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Pro-inflammatory cytokines in lung and blood during steroid-induced Pneumocystis carinii pneumonia in rats

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Abstract: To gain more insight into the role of cytokines in Pneumocystis carinii pneumonia (PCP) we followed pro-inflammatory cytokine profiles in rats with steroid-induced PCP at 2-week intervals. The cytokines measured were immunoreactive interleukin-1β (IL-1β), bioactive interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α). In vivo cytokine concentrations were determined in three compartments, i.e., bronchoalveolar lavage (BAL) fluid, lung homogenates, and plasma. Lipopolysaccharide (LPS)-stimulated cytokine production by alveolar cells and in whole-blood cultures was measured ex vivo. P. carinii load and host inflammatory response, as determined by lung/body weight ratio and 111In-IgG biodistribution were monitored throughout developing PCP. IL-1β was elevated in lung homogenates (600, range <20–1260 pg/mL) and IL-6 in BAL fluid (48, range <20–115 pg/mL), whereas the pro-inflammatory cytokine concentrations were not increased in plasma. Thus in rats with PCP elevated pro-inflammatory cytokine concentrations were found to be restricted to the lung compartments. Corticosteroids did not significantly influence cytokine concentrations, but showed profound inhibitory effects on ex vivo cytokine production. The LPS-stimulated cytokine production by alveolar cells gradually decreased during the 6 weeks after the start of the steroid injections, whereas the production in whole blood cultures was immediately and completely suppressed. J. Leukoc. Biol. 60: 710–715; 1996.

Key Words: corticosteroids · interleukin-1 · interleukin-6 · tumor necrosis factor α · 111In-IgG biodistribution · bronchoalveolar lavage · lung homogenates

INTRODUCTION

Pneumocystis carinii pneumonia (PCP) is a common pathogen in immunocompromised hosts. Cellular immunity, in particular CD4+ lymphocyte-dependent mechanisms, are considered important host defense mechanisms against Pneumocystis carinii [1, 2]. Evidence to date indicates that cytokines, the intercellular messengers involved in many inflammatory processes, also play a role in PCP. In vitro studies show that P. carinii possesses binding sites for tumor necrosis factor α (TNF-α) and that this cytokine is capable of killing P. carinii [3–5]. In mice TNF-α and interleukin-1β (IL-1β) are critical in the clearance of P. carinii and interleukin-6 (IL-6) was found to regulate pulmonary inflammation and antibody response during resolution of PCP [6–8]. P. carinii has been reported to stimulate in vivo and in vitro production of TNF-α by alveolar macrophages [9–14].

Corticosteroids down-regulate pro-inflammatory cytokine production and increase the risk for PCP in patients and in animal models [15–18]. We previously described cytokine patterns in immunosuppressed HIV seronegative and HIV seropositive patients with PCP [19, 20]. In these studies corticosteroids did not seem to influence plasma cytokine concentrations but we observed strong down-regulation of cytokine production in whole blood cultures of patients with severe PCP who had been given corticosteroids. However, since severe infection in itself can also down-regulate pro-inflammatory cytokine production, it was impossible to assess the relative contribution of corticosteroids and P. carinii pneumonia to the measured cytokine profiles [21, 22].

This prompted us to perform cytokine studies in immunosuppressed rats infected with P. carinii under carefully controlled experimental conditions.

MATERIALS AND METHODS

Animal model

Four- to six-week-old female Spraque-Dawley rats with body weights between 150 and 175 g were immunosuppressed by weekly subcuta-
ous injections of 25 mg hydrocortisone and an 8% protein-restricted diet. Amoxicillin (1 mg/mL) was added to the drinking water to prevent bacterial infections. PCP was induced by close cohabitation with P. carinii-infected rats (PCP rats), thereby exposing them passively to P. carinii, which is transmitted by air. Similar to other investigators, we lost 15–25% of the rats before endpoints were reached [23]. Of the rats who survived 4 weeks of cohabitation with infected littermates, more than 95% developed PCP. As we have shown before, the secondary rats develop a consistent and comparable PCP and the level of infection is standardized by the P. carinii density score (see below). Barrier-sustained rats (housed at another level of the animal quarters in cages covered with air filters) were subjected to the same immunosuppressive regime without development of PCP (steroid rats). Control rats were not immunosuppressed, were barrier-sustained, and were free of PCP (healthy rats). Viral coinfection was excluded by regular serological screening for common rodent viruses. Bacterial or fungal co-infection was excluded by microscopic examination of Giemsa-stained smears of the cut surface of the lung. Body weight was measured weekly.

