CYTOKINE RESPONSES IN PNEUMOCYSTIS CARINII PNEUMONIA

ROOS PERENBOOM

CYTOKINE RESPONSES
IN
PNEUMOCYSTIS CARINII PNEUMONIA

Roos M.Perenboom

Ontwerp omslag. Marie-Louise Perenboom-Hooymans, Wijchen

CIP data, Koninklijke Bibliotheek, Den Haag
ISBN 90-90099931

1996

CYTOKINE RESPONSES IN PNEUMOCYSTIS CARINII PNEUMONIA

een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor

aan de Katholieke Universiteit Nijmegen,

volgens besluit van het College van Decanen in het

openbaar te verdedigen op maandag 2 december 1996

des namiddags om 3.30 uur precies

door

Rosalinde Maria Perenboom

geboren op 15 oktober 1949

te Amsterdam

Promotor	Prof Dr J W M van der Meer
Co-promotoren	Dr R W Sauerwein
	Dr P Beckers
Manuscript Commissie	Prof Dr D J Ruiter
	Prof Dr E J Ruitenberg (RUU)
	Prof Dr C L A van Herwaarden

The studies presented in this thesis were supported by a grant from the Program coordination committee for AIDS Research (PccAO) in the Netherlands

The printing of this thesis was financially supported by the European Concerted Action group Biomed-I (Contract no BMH1-CT94-1118) on Pneumocystosis

voor mijn ouders

CONTENTS

Chapter 1

INTRODUCTION

HOST DEFENCE AGAINST P.CARINII

Pneumocystis carinii (P.carinii) is the major causative organism of opportunistic pneumonia in patients with the acquired immunodeficiency syndrome (AIDS), and an important pathogen in other immunocompromised patients. The major predisposing condition is impaired cellular immunity as induced by immunosuppressive drugs such as corticosteroids, protein malnutrition and primary immunodeficiency diseases. Animal experiments show the critical role of CD4+ T-lymphocytes in protection against P.carinii. [1,2]. Antibodies have been shown to play some role in host defenses against P.carinii [3-5]. Knowledge of other components of the host defense system is limited but there is evidence that macrophages can phagocytose and kill P. carinii, and that neutrophils are present in higher numbers in broncho-alveolar lavage fluid (BAL) in severe P.carinii pneumonia (PCP) [6,7]. Cytokines are involved as soluble mediators in the host defense against P.carinii, but their exact role in the infection remains to be determined. P.carinii was shown to stimulate production of tumor necrosis factor-α (TNF-α) by alveolar macrophages in-vivo and in-vitro [8-14]. Although in-vitro studies suggest that P.carinii possesses binding sites for TNF-α, it is controversial as to whether TNF-α is capable of directly killing P.carinii [8,15-17]. Nevertheless TNF-α and interleukin-1ß (IL-1ß) are considered to be important for the clearance of P.carinii in mice, while interleukin-6 (IL-6) regulates pulmonary inflammation and antibody response during resolution [18-20]. Increased TNF-α secretion and upregulated TNF-mRNA expression in alveolar macrophages have been shown in mice depleted of CD4+ T cells and infected with P.carinii [9]. In corticosteroid induced PCP in Wistar rats, TNF-α concentration in BAL increases during the first weeks of recovery and is inversely correlated with P.carinii cyst count [21]. In human immunodeficiency virus seropositive (HIVpos) patients with PCP, spontaneous TNF-α production by alveolar macrophages and to a lesser extent by peripheral blood monocytes is markedly increased [13].

Since cytokines mainly work in a paracrine or autocrine way, it can be expected that in a focal infection such as pneumonia, the cytokine pattern at the site of infection differs from the pattern in the circulation. In the lung, between the alveolar space and the blood circulation, there is a third compartment, the interstitial tissue. It is conceivable that even within the lung, cytokine profiles will be different in the various compartments, as differences in microenvironment have profound effects on the phenotype and capabilities

of macrophages, the main producers of TNF-α and IL-1ß.

PCP only develops in immunocompromised hosts. Immune deficiencies per se influence cytokine patterns; corticosteroids suppress pro-inflammatory cytokine production, while HIV infection is often associated with increased pro-inflammatory cytokine production [22-36]. Consequently, cytokine profiles in immunocompromised patients with PCP will be attributable to either PCP and/or the underlying immunosuppression.

OUTLINE OF THIS THESIS

The objective of this thesis was to increase insight in the role of cytokines in the pathogenesis of PCP by exploring the compartments in which the cytokine responses take place. Therefore, we present data on cytokine profiles (in-vivo concentrations and ex-vivo production) in various compartments in animals and humans with different underlying immunodeficiencies. We studied, in plasma as well as in BAL, concentrations of immunoreactive IL-1ß, TNF-α, IL-6 and their inhibitors, IL-1 receptor antagonist (IL-1RA) and the soluble TNF receptors (sTNF-R) in 2 patient groups, consisting of immunosuppressed HIV-seronegative (HIVneg) patients and HIVpos patients with PCP. In addition, immunoreactive IL-1ß and bio-active IL-6 and TNF-α were measured in corticosteroid-treated rats with PCP. In the rats, we studied cytokine profiles in BAL, plasma, and lung homogenates. Ex-vivo production by alveolar cells and in whole blood cultures were studied in patients as well as in rats.

To gain more insight in the role of cytokines during the course of PCP parameters were defined for P.carinii load and host inflammatory responses in corticosteroid-induced PCP in rats. In chapter two we present the rat PCP model. Whether P.carinii is able to stimulate in-vitro production of cytokines is explored in chapter three. The question of cytokine profiles in rats, HIVneg and HIVpos patients is addressed in respectively chapter four, five and six. Chapter seven explores the possible therapeutic role of local TNF modulation in experimental PCP. In the general discussion, in chapter eight, we summarize the common features of cytokine profiles in the different PCP models and present a hypothesis, explaining the differences between local and systemic cytokine profiles in PCP.

REFERENCES:

1. Harmsen AG, Stanckiewicz M. (1990) Requirement for CD4+ cells in resistance to Pneumocystis carinii pneumonia in mice. J Exp Med 172,937-945.
2. Shellito J, Suzara VV, Blumenfeld W, Beck JM, Steger HJ, Ermak TH (1990) A new model of Pneumocystis carinii infection in mice selectively depleted of helper T lymphocytes. J Clin Invest 85,1686-1693.
3. Roths JB, Sidman CL. (1993) Single and combined humoral and cell-mediated immunotherapy of Pneumocystis carinii pneumonia in immunodeficient scid mice. Infect Immun 61, 1641-1649.
4. Gigliotti F, Hughes WT. (1988) Passive immunoprophylaxis with specific monoclonal antibody converse partial protection against Pneumocystis carinii pneumonitis in animal models. J Clin Invest 81, 1666-1668.
5. Harmsen AG, Chen w, Gigliotti F. (1995) Active immunity to Pneumocystis carinii reinfection in T-cell-depleted mice. Infect Immun 63, 2391-2395
6. Von Behren LA, Pesanti EL. (1978) Uptake and degradation of Pneumocystis carinii by macrophages in vitro. Am Rev Respir Dis 118,1051-1059.
7. Mason JR, Hashimoto CH, Dickman PS, Voutty LF, Cobb CJ (1989) Prognostic implications of bronchoalveolar neutrophilia in patients with Pneumocystis carinii pneumonia and AIDS. Am Rev Respir Dis 139, 1336-1342.
8. Pesanti EL (1991) Interaction of cytokines and alveolar cells with Pneumocystis carinii in vitro. J Infect Dis 163, 611-616.
9. Kolls JK, Beck JM, Nelson S, Summer WR and Shellito J. (1993) Alveolar macro-phage release of Tumor Necrosis Factor during murine Pneumocystis carinii Pneumocystis AM J Respir Cell Mol Biol 8,370-376.
10. Tamburrini E, De Luca A, Ventura G et al. (1991) Pneumocystis carinii stimulates in vitro production of tumor necrosis factor-α by human macrophages Med Microbiol Immunol 180, 15-20.
11. Hoffmans OA, Standing JE and Limper AH. (1993) Pneumocystis carinii stimulates tumor necrosis factor-α release from alveolar macrophages through a ß-glucan-mediated mechanism. J Immunol 150,3932-3940
12. Theus SA, Linke MJ, Andrews RP and Walzer PD. (1993) Proliferative and cytokine responses to a major surface glycoprotein of Pneumocystis carinii Infect Immun 61,4703-4709
13. Krishnan VL, Meager A, Mitchell DM and Pinching AJ. (1990) Alveolar macrophages in AIDS patients increased spontaneous tumour necrosis factor-alpha production in Pneumocystis carinii pneumonia. Clin Exp Immunol 80,156-160
14. Kandil O, Fisman JA, Koziel H, Pinkston P, Rose RM, Remold HG (1994) Human immunodeficiency virus type 1 infection of human macrophages modulates the cytokine response to Pneumocystis carinii. Infect Immun 62,644-650.
15. Pesanti EL, Tomicic T, and Donta ST. (1991) Binding of ^{125}I-labelled tumor necrosis factor to Pneumocystis carinii and an insoluble cell wall fraction. J Protozool 38, 28S-29S (Abstract).

16. Donta ST and Pesanti EL.(1990) Tumor necrosis factor-α binds to specific receptors on Pneumocystis carinii. Clin Res 38, 352A (Abstract).

17. Koziel H, Armstrong MYK, Arena C. (1993) TNF-α does not affect viability of P.carinii. Am Rev Respir Dis 147, A34 (Abstract)

18. Chen W, Havell EA, Gigliotti F and Harmsen AG. (1993) Interleukin-6 production in a murine model of Pneumocystis carinii Pneumonia: Relation to resistance and inflammatory response. Infect Immun 61,97-102.

19. Chen W, Havell EA, Moldawer LL, et al (1992) Interleukin-1: An important mediator of host resistance against Pneumocystis carinii. J Exp Med 176,713-718.

