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

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Abstract. Concentrations and ex vivo production of interleukin 1β (IL-1), tumour necrosis α (TNF), interleukin 6 (IL-6), interleukin-1 receptor antagonist (IL-1RA) and TNF soluble receptors (sTNF-receptors, P55 and P75) were measured in bronchoalveolar lavage (BAL) fluid and blood in 23 HIV-seropositive (HIV+) patients with *Pneumocystis carinii* pneumonia (PCP) and compared with values found in healthy HIV-seronegative (HIV−) controls and asymptomatic HIV+ subjects. Concentrations of the proinflammatory cytokine IL-1β were increased in BAL fluid of HIV+ patients with PCP (184 ± 47 pg mL⁻¹) compared with undetectable levels in healthy control subjects (*P = 0.0001*). In plasma of these patients higher concentrations of the anti-inflammatory cytokine IL-1RA were found during acute PCP than after recovery (2.1 ± 0.7 vs. 0.5 ± 0.2 ng mL⁻¹, *P = 0.01*). No correlations could be found between cytokine concentrations and clinical severity of the infection. Corticosteroid treatment did not influence cytokine concentrations in BAL or blood, nor did it suppress the production of tumor necrosis factor-α (TNF) by alveolar cells. In whole-blood cultures, however, lipopolysaccharide (LPS)-stimulated production was significantly suppressed for IL-1 (1.3 vs. 5.5 ng mL⁻¹, *P = 0.009*) and for IL-6 (0.6 vs. 2.5 ng mL⁻¹, *P = 0.01*). The overall data show that in HIV+ patients with PCP (similar to what we had found previously in HIV− patients with PCP) proinflammatory cytokines are more prominently present in BAL, whereas anti-inflammatory reaction is predominant in the circulation.

Keywords. Cytokine, HIV, *Pneumocystis carinii* pneumonia, tumour necrosis factor α, soluble tumour necrosis factor receptor, interleukin 1, interleukin 1 receptor antagonist, interleukin 6.

Introduction

*Pneumocystis carinii* pneumonia (PCP) is the most frequently occurring severe opportunistic infection in patients infected with the human immunodeficiency virus (HIV). Clinical and experimental data suggest that CD4⁺ T lymphocytes are important for protection against PCP, although not directly as effector cells [1-3]. Macrophages have been shown to interact directly with *Pneumocystis carinii* (P. carinii) [4-7]. Cytokines are likely to be involved as soluble mediators in the host defence against *P. carinii*, as inducers of other mediators, as chemoattractants and as regulators of neutrophil adhesion, but their exact role remains to be established.

*P. carinii* is able to stimulate in vivo and in vitro production of tumor necrosis factor-α (TNF) by alveolar macrophages [8-13]. In vitro studies suggest that *P. carinii* possesses binding sites for TNF and that this cytokine is capable of killing *P. carinii* [14,15]. In mice, TNF and interleukin 1β (IL-1) are important for the clearance of *P. carinii*, whereas interleukin 6 (IL-6) regulates pulmonary inflammation and antibody response during resolution [16-18]. Thus, similar to a variety of other infections, IL-1, IL-6 and TNF seem to play a role in the pathogenesis of and protection against PCP.

To investigate the role of these proinflammatory cytokines in patients with PCP, we studied the profiles of IL-1, TNF, IL-6 and their inhibitors, IL-1 receptor antagonist (IL-1RA) and the soluble TNF receptors (sTNF-R) in blood as well as in bronchoalveolar lavage (BAL) fluid.