At 2-week intervals after the start of the immunosuppression, 111indium-IgG (111In-IgG) was given intravenously to a group of PCP, steroid, and healthy rats as described elsewhere [23]. Two days later the animals were killed by bleeding and a bronchoalveolar lavage (BAL) was performed [24]. Total lung weight before the BAL and weights of the separate lung lobes after BAL were measured. The uptake of 111In-IgG was measured in the right upper lung lobe and in a normal calf muscle sample for reference. P. carinii density was assessed in Giemsa and silver-stained impression smears and expressed in the logarithmic P. carinii density score as described by Bartlett [16]. Two individuals who were blinded to the condition of the animals examined the slides and rated them according to the following score: in one 1000× field > 100 cysts = 5, 11–100 = 4, 1–10 = 3, 2–9 = 2, 1 in 10 fields = 1, and 0 in 50 fields = 0.

**Processing BAL fluid and lung tissue**

Immediately after BAL the fluid was centrifuged at 500 g for 15 min. Supernatant was removed, aliquoted, and frozen at −80°C. The pellet was resuspended in Dulbecco’s modified Eagle’s medium at a concentration of 0.5 × 10^6 viable macrophages/mL; viability was assessed by trypan blue dye exclusion. This was subsequently incubated in 2-mL propylene tube and frozen at −80°C until cytokine analysis. To one tube of each dose was added 10 μg of LPS; to the other was added 10 μg of LPS plus 2 mmol phenylmethylsulfonyl fluoride, 1 mmol leupeptin, and 1 mg/mL pepstatin. Cytokine assay samples were analyzed by means of bioassays for IL-6 and TNF-α, respectively, and enzyme-linked immunosorbent assay (ELISA) for IL-1β. IL-6 was measured by use of the B9 hybridoma bioassay; TNF-α was measured by the L929 cytotoxicity assay [25, 26]. For IL-1 we used a specific rat ELISA (provided by Dr. S. Poole, National Institute of Biological Standards, Hertfordshire, UK). The detection limit of all three assays was 20 pg/mL.

**Radiopharmaceutical protocol**

111In-IgG scans and biodistribution are sensitive and quantitative methods to assess the severity of PCP [23, 27, 28]. 111In-IgG enters inflammatory sites via increased vascular permeability, after which the iodine is split off from the IgG and remains at that site. We have shown in an earlier report that 111In accumulation in the lung correlates well with P. carinii density scores and parameters of host inflammatory responses [23]. IgG diethylenetriamine penta-acetic bicyclie anhydride was conjugated to human, nonspecific, polyclonal IgG (Sandoglobalin, Sandoz AG, Neurenberg, FRG) according to the method described by Hnatovich and colleagues [29]. The conjugated solution was radio-labeled with 111In (indium chloride, Mallinkrodt Medical, Petten, The Netherlands) via citrate transchelation. Radiochemical purity was determined by TTRC-SG chromatography (Gelman Laboratories, Ann Arbor, MI) with 0.1 M citrate (pH 5) as the solvent. Labeling efficiency was always >95%.

For biodistribution studies, lung and muscle activity were measured 48 h after injection of 10 μg IgG labeled with 1 MBq 111In by counting the tissue samples in a shielded well-type gamma counter. To correct for radioactive decay and permit calculation of the uptake of the radiopharmaceuticals in each organ as a fraction of the injected dose, aliquots of the respective dose were counted simultaneously. The activity measured in tissues was expressed as percentage of injected dose per gram. Lung-to-muscle ratios were calculated.