20. Chen W, Havell EA, and Harmsen AG. (1992) Importance of endogenous tumor necrosis factor alpha and gamma interferon in host resistance against Pneumocystis carinii infection. Infect Immun 60, 1279-1284

21. Sukura A, Konttinen YT, Sepper R, Lindberg LA. (1995) Recovery from Pneumocystis carinii pneumonia in dexamethason-treated Wistar rats. Eur Respir J 8, 701-708.

22. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin Jr.AS. (1995) Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids Science 270, 283-286.

23. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M (1995) Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis Science 270, 286-290.

24. Peces R, Urra JM, Gorostidi M, Lopez-Larrea C. (1992) Role of maintenance immunosuppression and methylprednisone in OKT3-induced cytokine release. Transplant Proc 24,2596-2599.

25. Santos AS, Scheltinga MR, Lynch E, Brown EF, Lawton P, Chambers E, Browning J, Dinarello CA, Wolff SM, Wilmore DW. (1993) Elaboration of interleukin 1-receptor antagonist is not attenuated by glucocorticoids after endotoxemia.

26. Arch Surg 128,138-144. 26. Shieh JH, Peterson RHF, Moore MAS (1993) Cytokines and dexamethason modulation of IL-1 receptors on human neutrophils in vitro. J Immunol150, 3515-3524.

27. Amano Y, Lee SW, Allison AC. (1992) Inhibition by glucocorticosteroids of the formation of interleukin-1α and interleukin-1ß and interleukin-6. mediation by decreased mRNA stability. Molecular Pharmacology 43, 176-182.

28. Renz H, Henke A, Hoffman P, Wolff LJ, Schmidt A, Rüeschoff J, Gemsa D. (1992) Sensitization of rat aveolar macrophages to enhanced TNF-α release by in-vivo treatment with dexamethasone. Cellular Immunology 144, 249-257.

29. Chollet-Martin S, Simon F, Matheron S, Joseph CA, Elbim C, Gougerot-Pocidalo MA. (1994) Comparison of plasma cytokine levels in African patients with HIV-1 and HIV-2 infection. AIDS 8,879-884.

30. Godfried M, Poll van der T, Jansen J et al (1993) Soluble receptors for tumour necrosis factor a putative marker for disease progression in HIV infection. AIDS 7, 33-36.

31. Zangerle R, Gallati H, Sarcletti M, Wachter H, Fuchs D. (1994) Tumour necrosis fator alpha and tumour necrosis factor receptors in individuals with human immuno deficiency virus infection. Immunology letters 41, 229-234

32. Lafeuillade A, Poizot-Martin I, Quilichini R et al (1991) Increased interleukin-6 production is

associated with disease progression in HIV infection. AIDS 5, 1139-1140

33. Noronha IL, Daniel V, Schimpf K, Opelz G. (1992) Soluble IL-2 receptor and tumour necrosis factor-α in plasma in haemophilia patients infected with HIV. Clin Exp Immunol 87, 287-292.
34. Breen EC, Rezai AR, Kajima K et al (1990) Infection with HIV is associated with elevated IL-6 levels and production. J Immunol 144, 480-484.
35. Hober D, Haque A, Wattre P, Beaucaire G, Mouton Y, Capron A. (1989) Production of tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) in patients with AIDS. Enhanced level of TNF-α is related to a higher cytotoxic activity. Clin Exp Immunol 78, 329-333.
36. Jones PD, Shelley L, Wakefield D. (1992) Tumour necrosis factor-α in advanced HIV infection in the absence of AIDS related secondary infections. J Acquir Immune Defic Syndr 5, 1266-1271.

SERIAL ^{111}INDIUM-LABELLED IgG BIODISTRIBUTION IN RAT PNEUMOCYSTIS CARINII PNEUMONIA

A tool to monitor the course and severity of the infection.

Roos M Perenboom

Wim JG Oyen

Anita CHW van Schijndel

Pieter Beckers

Frans HM Corstens

Jos WM van der Meer

Published in European Journal of Nuclear Medicine, 1995, 122:1129-1132.

(reprinted with permission)

ABSTRACT

To study the effect of new therapeutic strategies, we developed an animal model to monitor course and severity of experimental Pneumocystis carinii pneumonia (PCP) in rats. Pneumocystis carinii (P.carinii) density scores in Giemsa stained impression smears were used to follow P.carinii load. ^{111}Indium (^{111}In) labelled IgGscintigraphy (IgG-scan) and biodistribution, histology of paraffine embedded tissue sections, lung/body weight (L/B wt) ratio and cell count and differentiation of broncho-alveolar lavage fluid were used as parameters of host inflammatory response.

Results: statistically significant differences in L/B wt ratio, number of neutrophils in BAL fluid, P.carinii density score, histologic extend of inflammation and ^{111}In-IgG accumulation in the lung were seen between the rats sacrificed at various timepoints. ^{111}In-IgG accumulation in the lung correlated well with L/B wt ratio, with P.carinii density score and correlated moderately with number of neutrophils in BAL fluid and with histologic extend of inflammation.

INTRODUCTION

Pneumocystis carinii pneumonia (PCP) is an important infection, causing morbidity and mortality in immunocompromised patients, especially AIDS patients [1]. Adverse reactions to effective drugs such as trimethoprim-sulfamethoxazol, clindamycine, pentamidine and trimetrexate have prompted a search for alternative therapeutic strategies [2].

To study the effect of new therapeutic strategies, we developed an animal model to monitor course and severity of experimental PCP in rats. The outcome of infection is decided by the balance between the offensive power of microbial pathogens and the defenses of the host. Sometimes the host succumbs because of an overwhelming invasion of microorganisms, at other times death is due to an overwhelming inflammatory response. To evaluate therapeutic strategies, it is therefore important to monitor the growth of the micro organisms as well as the magnitude of the host response. P.carinii density scores in Giemsa stained impression smears are a good method to follow P.carinii load, but do not give information on the inflammatory response of the host [3]. Histology of paraffin embedded tissue sections gives a good impression of the extent of inflamma-

tion, but is time consuming and difficult to quantify [4,5].

^{111}Indium (^{111}In) labelled IgG scintigraphy (IgG-scan) and biodistribution are effective methods to identify local inflammation in PCP [6,7]. These methods detect disease activity early in the course of the infection and are easy to quantify.

We describe a model to monitor course and severity of experimental PCP in rats, using ^{111}In-IgG biodistribution.

MATERIALS AND METHODS

Animal model

Four to six weeks old, female Spraque-Dawley rats, with bodyweights between 150-175 gram, were immunosuppressed with weekly injections of 25 mg hydrocortisone subcutaneously and a 8% protein restricted diet. Amoxicylline (1mg/ml) was added to their drinking water to prevent bacterial infections. PCP was induced by close cohabitation with P.carinii infected rats. Viral co-infection was excluded by regular serological screening on 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.

Radiopharmaceutical and imaging protocol

Diethylenetriaminepentaacetic bicyclic anhydride (bicyclic DTPA) was conjugated to HIV- and HBsAg-negative, human, nonspecific, polyclonal IgG (Sandoglobulin, Sandoz AG, Neurenberg, FRG) according to the method described by Hnatowich and colleagues (8). The conjugated solution was sterilized by membrane filtration with a 0.4μm millipore filter and aliquots of 0.5 ml were radiolabeled with ^{111}In (Indium chloride, Amersham International Ltd., Buckinghamshire, UK) via citrate transchelation. Radiochemical purity was determined by ITLC-SG chromatography (Gelman Laboratories, Ann Arbor, MI USA) with 0.1 M citrate (pH=5) as the solvent. Labeling efficiency was always higher than 95%.

For imaging purposes, a dose of approximately 10 μg of IgG-labeled with 6 MBq ^{111}In was injected intravenously via the tail vein 48 hours before IgG-scanning. Scintigraphic images were obtained with a Siemens Orbiter gamma-camera connected to a Scintiview image processor (Siemens Inc., Hoffmann Estates, IL). All images were collected in digital format. A quantitative analysis of intrapulmonary accumulation of ^{111}Indium was

calculated, using regions of interest in sequential IgG scans. The mean lung activity was normalized to background by dividing lung by head activity of the animals.

For biodistribution studies, lung and muscle activity was measured 48 hours after injection of 10 μg IgG labelled with 1 MBq ^{111}In 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.

Study design

In these studies a relative large number of rats is needed because in the steroid induced rat model a 15-25% mortality due to other causes that PCP has to be anticipated. In addition the severity of PCP may vary between rats.

In experiment A, we followed 50 individual rats longitudinally. IgG-scans were made at 2 week intervals. The animals were followed untill death, whereafter the lungs were removed and the lung weight measured. Giemsa stained impression smears of the cut surface of the left lobe were examined microscopically to determine numbers of P.carinii.

In experiment B, a transversal study on 32 rats, we compared lung/body weight ratio, number of broncho-alveolar cells, P.carinii density score, inflammation score and ^{111}In-IgG uptake in the lung as parameters during development of PCP. Every 2 weeks 5-8 rats were given ^{111}In-IgG intravenously. Two days later the animals were killed by bleeding and a broncho-alveolar lavage (BAL) was performed [9]. Total lung weight before the BAL and weights of the separate lung lobes after BAL were measured. The uptake of ^{111}In-IgG in the right upper lung lobe and in a normal calf muscle sample for reference were measured. P.carinii density was assessed in Giemsa and silver stained impression smears, extend of inflammation in paraffin embedded tissue sections and cell count and differentiation in cytocentrifuged BAL fluid samples. The logarithmic P.carinii density score was assessed according to the method of Bartlett [4]. In short, 2 individuals examined the slides, scoring at least ten fields per slide according to the following scheme: in one 1000xfield > 100 cysts =5, 11 to 100=4, 1 to 10=3, 2 to 9 in 10 1000xfield =2, 1 in 10 fields =1, and 0 in 50 fields =0.