Material and methods

Patients

Twenty-three adult HIV-positive (HIV+) patients with PCP who underwent BAL according to a standardized protocol (see below) at the four participating centres (three University Hospitals and one regional referral Hospital) were included in the study between October

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The diagnosis PCP was based on demonstration of the greatest radiological abnormality. In the control subjects, eight healthy non-smoking subjects who volunteered to undergo BAL were used as control subjects [21]. None of them used medication or had evidence of lung disease or a history of recent respiratory tract infection. Before BAL, a blood sample was taken for measurement of cytokines and white blood cell count (WBC). Blood was obtained for cytokine measurements from 25 asymptomatic ambulant HIV+ individuals with varying CD4 counts who visited the HIV clinic of the University Hospital Nijmegen. They were the asymptomatic HIV+ ambulant subjects for cytokine assessment in whole blood as described previously [22]. Briefly, venous blood was collected aseptically into three 5-mL-draw sterile vacuum tubes, containing 7.2 mg of lyophilized EDTA and 250 µL of aprotinin (Trasylo1 2500 KIU, Bayer, Leverkusen, Germany; final concentration 625 KIU/mL). One tube was immediately centrifuged at 1250×g for 10 min. Plasma was transferred to a polypropylene tube (Eppendorf, Sarstedt, Nümbrecht, Germany) and centrifuged at 15000×g for 1 min to remove platelets.

Processing whole blood

Venous blood was drawn from patients and control subjects for cytokine assessment in whole blood as described previously [22]. Briefly, venous blood was collected aseptically into three 5-mL-draw sterile vacuum tubes, containing 7.2 mg of lyophilized EDTA and 250 µL of aprotinin (Trasylo1 2500 KIU, Bayer, Leverkusen, Germany; final concentration 625 KIU/mL). One tube was immediately centrifuged at 1250×g for 10 min. Plasma was transferred to a polypropylene tube (Eppendorf, Sarstedt, Nümbrecht, Germany) and centrifuged at 15000×g for 1 min to remove platelets.

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 µL of LPS was added (final concentration 10 µg mL⁻¹) to stimulate cytokine production. This tube and the third tube were then incubated at 37°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 specific radioimmunoassays for IL-1, IL-1 receptor antagonist (IL-1RA) and TNF [23,24]. An Enzyme-linked binding assay (Hoffman-la Roche, Basel, Switzerland) was used for soluble receptors of TNF (sTNF-R; P55, P75), and an enzyme-linked immunoassay (ELISA) as described before for IL-6 (Inotherapy, Besançon, France) [25].

The detection limits of the various assays were: IL-1, 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 pg mL⁻¹.

**Statistical analysis**

Values are reported as medians and ranges. Differences between groups were analysed with the Wilcoxon rank-sum test. For paired samples the signed-rank test was used. Correlations between variables were estimated with Spearman’s rank correlation coefficient. P-values of 0·05 or less were considered significant.

The study was approved by the Hospital Ethics Committee and informed consent was obtained from all patients and healthy control subjects.

**Results**

**Clinical characteristics**

Clinical characteristics of patients and control subjects are shown in Table 1. PCP prophylaxis was used by four patients only (17%), all of them used monthly pentamidine. Mean duration of symptoms was 5 (± 5) weeks. Mean arterial oxygen pressure was 8·6 (± 1·5) kPa. Eight (35%) patients had cytomegalovirus early antigen in BAL fluid. Two (9%) patients needed a respirator and two (9%) died. Four of the 23 HIV+ patients with PCP (17%) had concomitant infections (two had cerebral...
PCP Control

Receptors were low in BAL fluid. In three HIV+ patients (>10 ng/mL), the difference was not significant. Production of IL-1RA tended to be higher in the acute phase than during recovery, the difference was however, not significant. Compared with asymptomatic HIV+ persons, proinflammatory cytokine production was slightly lower in the acute phase of PCP and higher during recovery. Compared with healthy control subjects, LPS-stimulated production of TNF in HIV+ patients in the acute phase of PCP was significantly suppressed (P=0.007, Fig. 2B) and IL-1RA production capacity increased (P=0.01, Fig. 2B).

Unstimulated production of proinflammatory cytokines was higher in HIV+ persons than HIV− control subjects and not influenced by the presence of PCP. Median concentrations in all HIV+ persons were 110 pg IL-1 mL−1 (range <20–640 pg mL−1) and 102 pg TNF mL−1 (range 60–282 pg mL−1), whereas concentrations in healthy control subjects were <20 pg IL-1 mL−1 (range <20–280 pg mL−1), and 80 pg TNF mL−1 (range 60–120 pg mL−1).