**Processing whole blood**

Blood was collected aseptically into three 2-mL polystyrene tubes containing 20 μL heparin (heparin sodium 5000 IU/mL, LEO Pharmaceuticals BV, Weesp, The Netherlands). One tube was immediately centrifuged at 1250 g for 10 min; the plasma was transferred to a polypropylene tube and frozen at −80°C until cytokine analysis. To one of the two remaining tubes LPS was added (final concentration 10 μg/mL) to stimulate cytokine production. This tube and the third tube were incubated at 37°C for 24 h. After incubation the tubes were centrifuged and the supernatant frozen at −80°C.

**Cytokine assays**

Plasma and BAL samples were analyzed by means of bioassays for IL-6 and TNF-α, respectively, and enzyme-linked immunosorbent assay (ELISA) for IL-1β. IL-6 was measured by use of the B9 hybridoma bioassay; TNF-α was measured by the L929 cytotoxicity assay [25, 26]. For IL-1 we used a specific rat ELISA (provided by Dr. S. Poole, National Institute of Biological Standards, Hertfordshire, UK). The detection limit of all three assays was 20 pg/mL.

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**Statistics**

Data on P. carinii density score, lung-to-body weight ratio, and 111In-IgG uptake, were normally distributed and are therefore given in means and standard error (±) and analyzed by Student’s t-test. Cytokine concentrations and production did not follow Gaussian distributions and are therefore given in medians and ranges and analyzed with the non-parametric Mann-Whitney test. Lung-to-body weight ratios, 111In-IgG uptake, and the various cytokine concentrations and production capacities were evaluated as parameters for inflammation using a one-way analysis of variance (ANOVA), including the Bonferroni correction to compensate for multipletesting. Linear regression coefficients were calculated.

The study was approved by the University Committee on Animal Experiments.

**RESULTS**

**Development of PCP and host inflammatory responses**

P. carinii density in lungs of rats exposed to P. carinii-infected littermates steadily increased during the 6 weeks after the start of immunosuppression (Fig. 1A). Similarly, host inflammatory reaction, expressed in terms of lung-to-body weight (L/B) ratio (Fig. 1B) and 111In-IgG lung-to-muscle biodistribution (L/M ratio, Fig. 1C) increased every 2 weeks. Both ratios were correlated with the P. carinii density score (r = 0.8, P < 0.0001).

Although the L/B ratio of steroid rats also slightly increased over time, the ratio was always higher in PCP rats. L/M ratios in PCP rats were significantly higher than in steroid rats or healthy rats at weeks 4 and 6.

*P. carinii* density score (see below).

Although the L/B ratio of steroid rats also slightly increased over time, the ratio was always higher in PCP rats. L/M ratios in PCP rats were significantly higher than in steroid rats or healthy rats at weeks 4 and 6.
Cytokine concentrations in BAL and lung homogenates

Immunoreactive IL-1β was present in BAL fluid of healthy rats at a concentration of 280 pg/mL (range <20–640 pg/mL; Fig. 2A). The median IL-1β concentrations in BAL in steroid and PCP rats were lower, these differences were, however, not significant.

In lung homogenates of PCP rats IL-1β concentrations at weeks 4 and 6 were significantly higher than in steroid or healthy rats (week 6: 600, range <20–1260 vs. 43, range 27–320, respectively, <20, range <20–380 pg/mL, P < 0.05; Fig. 2B).

Bioactive IL-6 could not be detected in BAL fluid of healthy rats or immunosuppressed rats. However, IL-6 was found in BAL fluid of rats with PCP at week 4 and 6, with the highest concentration at week 4 (Fig. 2C). Although these IL-6 concentrations were relatively low (48, range <20–115 pg/mL), the differences with healthy and steroid rats were significant (P < 0.05). IL-6 could not be found in lung homogenates of rats in any of these groups. Bioactive TNF-α could not be detected in BAL fluid or lung homogenates of rats in any group.