Extent of inflammation in the paraffin embedded lungsections was assessed by 2 examiners and scoring was defined as follows:0= normal lung histology, 1= mild alveolar and

	WEEK 0	WEEK 2	WEEK 4	WEEK 6
Lung-to-head ratio	1 59	1 81	2 53	3 40

Fig. 1. Serial ^{111}Indium-IgG scans and lung to-head ratios in *P carinii* pneumonia in rats N B Lung-to-head ratio = activity of chosen region of interest in the lung divided by activity of chosen region of interest in the head

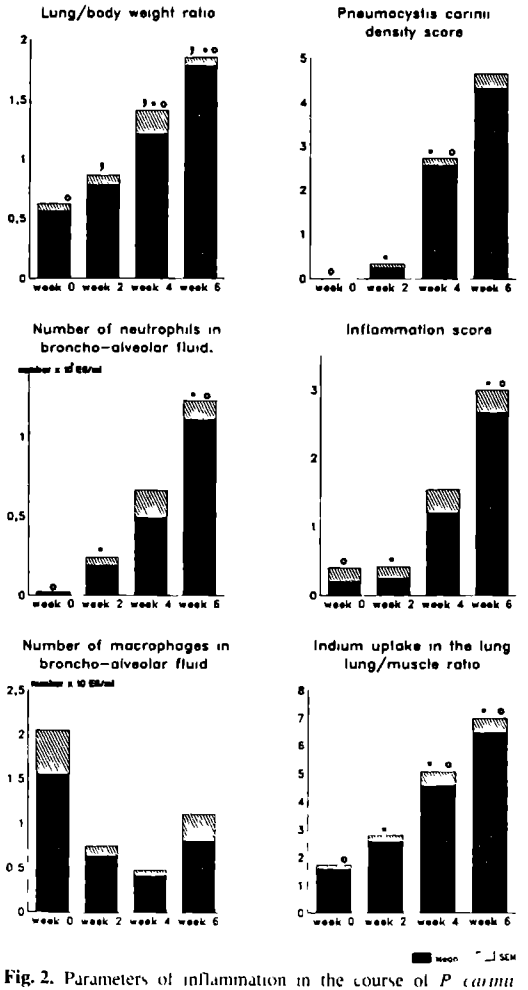


Fig. 2. Parameters of inflammation in the course of *P carinii* pneumonia in rats The appearance of similar signs (°, ■ and ,) above the columns denotes significant differences ($P<0.05$) at the time points in question (ANOVA)

interstitial cellular infiltrate with less than 10% of the alveoli involved, 2= moderate interstitial and alveolar cellular infiltrate, with between 10-75% of the alveoli involved and 3= severe interstitial and alveolar cellular infiltrate with more than 75% of the alveoli involved.

Statistics

The mean lung-to-head ratio at 0, 2, 4 and 6 weeks after start of the PCP inducing diet and corticosteroids were compared using a Student's T-test. Lung/body weight ratio's, number of macrophages and neutrophils in BAL fluid, P.carinii density scores, histology inflammation scores and ^{111}Indium uptake were evaluated as parameters for PCP severity using a one way analysis of variance (ANOVA). Linear regression coefficients were calculated.

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

RESULTS

Serial IgG scintigraphy

In experiment A, 8 rats (16%) died in the first 6 weeks or before an ^{111}In-IgG scan could be made, while 84% of the animals died with severe PCP, 6-11 (median 8) weeks after start of the experiment. From 2 weeks onward a steady increase of accumulation of radioactivity in the lungs was seen. The accumulation of radioactivity in

the head remained the same during the course of PCP. Figure 1 shows a typical example of serial scans and lung-to-head ratio in one rat. Although at visual interpretation, pulmonary uptake seemed to increase at the various timepoints, mean lung-to-head ratio at the various time points (0, 2, 4 and 6 weeks) were not statistically different.

Results of experiment B are presented in figure 2. Nine animals died before termination of the experiment or were excluded because of bacterial or fungal contamination. In the remaining 23 animals statistically significant differences in lung/body weight (L/B wt) ratio, number of neutrophils in BAL fluid, P.carinii density score, histologic extend of inflammation and ^{111}In-IgG accumulation in the lung were seen between the rats sacrificed at various timepoints. The mean ^{111}In-IgG accumulation in the muscles was not different at the various time points. ^{111}In-IgG accumulation in the lung correlated well with L/B wt ratio (correlation coefficient (cc) 0.8, $p<0.0001$), with P.carinii density score (cc 0.8, $p<0.0001$) and correlated moderately with number of neutrophils in BAL fluid (cc 0.6, $p=0.008$) and with extend of inflammation (cc 0.6, $p=0.0026$). It did not correlate with number of macrophages in BAL fluid (cc -0.38, $p=0.149$).

DISCUSSION

This study shows that IgG-scans and ^{111}Indium biodistribution are sensitive and quantitative methods to assess the severity of PCP. An early event in PCP is the alveolar-capillary leak, which follows attachment of P.carinii to the type 1 pneumocyte and its subsequent decrease of surface glycocalix [10]. As shown recently ^{111}In-IgG scintigraphy visualises this leak. The mechanism by which ^{111}In-IgG accumulates in focal sites of infection and inflammation was recently elucidated by Claessen et al [11]. It was shown that ^{111}In-IgG enters the inflammatory site via an increased vascular permeability. In the inflammatory field, the Indium appears to be split off from the IgG and remains at that site. The IgG has been shown to leave the inflammatory site. Our data corroborate the findings by Fishman et al. in rat PCP and by Buscombe et al. in human PCP, that serial IgG scans are useful to monitor the course of PCP [6,12,13]. The present study gives a more precise quantification of the inflammatory parameters and demonstrates that the various parameters correlate with one another. Quantitative analysis of the inflammation can be done by comparing the ratio of lung activity to that of another (non involved) part of the body and was shown to be a reliable quantitative tool in human respiratory infections [10]. In rat

PCP however, this analysis in sequential scans was hampered by the difficulty to draw regions due to interfering blood pool, i.e. heart activity. The tiny lung area of the rat precluded reliable assesment and most probably caused the inability to show statistically significant increases in lung activity. However ^{111}Indium biodistribution proved to be a good quantitative tool to monitor the magnitude of the alveolar-capillary leak. This parameter significantly increases at each 2-week interval and correlates well with various other parameters of severity of the infection. Lung/body weight ratio and ^{111}Indium uptake both mirror the magnitude of alveolar-capillar leakage and correlate well with one another.

We could confirm earlier reports that the number of neutrophils increase during the course of a PCP and found that it correlates well with severity of infection.

In PCP, P.carinii density score in Giemsa stained impression smears, have been shown a reliable and reproducable method to assess P.carinii load [2]. In the present study, P.carinii density score increases gradually during infection and the score was significantly different at the time points chosen (0, 2, 4 and 6 weeks).

Histology of paraffin embedded tissue sections gives a good impression of the extend of inflammation (the host response), but it is time consuming and difficult to quantify. The histological scoring system showed, in our hands, considerable intra- and inter-individual variation, especially early in the infection (data not shown). As the infection is patchy in that period, sampling errors are probably partly responsible. Difficulties in quantification of the inflammation, the work and time needed to prepare and evaluate the tissue sections make this method less suitable to monitor the magnitude of host response in PCP.

In conclusion, we showed that combining data on lung/body weight ratio, number of neutrophils in BAL fluid, P.carinii density score in Giemsa stained impression smears and ^{111}Indium-IgG biodistribution in rat PCP is a good way to monitor course and severity of PCP. Future studies have to show whether this method is useful to evaluate therapeutic strategies in PCP.

Acknowledgements:

The skilful assistance of Gerry Grutters and Henny Eikholt of the Central Animal Laboratory, of Emile Koenders of the Nuclear Medicine Department and of Tita Oettinger of the Department of Parasitology are greatfully acknowledged.

REFERENCES

1. Glatt AE, Chirgwin K. Pneumocystis carinii pneumonia in human immunodeficiency virus-infected patients. Arch Intern Med 1990,150:271-279
2. Koopmans PP, van der Ven AJAM, Vree TB, van der Meer JWM. Pathogenesis of hypersensitivity reaction to drugs in patients with HIV infection: allergic or toxic. AIDS 1995;13:217-223
3. Bartlett MS, Queener SF, Jay MA, Durkin MM, Smith JW. Improved rat model for studying Pneumocystis carinii pneumonia. J Clin Microbiol 1987;25:480-484
4. Kim CK, Foy JM, Cushion MT, Stanforth D, Linke MJ, Hendrix HL, Walzer PD. Comparison of histologic and quantitative techniques in evaluation of therapy for experimental Pneumocystis carinii pneumonia. Antimicrob Agents Chemother 1987;31:197-201
5. Walzer PD, Powell RD, Yoneda K, Rurtledge ME, Milder JE. Growth characteristics and pathogenesis of experimental Pneumocystis carinii pneumonia. Inf Immun 1980;27:928-937
6. Fishman JA, Strauss HW, Fischman AJ, Nedelman M, Callahan R, Kwah BA, Rubin RH. Imaging of Pneumocystis carinii pneumonia with ^{111}In-labelled non-specific polyclonal IgG: an experimental study in rats. Nucl Med Commun 1993,65:147-157
7. Oyen WJG, Claessens RAJM, van der Meer JWM, Corstens FHM. Biodistribution and kinetics of radiolabelled proteins in rats with focal infection. J Nucl Med 1992,33:388-394
8. Hnatowich DJ, Childs RL, Lanteigne D, Najafi A. The preparation of DTPA-coupled antibodies radiolabeled with metallic radionuclides: an improved method. J Immun Meth 1983;65:147-157
9. Mauderly JL. Bronchopulmonary lavage of small laboratory animals. Laboratory Animal Science 1977;27:255-261
10. Yoneda K, Walzer PD. Interaction of Pneumocystis carinii with host lungs: an ultrastructural study Infect Immun 1980;29:692-703
11. Claessens RAMJ, Koenders EB, Boerman OC, Oyen WJG, Boom GF, van der Meer JWM, Corstens FHM. Dissociation of indium from In-111 labelled DTPA conjugated nonspecific polyclonal human immunoglobulin in inflammatory foci. Eur J Nucl Med 1996;37:1392-1397
12. Buscombe JR, Oyen WJG, Grant A, Claessens RAJM, van der Meer JWM, Corstens FHM, Ell PJ, Miller RF. Indium-111-labelled polyclonal human immunoglobulin: Identifying focal infection in patients positive for human immunodeficiency virus. J Nucl Med 1993;34:1621-1625
13. Buscombe JR, Khalkhall I, Mason GR, Rauh D, Meatherall, Oyen WJG, Corstens FMH. Indium-111 labelled pooled human immunoglobulin imaging to monitor the efficacy of specific therapy for Pneumocystis carinii pneumonia. Eur J Nucl Med, 1994;21:1148-1150.