Effects of glucocorticosteroids and/or concomitant infections on cytokines in HIV+ patients with PCP

Corticosteroids (40–60 mg of prednisone) were given to eight patients, 2–24 h before BAL. Concentrations of cytokines in BAL and plasma did not differ from the 15 patients who were not treated with corticosteroids. LPS-stimulated production in alveolar cells also did not differ significantly between these two groups (data not shown).

Cytokine concentrations in BAL fluid and in plasma

BAL. BAL was performed in the acute phase of PCP and in healthy control subjects. Concentrations of all three proinflammatory cytokines were generally higher in the acute phase of PCP than in healthy control subjects, but significance was only obtained for IL-1 (184 ± 47 pg mL−1 vs. no IL-1 detected, P=0.0001, Fig. 1A). Although concentrations of IL-1RA were high in three HIV+ patients (>10 ng/mL), the difference with healthy control subjects did not reach statistical significance (Fig. 1B). Both types of soluble TNF receptors were low in BAL fluid.

Plasma. With the exception of IL-1RA (2.1 ± 0.7 in the acute phase vs. 0.5 ± 0.2 ng mL−1 during recovery, P=0.01), plasma concentrations of proinflammatory cytokines and anti-inflammatory mediators did not differ between the acute and recovery phase in HIV+ patients with PCP (Fig. 1D).

HIV+ patients with PCP had higher circulating concentrations of IL-1, IL-1RA and P75 than asymptomatic HIV+ patients (124 vs. 98 pg mL−1 respectively, P=0.002, Fig. 1C; 1.3 vs. 0.8 ng mL−1, P=0.03; 8.5 vs. 5.8 ng mL−1, P=0.01, Fig. 1D).

Compared with healthy control subjects, HIV+ patients with PCP had significantly higher concentrations of proinflammatory cytokines (P<0.002, Fig. 1C) and of sTNF-receptors (P<0.0006, Fig. 1D), whereas IL-1RA did not differ between the two groups.

Ex vivo production of cytokines

Alveolar cells. During the acute phase of PCP, LPS-stimulated production of proinflammatory cytokines by alveolar cells of HIV+ patients did not differ from the production of healthy control subjects (Fig. 2A), whereas LPS-stimulated production of IL-1RA was significantly higher (29.8 ± 4.9 vs. 10.4 ± 1.8, P=0.01, Fig. 2A). Similarly, unstimulated ex vivo production showed higher production of IL-1RA in HIV+ patients with PCP, median 12.5, range 1.6–95 ng mL−1 vs. median 6.5, range 0.8–9.7 ng mL−1 in healthy control subjects (P<0.05).

Whole-blood cultures. LPS-stimulated production of IL-6 and TNF was suppressed in the acute phase of PCP, and restored during recovery (Fig. 2B). Although the same trend was seen for production of IL-1, the difference was not significant. Production of IL-1RA tended to be higher in the acute phase than during recovery, the difference was however, not significant. Compared with asymptomatic HIV+ persons, proinflammatory cytokine production was slightly lower in the acute phase of PCP and higher during recovery. Compared with healthy control subjects, LPS-stimulated production of TNF in HIV+ patients in the acute phase of PCP was significantly suppressed (P=0.007, Fig. 2B) and IL-1RA production capacity increased (P=0.01, Fig. 2B).

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cytokines in HIV+ patients with PCP

Table 2. Effect of corticosteroids on (anti-)inflammatory mediators. Plasma concentrations and LPS-stimulated production of (anti-)inflammatory mediators were measured in two HIV+ patients with PCP before administration of 40 mg of prednisone (C—) and 24 h after the first dose (C+). Data are presented in ng mL⁻¹