Cytokine concentrations in plasma

IL-6 was present in plasma (21–136 pg/mL) from 6 of the 19 immunosuppressed rats in week 4 and 6 irrespective of the presence of PCP. Although IL-6 could not be detected in the plasma of healthy rats, the differences between PCP and healthy rats were not significant. Bioactive TNF-α and immunoreactive IL-1β could not be detected in the plasma of the rats in any group.

Ex vivo cytokine production by alveolar cells

LPS-stimulated production of IL-1β, IL-6, and TNF-α gradually decreased over the 6-week period in both steroid and PCP rats, with a slower decline in PCP rats (Fig. 3).

At week 4 the production of pro-inflammatory cytokines was higher in PCP than in steroid rats, although this difference was only significant for IL-6. In the absence of LPS, ex vivo cytokine production of alveolar cells was generally low (<400 pg/mL); however, in week 4 significant amounts of IL-6 (747, range 20–2650 pg/mL) and TNF-α (545, range <20–2400 pg/mL) secretion was found in the non-stimulated culture supernatant in PCP rats.

Ex vivo production in whole-blood cultures

In healthy rats, LPS-stimulated production of IL-1β was 500, range 200–786 pg/mL and of IL-6 2250, range 600–5000 pg/mL. Both in PCP and steroid rats IL-1β and IL-6 production was almost completely suppressed from week 2 onward (Fig. 3, D and E).

In the absence of LPS ex vivo production of pro-inflammatory cytokines was around the detection limit, with an exception for IL-6 in steroid rats at weeks 4 and
Correlations between cytokine patterns and parameters of inflammation

Cytokine concentrations in BAL, lung homogenates, or plasma did not correlate with the other inflammatory parameters. The LPS-stimulated production of TNF-α and IL-6 by alveolar cells in steroid rats was inversely correlated with L/B ratios \( (r = -0.63, P = 0.01, \text{respectively, } r = -0.72, P = 0.002) \). There was no correlation with L/M ratios. The LPS-stimulated production of IL-6 by alveolar cells in PCP rats was inversely correlated with L/B ratios \( (r = -0.58, P = 0.02) \) as well as with L/M ratios \( (r = -0.61, P = 0.01) \). Production in whole-blood cultures did not correlate with other inflammatory parameters.

DISCUSSION

In this study on experimental PCP in rats, pro-inflammatory cytokine concentrations were followed in BAL fluid, lung homogenates, and plasma and were compared with those in rats subjected to the same immunosuppressive regime but without PCP (steroid rats) and with those of healthy rats. In rats with PCP, elevated pro-inflammatory cytokine concentrations were found in the lung compartments at weeks 4 and 6, i.e., IL-1β in lung homogenates and IL-6 in BAL fluid. Why different pro-inflammatory cytokines are present in the different lung compartments requires further study. The explanation is most likely multifactorial and may reflect relative differences in cytokine-producing cell populations, presence of other inflammatory proteins, and presence or absence of surfactant in the different lung compartments. The presence of pro-inflammatory cytokine concentrations in the lung is supported by the results obtained with ex vivo production of cytokines. At week 4, when the infection is clearly established and all PCP rats have elevated \( P. carinii \) density scores, alveolar cells of PCP rats spontaneously produced substantial amounts of IL-1β and IL-6 and their LPS-stimulated production was higher than in steroid rats. This could point to activation of these cells in vivo. Previously we observed a similar pattern in patients with PCP, showing increased pro-inflammatory cytokine concentrations in BAL fluid \([19, 20]\). In HIV-seronegative/PCP-positive patients on long-term corticosteroids, immunoreactive TNF-α was found to be increased in BAL fluid, whereas in HIV-seropositive patients IL-1β was found to be increased. In this study on rat PCP, we used a TNF-α bioassay and the undetectable concentrations of TNF-α in BAL and lung homogenates may be due to the presence of inhibitors, such as soluble TNF-α receptors. A reliable immunoassay for rat-TNF-α was not available to us.

Our finding of undetectable bioactive TNF-α levels in whole-blood cultures, even following LPS stimulation, differs from the findings of Wright et al. \([30]\). Differences in animal model (rabbit vs. rat) and bioassay (WEHI vs. L929 bioassay) may explain the discrepancy.