PNEUMOCYSTIS CARINII AND IN-VITRO PRODUCTION OF CYTOKINES

Roos M Perenboom

Robert W Sauerwein

Pieter Beckers

Anita CHW van Schijndel

Jos WM van der Meer

INTRODUCTION

Induction of cytokine responses in cells of the defense system is an important event in cellular immunity and inflammation. Since TNF-α, IL-1ß and IL-6 seem to play a role in the defense against PCP, we studied the capacity of P.carinii cysts to induce in-vitro production of these cytokine in peripheral blood mononuclear cells and alveolar cells [1-4].

MATERIAL AND METHODS

P.carinii cysts and trophozoites were incubated with peripheral blood mononuclear cells (PBMC's) and alveolar cells (obtained by bronchoalveolar lavage) from 8 healthy volunteers for 24 hours. The P.carinii cysts and trophozoites were isolated on a discontinuous Percoll gradient (Sigma Chemical) from enzyme treated (0.2% collagenase and 0.2% hyaluronidase, Sigma) lungs of P.carinii infected rats and a HIV seronegative patient. The cultures were carried out in duplicate with polymyxine B (Sigma, final concentration 1 μg/ml) to neutralize the possible presence of contaminating lipopolysaccharide (LPS). Parallel cultures of the same cells were performed without any additions, with LPS (final concentration 10 μg/ml) and with supernatants of uninfected rat lung preparations. Immunoreactive TNF-α, IL-1ß, and IL-1RA were measured in the supernatants, using specific radio-immuno assays (RIA) [5,6].

RESULTS AND DISCUSSION

LPS-stimulated cultures of PBMC's and alveolar cells showed a significant increase in cytokine production as compared to unstimulated cultures. Cytokine production by PBMC's and alveolar cells co-cultured with P.carinii cysts was not different from the production of cells incubated with uninfected lungs (table 1).

Tabel 1a. In-vitro cytokine production by alveolar cells

	IL-1ß, pg/ml	TNF-α, pg/ml	IL-1RA, ng/ml
No additions	<80 (<80-2280)	900 (390-12.060)	6.5 (<0.8-9.7)
LPS, 10μg/ml	3580 * (360-5440)	22.700 * (5000->33.000)	10.2 (1.4-17.3)
uninfected rat lung	<80 (<80-80)	1300 (660-4100)	3.5 (2.6-7.8)
Human P.carinii	<80 (<80-240)	1430 (680-3780)	4.3 (1.8-9.2)
Rat P.carinii	<80 (<80-100)	1680 (<80-3080)	6.2 (0.8-9.0)

Tabel 1b. In-vitro cytokine production by peripheral blood mononuclear cells.

	IL-1ß, pg/ml	TNF-α, pg/ml	IL-1RA, ng/ml
No additions	<80 (<80-160)	600 (<80-1420)	1.2 (<0.8-6.2)
LPS, 10μg/ml	3760 * (1720- >25.000)	2360 * (440-9620)	3.6 (0.8-11)
uninfected rat lung	<80 (<80-740)	380 (<80-820)	1.1 (<0.8-2.4)
Human P.carinii	<80 (<80-80)	600 (100-940)	0.95 (<0.8-1.1)
Rat P.carinii	<80 (<80)	320 (80-820)	1.4 (<0.8-3.1)

Tabel 1. In-vitro cytokine production by alveolar cells and peripheral blood mononuclear cells (PBMC's) from 8 healthy volunteers. Data are given in median and range.

* Denotes statistical significance ($P<0.05$, Wilcoxon rank test) from unstimulated controls. All cell suspensions contained $0.5x10^6$ cells/ml, all P.carinii cysts suspensions contained 10^6 cysts /ml. To avoid the effects of contaminating lipopolysaccharide (LPS), P carinii cysts and uninfected rat lung suspension were diluted in medium containing 1μg/ml of polymyxin B.

The high production of cytokines in the LPS-stimulated cultures proved that the cells in our system were viable and able to produce cytokines. P.carinii organisms however, could not stimulate the production of TNF-α, IL-1ß and IL-1RA by human PBMC's and alveolar cells. Helmke and Hidalgo also reported that rat alveolar macrophages failed to produce TNF-α when exposed in-vitro to P.carinii organisms [7], but several others did report in-vitro TNF-α release in similar experiments (1-4). As the source and number of P.carinii used, the cell concentrations and the concentration of polymyxine B in our experiments are similar to the conditions used by Tamburrini and Pesanti we cannot explain the discrepancies. These results may be explained by differences in cell selection (unselected alveolar cells versus adherent cells), culture conditions and the presence or abscence of contaminants in the P.carinii preparations.

REFERENCES:

1. Pesanti EL. (1991) Interaction of cytokines and alveolar cells with Pneumocystis carinii in vitro. J Infect Dis 163, 611-616.
2. Tamburrini E, De Luca A, Ventura G et al. (1991) Pneumocystis carinii stimulates in vitro production of tumor necrosis factor-α by human macrophages. Med Microbiol Immunol 180, 15-20.
3. Hoffmans OA, Standing JE and Limper AH. (1993) Pneumocystis carinii stimulates tumor necrosis factor-α release from alveolar macrophages through a ß-glucan-mediated mechanism. J Immunol 150,3932-3940.
4. Theus SA, Linke MJ, Andrews RP and Walzer PD. (1993) Proliferative and cytokine responses to a major surface glycoprotein of Pneumocystis carinii. Infect Immun 61,4703-4709.
5. Nerad JL, Griffith K, van der Meer JWM et al. (1992) Interleukin-1ß(IL-1ß), IL-1 receptor antagonist and TNF-α production in whole blood. J Leucoc Biol 52: 687-692.
6. Van der Meer JWM, Endres S, Lonneman G, et al. (1988) Concentrations of immunoreactive human tumor necrosis factor alpha produced by human mononuclear cells in vitro. J Leucoc Biol 43:16-23.
7. Helmke R, Hidalgo H. (1992) Failure of Pneumocystis carinii to induce tumour necrosis factor and interleukin-6 in alveolar macrophages. FASEB J 6, A1614 (Abstract).

PRO-INFLAMMATORY CYTOKINES IN LUNG AND BLOOD DURING STEROID INDUCED PNEUMOCYSTIS CARINII PNEUMONIA IN RATS

Roos M.Perenboom

Pieter Beckers

Jos W.M. van der Meer

Anita C.H.W. van Schijndel

Wim J.G.Oyen

Frans H.M.Corstens

Robert W. Sauerwein

Journal of Leucocyte Biology, in press

ABSTRACT

To gain more insight in the role of cytokines in Pneumocystis carinii pneumonia (PCP) we followed pro-inflammatory cytokine profiles in rats with steroid induced PCP at 2-weekly intervals. The cytokines measured were immunoreactive interleukin-1 (IL-1) and bioactive interleukin-6 (IL-6) and tumor necrosis factor-α (TNF). In-vivo cytokine concentrations were determined in 3 compartments, i.e. BAL fluid, lung homogenates and plasma. Lipopolysaccharide (LPS)-stimulated cytokine production by alveolar cells and in whole blood cultures was measured ex-vivo. Pneumocystis carinii (P.carinii) load and host inflammatory response, as determined by lung/bodyweight ratio and ^{111}-IgG-Indium-biodistribution were monitored throughout developing PCP.

IL-1ß 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 cytokines 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 start of the steroid injections, while the production in whole blood cultures was immediately and completely suppressed.

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 P.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) and that this cytokine is capable of killing P.carinii [3-5]. In mice TNF and interleukin-1ß (IL-1ß) 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 by alveolar macrophages [9-14].

Corticosteroids downregulate 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 (HIVneg) and HIV seropositive (HIVpos) patients with PCP [19,20]. In these studies corticosteroids did not seem to influence plasma cytokine concentrations, but we observed strong downregulation 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 downregulate 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.

MATERIAL AND METHODS

Animal model

Four to six weeks old, female Spraque-Dawley rats, with body weights between 150-175 gram, were immunosuppressed by weekly subcutaneous injections of 25 mg hydrocortisone and a 8% protein restricted diet (Table 1.). Amoxicilline (1mg/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 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 P.carinii density score (see below) Barrier sustained rats (housed at another level of the animal quarters, in cages covered with airfilters) were subjected to the same immunosuppressive regime without development of PCP (steroid rats). Control rats were not immunosuppressed, barrier sustained and free of PCP (healthy rats). Viral co-infection 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 weekly intervals after start of the immunosuppression, ^{111}Indium-IgG (^{111}In-IgG) intravenously was given to a group of PCP, steroid and healthy rats, as described elsewhere [23]. Two days later the animals were killed by bleeding and a broncho-alveo-

lar 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 ^{111}In-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]. Blinded to the condition of the animals, two individuals examined the slides, according to the following score: in one 1000xfield > 100 cysts =5, 11 to 100=4, 1 to 10=3, 2 to 9 in 10 1000xfield =2, 1 in 10 fields =1, and 0 in 50 fields =0.