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<tr>
<th></th>
<th>Plasma concentration</th>
<th>Ex vivo production</th>
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<tr>
<td></td>
<td>C—</td>
<td>C+</td>
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<tr>
<td><strong>Patient A</strong></td>
<td></td>
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</tr>
<tr>
<td>IL-1</td>
<td>0·12</td>
<td>0·11</td>
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<tr>
<td>IL-6</td>
<td>&lt;0·02</td>
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<td>TNF</td>
<td>0·11</td>
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<td>IL-1RA</td>
<td>1·0</td>
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<td>sTNF-R, p. 55</td>
<td>3·0</td>
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<td>sTNF-R, p. 75</td>
<td>7·5</td>
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<tr>
<td><strong>Patient B</strong></td>
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<td>IL-1</td>
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<td>sTNF-R, p. 75</td>
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However, LPS-stimulated ex vivo production in whole blood showed significantly reduced IL-1 (median 300, range 100–7400 pg mL⁻¹) and IL-6 (median 180, range 30–2960 pg mL⁻¹) production in corticosteroid-treated patients as compared with untreated patients (IL-1 mean 5700, range 700–30300 pg mL⁻¹, P = 0.009; and IL-6 mean 2060, range 160–4950 pg mL⁻¹, P = 0.01); there was no difference in TNF and IL-1RA synthesis.

In two patients circulating concentrations of cytokines were not different before and within 24 h after steroid administration. However, profound effects were seen on whole-blood cytokine production in one patient, whereas in the other patient the production was already reduced before the administration of corticosteroids (Table 2).

Unexpectedly, cytokine concentrations and production in the four HIV+ patients with PCP and another opportunistic infection (cerebral toxoplasmosis in two, cryptococcal pneumonia in one and salmonella septicaemia in one) were not different from those without concomitant infections (data not shown). We were also unable to show that cytomegalovirus in BAL fluid, use of prophylactic pentamidine inhalations or prior use of anti-Pneumocystis carinii therapy affected cytokine profiles.

Discussion

HIV+ patients with PCP showed higher concentrations of the proinflammatory cytokine IL-1 in BAL fluid than healthy control subjects. In the circulation, higher concentrations of the anti-inflammatory cytokine IL-1RA were found compared with the situation during recovery.

It is tempting to hypothesize that high concentration of anti-inflammatory mediators in the circulation ensures that the effect of proinflammatory cytokines remains limited to the focus of infection. The host is subsequently protected against the deleterious systemic effects of proinflammatory cytokines, including damage to the endothelium, shock and mortality. At the site of infection, these cytokines are important as early response mediators regulating cellular function, activating the host response against P. carinii and dictating events leading to repair of tissue injury. Their presence in BAL in patients with PCP is in accordance with the findings in in vitro and animal studies showing that TNF and IL-1 play a role in the acute phase of PCP [12,13,17,18].

Increased concentrations of proinflammatory cytokines in BAL fluid and lungs were also found in HIV-negative (HIV−) immunocompromised patients and steroid-treated rats with PCP [21,26]. TNF was significantly increased in BAL fluid of HIV− patients, IL-6 in BAL fluid of rats. In lung homogenates of rats, concentrations of IL-1 were increased. Thus, in PCP at least one of the proinflammatory cytokines is present at the site of infection, although this is not always the same mediator. Why different proinflammatory cytokines are present in BAL in the various PCP models (HIV+ patients, immunosuppressed HIV− patients and steroid-treated rats) needs further study. The explanation for these differences is most probably multifactorial and may reflect different underlying immune defects or disease stages. It is remarkable that TNF, which is most frequently mentioned in relation to HIV and P. carinii, is only increased in BAL in HIV− patients. These data do not corroborate findings by Millar et al. [27], who reported increased production of TNF by alveolar macrophages in HIV+ patients with PCP. Differences in cell populations (adherent cells vs. unselected BAL cells in our study), incubation time (4 vs. 24 h) and assays (immunoassay vs. radioimmunoassay) might be responsible for these discrepancies. Our data are, however, in accordance with the results by Kandil et al. [13]. They show that HIV infection modulated the response to P. carinii; monocyte-derived macrophages in vitro infected with HIV and

co-incubated with \textit{P. carinii}, produce less TNF and IL-1 than non-HIV infected cells.

