyrogenic cytokines TNF, IL-1 β and IL-6. Thus, it remains unclear how the febrile response during PCP is mediated. It is remarkable that even IL-6, a pyrogenic cytokine that reaches high circulating concentrations under many clinical conditions of systemic inflammation, was not detectable.

The high concentrations of the anti-inflammatory mediators IL-1RA and sTNF-R-p75 (actual concentration as well as the concentration corrected for

renal function) in the blood and IL-1RA in BAL fluid in the acute phase of the PCP suggest that counter-regulation of proinflammatory cytokines occurs in PCP. During convalescence, IL-1RA and sTNF-R-p75 decrease but remain above the levels seen in volunteers. What causes the high concentration of IL-1RA and p75 in these 'healthy' immunosuppressed patients needs further study. In our patients the LPS-stimulated *ex vivo* production of the proinflammatory cytokines in blood cells was suppressed in the acute phase and improved during convalescence, whereas the production capacity of the anti-inflammatory cytokine IL-1RA was preserved. Previously we have observed a similar pattern in patients with severe meningococcal infection and in patients with typhoid fever [23,24]. Other investigators also reported suppression of proinflammatory cytokine after endotoxin infusion and during other acute disease states [25,26].

We hypothesize that, during an acute infection, blood cells switch from a balanced proinflammatory and anti-inflammatory repertoire (such as seen in healthy volunteers) to an anti-inflammatory repertoire. During the recovery phase the cells gradually regain the balanced status.

The down-regulation of proinflammatory cytokine production of cells in the circulation would not necessarily imply a similar down-regulation at the level of the tissues and at the site of infection. However, in this study we found such down-regulation of proinflammatory cytokines also at the level of the alveolar macrophages, suggesting a more generalized phenomenon.

An intriguing finding is the disparity found between IL-1RA production by blood cells and alveolar cells. In volunteers, unstimulated alveolar cells readily produced IL-1RA, whereas unstimulated blood cells did not produce detectable amounts of this cytokine inhibitor. These findings are in agreement with observations of Kline *et al.* [27] and Moore *et al.* [28] who found that alveolar macrophages express steady-state levels of IL-1RA mRNA under unstimulated culture conditions, whereas peripheral blood cells do so only in response to LPS or adherent IgG. In addition, we found differential regulation of IL-1RA between blood and alveolar cells in the acute phase of PCP: here IL-1RA *ex vivo* production could be stimulated in the blood, but not in alveolar cells. Whether this down-regulation of the anti-inflammatory response has consequences, e.g. in terms of pulmonary damage, needs further study.

It is of interest that, despite the low TNF production capacity of the alveolar cells, TNF is present in the BAL fluid of 60% of the patients with PCP. The cellular source of this TNF is currently unclear, but it is unlikely to be derived from alveolar macrophages, since these cells are refractory in terms of TNF production. What accounts for the down-regulation of proinflammatory cytokine production with relatively preserved IL-1RA production in the course of an infection needs further research. Interleukin-4, a T-lymphocyte-derived

cytokine known to block the production of IL-1, IL-6 and TNF by monocytes, is a potent stimulus for IL-1RA production [29,30]. It could therefore be involved in the switch. Likewise, the cytokines IL-10 or TGF- β could be involved [31,32].

An important question is what role immunosuppression and the concomitant infections play in our study. Several investigators indicate that glucocorticosteroids and cyclosporin A decrease proinflammatory cytokine production [33–35]. With regard to IL-1RA production, Santos *et al.* [36] have reported a minimal influence of hydrocortisone on endotoxin-stimulated IL-1RA production in volunteers. In our study however, the pattern of cytokine production did not appear to be influenced by prednisone dose (ranging from 0 to 80 mg in the acute phase and from 0 to 20 mg in convalescence) and cyclosporin A treatment. The concomitant infections occurring in our patients did seem to influence cytokine patterns in the same direction as PCP, since the cytokine patterns of the six patients without concomitant infections were not different from the others. Although it is reasonable to presume that the immunosuppressive drugs and the concomitant infections in these patients interfered with the magnitude of the cytokine production, the observed cytokine patterns do not seem to be greatly influenced by either these drugs or the concomitant infections.

In conclusion, we found elevated TNF concentrations in BAL in acute PCP and elevated plasma levels of the anti-inflammatory cytokine IL-1RA and the soluble TNF receptors in the acute phase of a PCP in 10 HIV-seronegative patients, with at the same time a suppressed production of proinflammatory cytokines, in the blood as well as at the site of infection. During convalescence the blood cells return to a balanced proinflammatory status. IL-1RA production in acute PCP was down-regulated in alveolar cells and preserved in blood cells.

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