Processing BAL fluid and lung tissue

Immediately following BAL the fluid was centrifuged at 500g for 15 minutes. 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 X 10^6 viable macrophages per ml; viability was assessed by trypan blue dye exclusion. This was subsequently incubated in 2-ml closed polystyrene tubes (Greiner, Alphen, The Netherlands) in the presence or absence of lipopolysaccharide (LPS, E Coli serotype 055;B5, Sigma) at a final concentration of 10 μg/ml. All incubations were done at 37° C for 24 hours in a CO_2 incubator. Following incubation the tubes were centrifuged at 1200g for 10 minutes and the supernatant and cell pellets separately frozen at -80°C until cytokine analysis. The left upper lung lobe was homogenized in a blender and sonificated in a 10 ml PBS solution of protease inhibitors, containing 2 mMol Phenylmethylsulfonyl fluoride, 1mg/L antipaine, 1mg/L aprotinine, 1mg/L leupeptine and 1mg/L pepstatin. The sonificated homogenate was centrifuged at 1200g for 15 minutes and the supernatant frozen until cytokine analysis.

Processing whole blood

Blood was collected aseptically into three 2-ml polystyrene tubes, containing 20 μl heparine (Heparine sodium 5000 IU/ml, LEO Pharmaceuticals BV, Weesp, The Netherlands). One tube was immediately centrifuged at 1250g for 10 minutes, the plasma transferred to a polypropylene tube and frozen at minus -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 hours. After incubation the tubes were centrifuged and the supernatant frozen at -80°C.

Cytokine assays

Plasma and BAL samples were analyzed by means of bio-assays for IL-6 and TNF respectively and ELISA for IL-1ß. IL-6 was measured using the B9 hybridoma bio-assay, TNF by the L929 cytotoxicity assay [25,26]. For IL-1 we used a specific rat ELISA (provided by Dr.S. Poole, National Institute Biological Standards, Hertfordshire, England). The detection limit of all three assays was 20 pg/ml.

Radiopharmaceutical protocol

^{111}In-IgG scans and biodistribution are sensitive and quantitative methods to assess the severity of PCP [23,27,28]]. ^{111}In-IgG enters inflammatory sites via an increased vascular permeability, whereafter the indium is split off from the IgG and remains at that site. We have shown in an earlier report that ^{111}In cumulation in the lung correlates well with P.carinii density scores and parameters of host inflammatory responses [23]. IgG Diethylene triamine penta-acetic bicyclic anhydride (bicyclic DTPA) was conjugated to human, nonspecific, polyclonal IgG (Sandoglobulin, Sandoz AG, Neurenberg, FRG) according to the method described by Hnatowich and colleagues [29]. The conjugated solution was radiolabeled with ^{111}In (Indium chloride, Mallinckrodt Medical, Petten, The Netherlands) via citrate transchelation. Radiochemical purity was determined by ITLC-SG chromatography (Gelman Laboratories, Ann Arbor, MI USA) with 0.1 M citrate (pH=5) as the solvent. Labeling efficiency was always higher than 95%.

For biodistribution studies, lung and muscle activity was measured 48 hours after injection of 10 μg IgG labelled with 1 MBq ^{111}In 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.

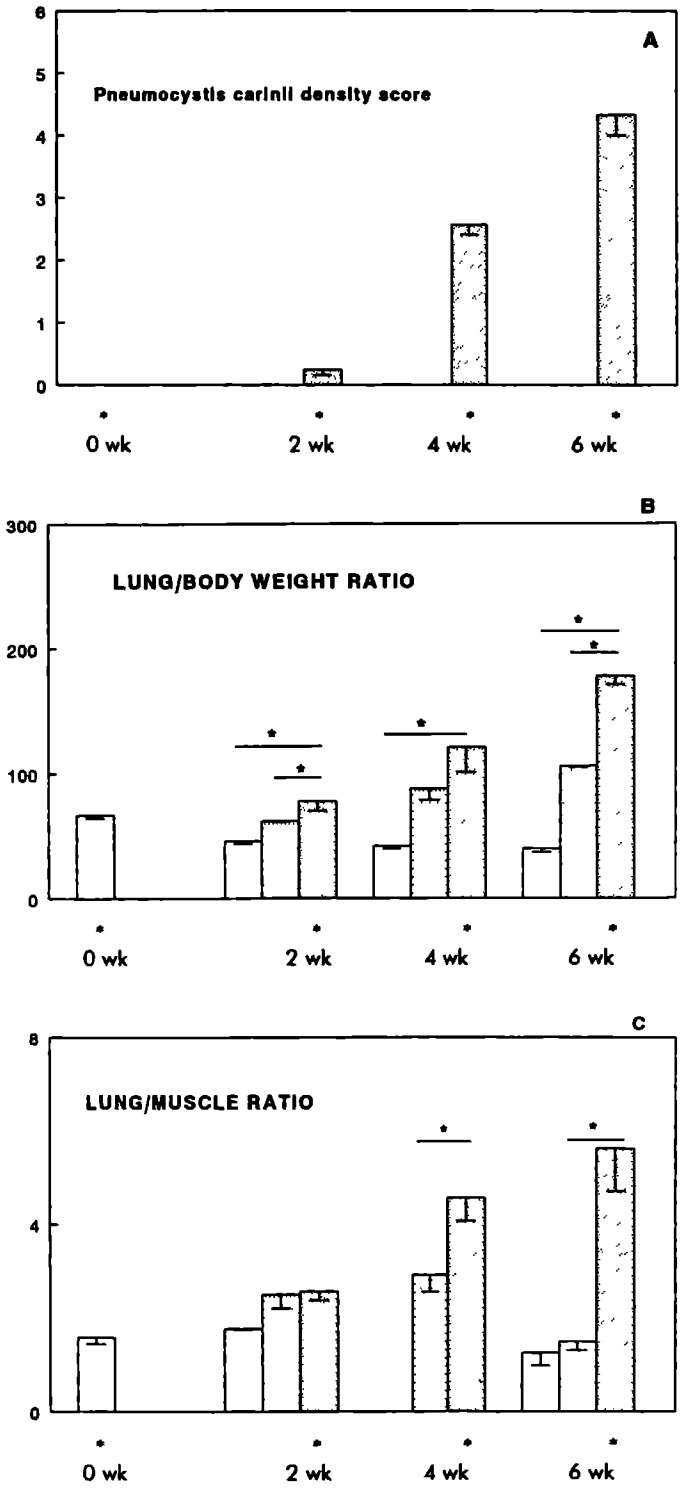


Fig.1. Parameters of inflammation in steroid induced Pneumocystis carinii pneumonia in rats; P.carinii density in the lung (A), relative lungweight (B) and relative uptake of ^{111}Indium-IgG in the lung (C). Steroid rats ( ) are rats immunosuppressed with weekly injections of hydrocortisone and a protein restricted diet, and are free of PCP. PCP rats ( ) are rats subjected to the same immunosuppression regimen with severe PCP at week 6. Healthy control rats ( ) were not immunosuppressed and free of PCP. Number of rats sacrificed: control rats 8; PCP rats 7 at week 2, 6 at week 4, 5 at week 6; steroid rats 4 at each time point. * : indicates statistical significance ($p < 0.05$, Students t test).

Statistics

Data on P.carinii density score, Lung/body weight ratio and ^{111}-IgG-Indium uptake were normally distributed and are therefore given in means and standard error of the means (SEM) and analyzed by Students'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/body weight ratio's, ^{111}-IgG-Indium uptake and the various cytokine concentrations and production capacities were evaluated as parameters for inflammation using a one way analysis of variance (ANOVA). 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 start of immunosuppression (Fig.1A). Similarly, host inflammatory reaction, expressed in terms of lung/body weight (L/B) ratio (Fig.1B) and ^{111}In-IgG lung/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 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 week 4 and 6.

Cytokine concentrations in BAL and lung homogenates

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

In lung homogenates of PCP rats IL-1ß concentrations at week 4 and 6 were significantly higher than in steroid or healthy rats (week 6: 600, range <20-1260 versus 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