Anti-inflammatory mediators in BAL fluid of HIV– and HIV+ patients with PCP show low concentrations of s-TNF receptors, whereas IL-1RA is significantly increased in the HIV– patients and marginally increased in HIV+ patients. Although in some HIV+ patients the increased concentrations of IL-1 were accompanied by a local increase in IL-1RA, this was not the case in all patients. Why in some HIV+ patients with PCP IL-1RA levels were higher in the acute phase of PCP than during recovery.

For cytokine patterns in the lung, we had to compare HIV+ patients with healthy control subjects because a BAL procedure was not considered eligible during recovery or in asymptomatic HIV+ persons. For cytokines in the circulation, acute phase was compared with recovery in the same patients to match for the presence of HIV infection. These data must be interpreted with caution because of the use of different control subjects. Another problem in our study was the uneven distribution of smokers. Smoking has been shown to decrease IL-6 concentration in BAL fluid as well as \textit{ex vivo} production of TNF and IL-6 by alveolar macrophages. In our study 39% of the HIV+ patients with PCP smoked, whereas all healthy control subjects were non-smokers. It is therefore possible that without this confounding factor, concentrations of proinflammatory cytokines in BAL fluid in PCP patients would be even higher. Moreover, our principle conclusion of divergence of local and systemic cytokine profile is supported by similar data in HIV– patients with PCP and a rat model.

Correlations between inflammatory mediators and disease activity were not found in our study (data not shown). This is in contrast to findings of Benfield et al. (29,30), who showed a significant correlation between the chemotactic cytokine IL-8 and severity of infection, as determined by \textit{Po2} and BAL neutrophilia, TNF and IL-1, which are able to induce IL-8, may have been present early in the disease process but may have disappeared at the first presentation of the patient. From other infectious diseases, e.g., meningococcal sepsis, we have found that IL-8 remains much longer in the circulation than IL-1 and TNF (31). The high circulating concentrations of proinflammatory cytokines in HIV+ patients with PCP should be interpreted against the background of cytokine abnormalities in HIV. We were able to confirm that HIV infection \textit{per se} results in high plasma concentrations of proinflammatory cytokines and s-TNFRs, and also in increased unstimulated proinflammatory cytokine production (32–35). PCP did not further increase these plasma concentrations and unstimulated production. Plasma IL-1 concentration in the acute phase of PCP in HIV+ patients was not significantly increased as compared with recovery, but was significantly higher than in asymptomatic HIV+ persons, which might be explained by the more severe HIV infection in the PCP patients; median CD4 count 30 vs. 100 in the asymptomatic persons (Table 1).

In a recent study, Huang & Eden (36) found diminished LPS-stimulated production of IL-1 and TNF by alveolar cells of AIDS patients with PCP who received corticosteroids and suggested that the beneficial effect of steroids in PCP may be due to a local reduction in proinflammatory cytokine production. In our study, however, production of proinflammatory cytokines by alveolar cells in HIV+ patients with PCP was not reduced in patients who received high-dose corticosteroids 2–24 h before BAL. It is therefore improbable that the beneficial effect is due to suppression of local production of proinflammatory cytokine, unless one assumes that increased proinflammatory cytokine production in severe PCP is counteracted by suppression of corticosteroids.

Our data from steroid-induced PCP in a rat model do not support the latter hypothesis (26).

Both HIV– patients and HIV+ patients with PCP treated with steroids showed suppressed production of proinflammatory cytokines in whole-blood cultures in the acute phase. In contrast, proinflammatory cytokine production was not suppressed in the HIV+ patients with PCP, who did not receive steroids. Production in the latter group did not differ from that in recovery, nor from that in asymptomatic HIV+ individuals. The high dosages of corticosteroids were only given to patients with \textit{Po2} \textless \text{8 kPa}. Thus, severe infection could also be responsible for suppression of proinflammatory cytokine production (31,37,38). In four of four patients with PCP (two HIV+ from the present series and two HIV– [20]), blood was taken before and after high-dose corticosteroids; in three of these patients we found that cytokine production was already suppressed before administration of steroids. As all four patients had severe PCP with low \textit{Po2}, severe infection rather than corticosteroids seems to lead to down-regulation of systemic proinflammatory cytokine production.

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