Bioactive TNF-α could not be detected in whole-blood cultures.

Fig. 2. Concentrations of immunoreactive IL-1β in BAL fluid (A) and lung homogenates (B) and of bioactive IL-6 in BAL (C) in PCP rats (filled triangles), steroid rats (filled circles), and control rats (open circles). Data are given in median (symbol) and range (vertical lines). Steroid rats are rats immunosuppressed by weekly injections of hydrocortisone and a protein-restricted diet, free of PCP. PCP rats are rats subjected to the same immunosuppressed regimen with severe PCP at week 6. Number of rats killed: control rats, 8; PCP rats, 7 at week 2, 6 at week 4, 5 at week 6; steroid rats 4 at each time point. *Statistical significance \( P < 0.05, \text{Mann-Whitney test} \).

6 (150, range <20–365 pg/mL, respectively, 340, range <20–570 pg/mL).

Bioactive TNF-α could not be detected in whole-blood cultures.
centrations of pro-inflammatory cytokines were not elevated in PCP rats, pointing to a compartmentalization of the pro-inflammatory cytokines in the lungs. One may speculate that the pro-inflammatory cytokines are needed in the lung to neutralize *P. carinii*, whereas the body should be protected from the deleterious effects of pro-inflammatory cytokines in the circulation.

Apparently the effect of steroids on systemic and local cytokine concentrations are limited, as these concentrations were not significantly different between healthy and steroid rats. These findings are consistent with our data in patients with PCP, which showed that cytokine concentrations did not differ between steroid-treated and non-steroid-treated patients \[19, 20\].

The ex vivo production, however, was profoundly influenced by corticosteroids administered in vivo. LPS-stimulated pro-inflammatory cytokine production by alveolar cells in both PCP rats and steroid rats was not immediately suppressed, since it took 6 weeks before complete suppression was reached. These findings refute the suggestion of Huang et al. that the beneficial effect of steroids on the clinical course of PCP is due to immediate suppression of the pro-inflammatory cytokine production by alveolar cells \[31\].

Although ex vivo cytokine production in whole blood cultures was also influenced by corticosteroids, the time course was different; in these cultures the cytokine production was immediately and profoundly suppressed. No production could be elicited at week 2, whereas a slight recovery was seen at weeks 4 and 6. This pattern was similar in PCP and steroid rats, suggesting a profound influence of steroids on the systemic cytokine response.

The differential effect of steroids on cytokine production in alveolar cells and blood is in agreement with the
This study was supported by a grant from the Program coordination committee for AIDS research (PccAO) in the Netherlands.

Action group Biomed-I (contract no. BMH1-CT94-1118) and of Pneumocystis for their help in obtaining permission to use the course of PCP. TNF-α (L/M ratio) seems to be a better parameter of inflammation than the relative body weight, as it discriminates better between steroid-treated rats with and without PCP and hardly differs between healthy and uninfected steroid rats.

Although concentrations of IL-6 and IL-1β were elevated in weeks 4 and 6, when there was established PCP, they did not correlate with the other inflammatory parameters, L/B and 111-In-IgG L/M ratios. This was similar to our findings in patients, showing no correlation between cytokine concentrations and clinical severity of the disease [19, 20].

Despite some differences, the steroid-induced PCP in the rat is a good model for human steroid-induced PCP and suitable to study the effect of cytokine modulation on the course of PCP.

ACKNOWLEDGMENTS

This study was supported by a grant from the Program coordination committee for AIDS research (PccAO) in the Netherlands.

We would like to express our gratitude for the expert help of Gerry Gutters and Henny Eikholt of the Central Animal Laboratory, of Emiel Koenders of the Netherlands Medicine Department and of Tita Oettinger of the Department of Parasitology. We thank the European Concerted Action group Biomed-I (contract no. BMH1-CT94-1118) on Pneumocystosis for their help in obtaining P. carinii-infected rats and the fruitful discussions during the regular meetings.

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