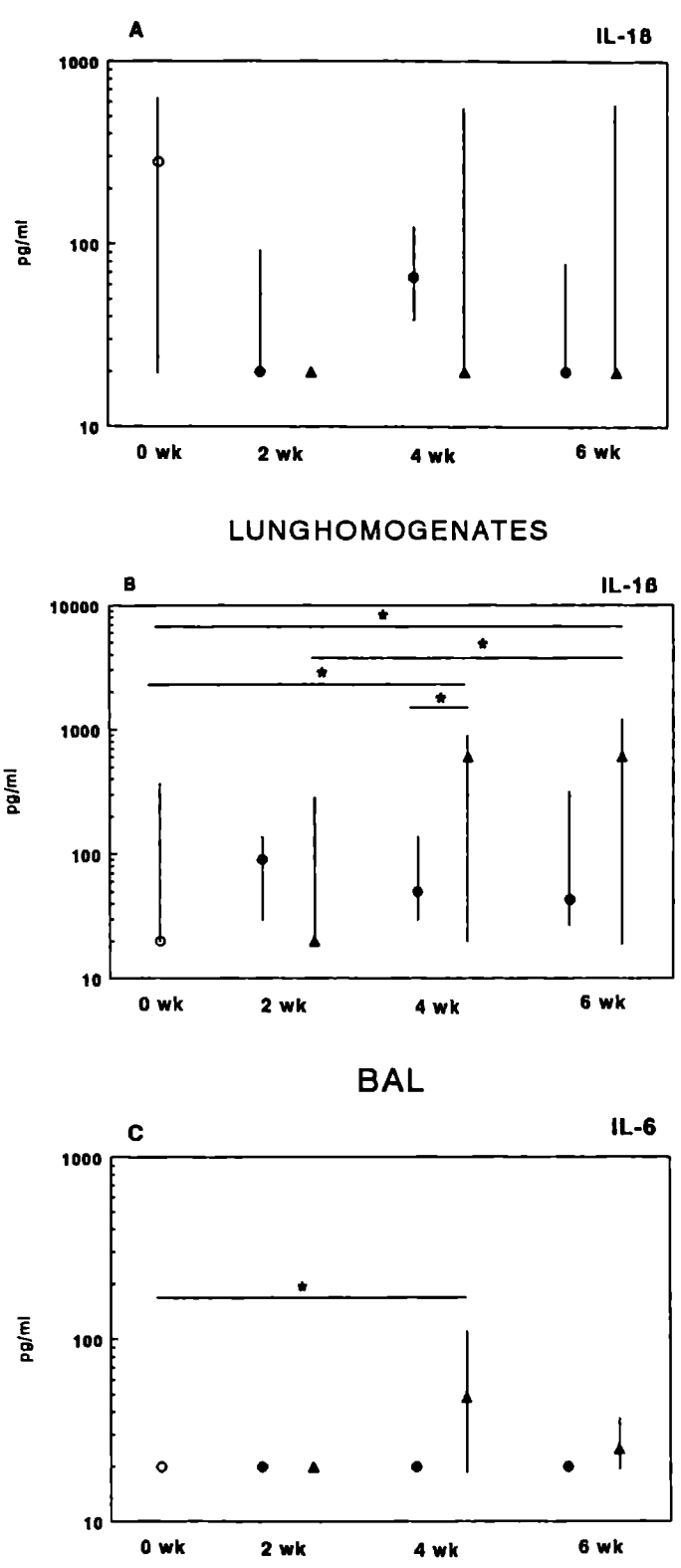


Fig.2. Concentrations of immunoreactive IL-1ß in BAL fluid (A) and lung homogenates (B) and of bio-active IL-6 in BAL (C) in PCP rats (▲), steroid rats (●) and control rats (o). Data are given in median (symbol) and range (|). Steroid rats are rats immunosuppressed by weekly injections of hydrocortisone 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 sacrificed: control rats 8; PCP rats 7 at week 2, 6 at week 4, 5 at week 6; steroid rats 4 at each time point. *: indicates statistical significance ($p < 0.05$, Mann-Whitney test).

were significant ($p < 0.05$). IL-6 could not be found in lung homogenates of rats in any of these group. Bioactive TNF 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 the presence of PCP. Although IL-6 could not be detected in the plasma of healthy rat, the differences between PCP and healthy rats were not significant. Bioactive TNF and immunoreactive IL-1ß could not be detected in plasma of the rats in any group.

Ex-vivo cytokine production by alveolar cells

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

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 week 4 and week 6 (150, range <20-365 pg/ml respectively 340, range <20-570 pg/ml).

Bio-active TNF could not be detected in whole blood cultures.

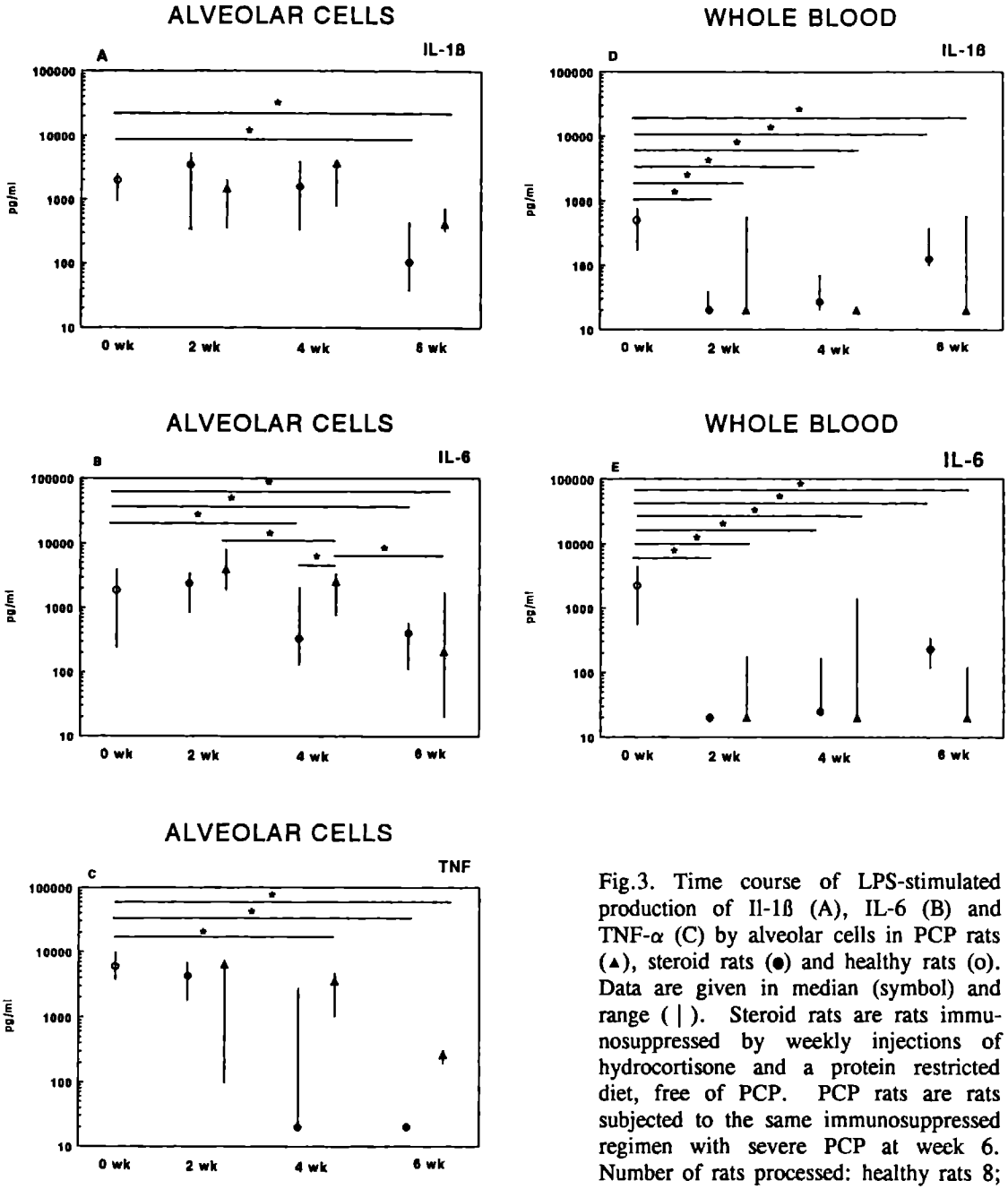


Fig.3. Time course of LPS-stimulated production of Il-1ß (A), IL-6 (B) and TNF-α (C) by alveolar cells in PCP rats (▲), steroid rats (●) and healthy rats (o). Data are given in median (symbol) and range (|). Steroid rats are rats immunosuppressed by weekly injections of hydrocortisone and a protein restricted diet, free of PCP. PCP rats are rats subjected to the same immunosuppressed regimen with severe PCP at week 6. Number of rats processed: healthy rats 8; PCP rats 7 at week 2, 6 at week 4, 5 at week 6; steroid rats 4 at each time point. * : indicates statistical significance ($p < 0.05$, Mann-Whitney test).

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 and IL-6 by alveolar cells in steroid rats was inversely correlated with L/B ratios ($r = -0.63$, $P=0.01$ 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 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 week 4 and 6, i.e., IL-1ß in lung homogenates and IL-6 in BAL fluid. Why different pro-inflammatory cytokines are present in the different lungcompartments needs 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 lungcompartments. 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-1ß 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 patients with PCP on longterm corticosteroids, immunoreactive TNF was found to be increased in BAL fluid, while in HIV-seropositive patients IL-1ß was found to be increased. In this study on rat PCP, we used a TNF bioassay and the undetectable concentrations of TNF in BAL and lung homogenates may be due to the presence of inhibitors, such as soluble TNF-receptors. A reliable immunoassay for rat-TNF was not available to us. Our finding of undetectable bio-active TNF levels in whole blood cultures, even following LPS stimulation, differs from the findings of Wright

et al.[30]. Differences in animal model (rabbit versus rat) and bio-assay (WEHI versus L929 bio-assay) may explain the discrepancy.

Similar to our data in humans with PCP, plasma concentrations 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, while a slight recovery was seen at week 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 findings in patients with PCP. In steroid-treated HIV-seropositive patients with PCP, production of immunoreactive IL-6 and TNF in whole blood cultures was suppressed while the production in alveolar cells was not affected [20]. It also accords with the in vitro findings of Strieter et al, who showed that the effects of steroids on TNF production was more pronounced in peripheral blood monocytes than in alveolar macrophages [32]. In PCP in rats, the relative ^{111}In-IgG uptake in the lung (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-1ß were elevated in week 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.

Acknowledgements

We like to express our gratitude for the expert help of Gerry Grutters and Henny Eikholt of the Central Animal Laboratory, of Emiel Koenders of the Nuclear 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.

REFERENCES

1. Harmsen AG, Stanckiewicz M (1990) Requirement for CD4+ cells in resistance to Pneumocystis carinii pneumonia in mice J.Exp.Med.172,937-945.
2. Shellito J, Suzara VV, Blumenfeld W, Beck JM, Steger HJ, Ermak TH (1990) A new model of Pneumocystis carinii infection in mice selectively depleted of helper T lymphocytes. J.Clin. Invest. 85,1686-1693.
3. Donta ST and Pesanti EL. (1990) Tumor necrosis factor-α binds to specific receptors on Pneumocystis carinii. Clin Res 38,352A (Abstract).
4. Pesanti EL. (1991) Interaction of cytokines and alveolar cells with Pneumocystis carinii in vitro. J Infect Dis 163, 611-616.
5. Pesanti EL, Tomicic T, and Donta ST. (1991) Binding of ^{125}I-labelled tumor necrosis factor to Pneumocystis carinii and an insoluble cell wall fraction. J Protozool 38, 28S-29S (abstract).
6. Chen W, Havell EA, Gigliotti F and Harmsen AG. (1993) Interleukin-6 production in a murine model of Pneumocystis carinii Pneumonia· Relation to resistance and inflammatory response. Infect Immun 61,97-102.
7. Chen W, Havell EA, Moldawer LL, et al (1992) Interleukin-1 An important mediator of host resistance against Pneumocystis carinii J.Exp Med 176,713-718.

8. Chen W, Havell EA, and Harmsen AG. (1992) Importance of endogenous tumor necrosis factor alpha and gamma interferon in host resistance against Pneumocystis carinii infection. Infect.Immun.60, 1279-1284
9. Hoffman OA, Standing JE and Limper AH. (1993) Pneumocystis carinii stimulates tumor necrosis factor-α release from alveolar macrophages through a ß-glucan-mediated mechanism. J.Immunol.150, 3932-3940.
10. Kandil O, Fisman JA, Koziel H, Pinkston P, Rose RM, Remold HG. (1994) Human immunodeficiency virus type 1 infection of human macrophages modulates the cytokine response to Pneumocystis carinii. Infect.Immun.62, 644-650
11. Kolls JK, Beck JM, Nelson S, Summer WR and Shellito J. (1993) Alveolar macrophage release of Tumor Necrosis Factor during murine Pneumocystis carinii Pneumocystis. AM J Respir Cell Mol Biol. 8,370-376.
12. Krishnan VL, Meager A, Mitchell DM and Pinching AJ (1990) Alveolar macrophages in AIDS patients:increased spontaneous tumour necrosis factor-alpha production in Pneumocystis carinii pneumonia. Clin.exp.Immunol. 80,156-160.
13. Tamburrini E, De Luca A, Ventura G et al. (1991) Pneumocystis carinii stimulates in vitro production of tumor necrosis factor-α by human macrophages Med Microbiol Immunol.180, 15-20.
14. Theus SA, Linke MJ, Andrews RP and Walzer PD. (1993) Proliferative and cytokine responses to a major surface glycoprotein of Pneumocystis carinii. Infect Immun. 61.4703-4709.
15. Amano Y, Lee SW, Allison AC. (1992) Inhibition by glucocorticosteroids of the formation of interleukin-1α and interleukin-1ß and interleukin-6: mediation by decreased mRNA stability. Molecular Pharmacology 43, 176-182.
16. Bartlett MS, Queener SF, Jay MA, Durkin MM, Smith JW. (1987) Improved rat model for studying Pneumocystis carinii pneumonia. J Clin Microbiol 25, 480-484.
17. Sepkowitz KA. (1993) Pneumocystis carinii pneumonia in patients without AIDS. Clin Infect Dis 17 (Suppl 2), S416-422
18. Peces R, Urra JM, Gorostidi M, Lopez-Larrea C. (1992) Role of maintenance immunosuppression and methylprednisone in OKT3-induced cytokine release. Transplant Proc 24,2596-2599
19. Perenboom RM, Schijndel van JCHW, Beckers P et al (1996) Cytokine profiles in BAL and blood in HIV seronegative patients with Pneumocystis carinii pneumonia Eur J Clin Invest 26,159-166.
20. Perenboom RM, Sauerwein RW, Beckers P et al (1995) Cytokine profiles in broncho-alveolar lavage fluid and blood in HIV negative and HIV seropositive patients with PCP Cytokine 7, 601 (Abstract).
21. Keuter M, Dharmana E, Hussein Gasem M et al (1994) Patterns of proinflammatory cytokines and inhibitors during typhoid fever J Infect Dis 169.1306-1311.
22. Van Deuren M, van der Ven-Jongekrijg J, Demacker PNM et al (1994) Differential expression of proinflammatory cytokines and their inhibitors during the course of meningococcal infections. J Infect Dis 169:157-61.
23 Perenboom RM, Oyen WJG, Schijndel van JCHW, Beckers P, Corstens FHM, Meer van der JWM. (1995) Serial ^{111}In-labelled IgG biodistribution in rat Pneumocystis carinii pneumonia: A tool to monitor

the course and severity of the infection. Eur J of Nuclear Medicine. 122:1129-1132.

24. Mauderly JL. (1977) Bronchopulmonary lavage of small laboratory animals. Laboratory Animal Science 27:255-261
25. Aarden LA, de Groot ER, Schaap DL, Lansdorp PM. (1987) Production of hybridoma growth factor by human monocytes. Eur J Immunol 17:1411-1416.
26. Aggarwal BB. (1985) Human lymphotoxin. Methods Enzymol. 116:441-444
27. Fishman JA, Strauss HW, Fishman AJ, Neddelman M, Callahan R, Kwah BA, Rubin RH. (1993) Imaging of Pneumocystis carinii pneumonia with ^{111}In-labeled non-specific polyclonal IgG: an experimental study in rats. Nucl Med Commun 65:147-157.
28. Claessens RAJM, Koenders EB, Boerman OC, Oyen WJG, Borm GF, van der Meer JWM, Corstens FHM. (1995) Dissociation of indium from indium-111 labeled diethylene triamine pentaacetic acid conjugated non-specific polyclonal human immunoglobulin G in inflammatory foci. Eur J Nucl Med 22:212-219.
29. Hnatowich DJ, Childs RL, Lanteigne D, Najafi A. (1983) The preparation of DTPA-coupled antibodies radiolabeled with metallic radionuclides· an improved method. J Immun Meth 65:147-157
30. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249: 1431-1433.
31. Huang ZB, Eden E. (1993) Effect of corticosteroids on IL-1ß and TNF-α release by alveolar macrophages from patients with AIDS and PCP. Chest 104. 751-755.
32. Strieter RM, Remick DG, Lynch JP et al. (1989) Differential regulation of tumour necrosis factor-alpha in human alveolar macrophages and peripheral blood monocytes· a cellular and molecular analysis. Am J Respir Cell Mol Biol 1.57-63

CYTOKINE PROFILES IN BAL AND BLOOD IN HIV-SERONEGATIVE PATIENTS WITH PNEUMOCYSTIS CARINII PNEUMONIA

Roos M.Perenboom

Anita C.H.W.van Schijndel

Pieter Beckers

Robert W.Sauerwein

Henk W.van Hamersvelt

Jan Festen

Harald Gallati

Jos W.M.van der Meer

Published in European Journal of Clinical Investigation, 1996;26:159-166.

(reprinted with permission)

ABSTRACT

Concentrations and ex-vivo production of interleukin-1ß (IL-1ß), tumor necrosis factor α (TNF), interleukin 6 (IL-6), interleukin 1 receptor antagonist (IL-1RA) and TNF soluble receptors were followed in broncho-alveolar lavage (BAL) fluid and blood in 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-1ß was detectable in BAL fluid. At that time, plasma concentrations of the pro-inflammatory cytokines were low, while plasma concentrations of the anti-inflammatory cytokines were high. The ex-vivo production capacity of pro-inflammatory 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. IL-1RA production capacity of the alveolar cells was also suppressed in the acute phase, but preserved in blood cells.

INTRODUCTION

Pneumocystis carinii is an intriguing pathogen, mainly causing pulmonary infections in immunocompromised hosts. Allthough 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, are likely to be involved in the host defense against Pneumocystis carinii (P.carinii), but their exact role in PCP is not yet clear.

In-vitro studies showed that P.carinii possesses binding sites for tumor necrosis factor-α (TNF-α) and that this cytokine was capable of killing P.carinii [6-8].

In mice TNF-α and interleukin-1ß (IL-1ß) 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-α by alveolar macrophages [12-17].

Thus, like in many bacterial and protozoal infections, IL-1ß, IL-6, and TNF-α 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 still is an important opportunistic infection in other immunocompromised patients, notably organ transplant patients and patients with lymphatic malignancies.

A series 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.

MATERIAL AND METHODS

Patients

All HIV-seronegative adult patients with PCP, diagnosed by BAL, between October 1992 and March 1993 were included in the study. PCP diagnosis was based on demonstration of P.carinii organisms on Churukian-Schenk ammoniacal-silver stained and Giemsa stained BAL specimens [18].

At the time of BAL, blood samples of all patients were taken for measurement of circulating and ex-vivo produced cytokines and inhibitors; an other sample was obtained after full recovery. Nine patients were non smoking. All patients were proven seronegative for HIV. None of the patients received non-steroidal anti-inflammatory drugs or pentoxyfyllin.

The study was approved by the Hospital Ethical Committee and informed consent was obtained from all patients and healthy controls.

Controls

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 infection. Prior to the BAL, a blood sample was taken for measurement of cytokines and white bloodcell count.

Broncho-alveolar 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 radiologic abnormality. In the controls the lavage was done in the right middle lobe. BAL was performed by injecting and aspirating 6-8 aliquots of 20 ml 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 following BAL, the flask was put on ice and transported to the laboratory. Thirty μl was taken for cell count and cell viability was assessed by trypan blue dye exclusion. The remaining fluid was centrifuged at 500g for 15 minutes. The supernatant was removed and 1% bovine serum albumin (BSA Sigma, St Louis, USA) was added. Then the fluid was filter sterilized, aliquoted and frozen at -80°C until assay.

The pellet was resuspended in Dulbecco's modified Eagle's medium at a concentration of 0.5 X 10^6 viable macrophages per ml; 100 μl 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, Holland) without any addition (unstimulated culture) and the other half was incubated with lipopolysaccharide (LPS, E Coli serotype 055;B5, Sigma) at a final concentration of 10 μg/ml. All incubations were done at 37°C for 24 hours. Following incubation the tubes are centrifuged at 1200g for 10 minutes 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 before [19]. In short, venous blood was collected aseptically into three 5-ml-draw sterile vacuum tubes, containing lyophilized 7.2 mg EDTA and 250 μl aprotinin (Trasylol 2500 KIU, Bayer, Leverkussen, Germany;final concentration 625

KIE/ml).

One tube was immediately centrifuged at 1250g for 10 minutes, then plasma was transferred to a polypropylene tube (Eppendorf, Sarstedt, Nümbrecht, Germany) and centrifuged at 15000g for 1 minute in order to remove the platelets. This platelet-poor plasma was pipetted into another sterile polypropylene tube and frozen at minus -80 °C until cytokine analysis.

In one of the two remaining tubes 50 μl LPS was added (final concentration 10 μg/ml) to stimulate cytokine production. This tube and the third tube were then incubated at 37 °C for 24 hours. After incubation the tubes were centrifuged and the plasma frozen at -80°C as described above.

Cytokine assays

Plasma and BAL samples were analyzed by means of specific radioimmunoassays for Il-1ß , IL-1 receptor antagonist (IL-1RA) and TNF-α [20-21]. An Enzyme Linked Binding assay (ELIBA, Hoffman la Roche, Basel, Switserland) was used for soluble receptors of TNF-α (sTNF-R;P55, P75), and a bioassay (B9 hybridoma cell-line) for IL-6.

The detection limits of the various assays were: IL-1ß, 20 pg/ml; IL-1RA, 800 pg/ml; TNF, 20 pg/ml; sTNF-R:P55, 80 pg/ml, P75, 300 pg/ml; and IL-6, 20 U/ml.

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 : (actual sTNF-R concentration) x (creatinine clearance) x 1/100 [22]

Statistical analysis

Values are reported as medians and range. Differences between medians were analyzed using the Wilcoxon signed-rank test for paired and unpaired samples. $P<0.05$ was considered significant. The 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 controls

All 10 patients (median age: 45 years;range 29-69, 4 males, 6 females) had fever, dry

cough and/or dyspnoea. Nine patient had interstitial or alveolar infiltrates on the chest X ray, in one patient (number 4) the chest X ray was normal.

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

	BAL			Peripheral blood		
Healthy control subjects	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)

Of the patients 8 were renal transplant recipients with a median interval between transplantation and PCP of 3 months (range 2-30). One patient developed PCP while using prednisone for an undetermined auto-immune disease and one patient was treated with chemotherapy (including prednisone) for a 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 8 healthy volunteers, 2 males and 6 females, was 24 years (range 20-25).

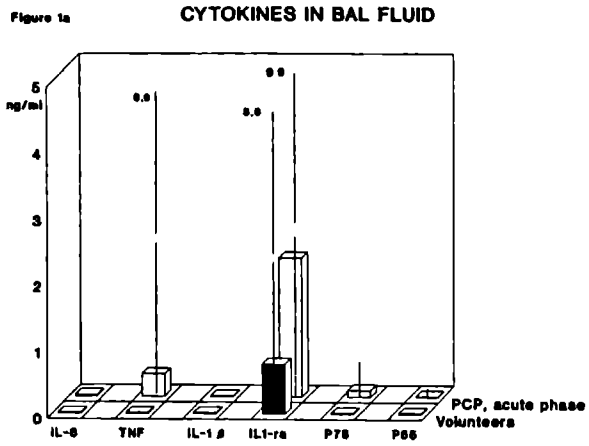
Table 2 Clinical characteristics of patients with PCP

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	*Staphylococcur aureus* sepsis	–		CMV		–	–	CMV	–
Pao_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 AZA	CsA AZA		CsA AZA
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

Cytokines in BAL and blood

In BAL fluid of only one of the 10 PCP patients a measurable concentration of IL-6 (446 U/ml) was present (Fig.1a). TNF-α concentrations in the BAL fluid were high (6.1 ng/ml) in one patient (number 7), detectable (0.1-0.6 ng/ml) in 5 patients and below the detection limit in 4 patients. IL-1ß concentrations were detectable in 3 patients (0.16-0.24



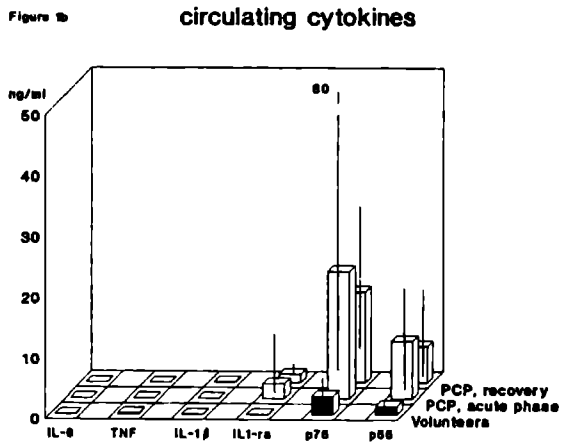


Figure 1. Concentration (median and range, in ng mL $^{-1}$) of cytokines in BAL fluid (a) and plasma (b) in volunteers and in HIV-seronegative patients with PCP during the acute phase and in recovery (for plasma only). Bars = ranges.

ng/ml) and below the detection limit in 7. IL-1RA concentration in the BAL fluid of the PCP patients was 2.1 (0.4-9.9) ng/ml (Fig.1a). In 4 of the 10 patients, IL-1RA concentration in BAL fluid was above the upper normal limit of healthy volunteers (≥ 95% percentiles). The sTNF-R's could not be detected in BAL fluid, except in one (number 10), where values just above the detection limit were found (p55:1.2 ng/ml, p75:1.3 ng/ml).

IL-1RA in BAL fluid of healthy volunteers was 0.75 (<0.4-6.6) ng/ml, which was significantly lower than in patients ($p=0.03$, fig.1a.).

The concentrations of IL-6, TNF-α, IL-1ß and sTNF-R's in the BAL fluid of volunteers were below the detection limit.

In the patients, the plasma concentrations of the pro-inflammatory cytokines, IL-6, TNF-α, IL-1ß 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, for sTNF-R-p55 10.8 (6.6-17.7) ng/ml and for sTNF-R-p75 23 (14-36) ng/ml (Fig.1b).

(a)

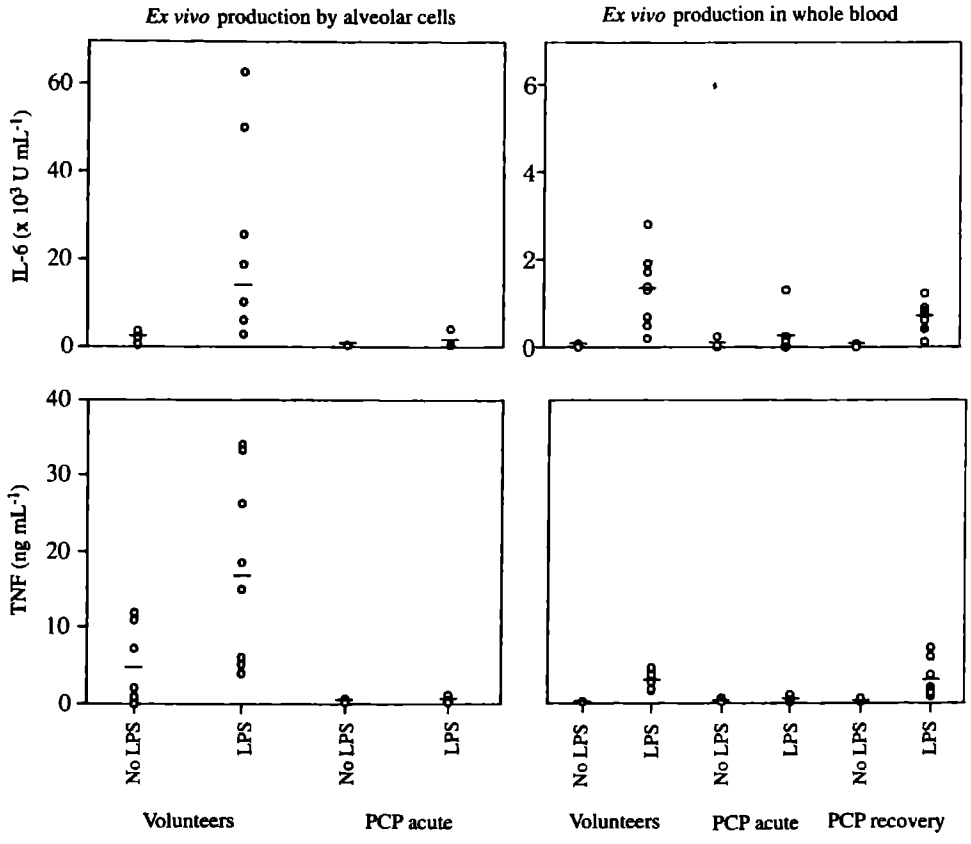


Figure 2. Unstimulated and LPS-stimulated ex-vivo production of cytokines, (IL-6 and TNF, IL-1 and IL-1RA) by alveolar cells and in whole-blood cultures from volunteers and HIV-seronegative patients with PCP in the acute phase and at recovery -, Median. ●, patients without concomitant infections, O,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

At recovery, while using low maintenance dose (10-20 mg) 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 compared to values of healthy volunteers, both in the acute phase, p55 :3.5 (1.5-7.4), p75:9.1 (3.7-19.5) ng/ml and in recovery, p55 2.7 (0.8-8.7), p75 3.9 (2.6-15.2) ng/ml. Corrected for renal function, p75 concentration was higher in the acute phase ($p=0.04$) than in recovery, while for p55 concentration the difference in the acute phase and in recovery 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 bloodcells

In 6 patients (patients 1,2,5,7,8 and 10) adequate BAL material was obtained for assessment of ex-vivo production by alveolar cells.

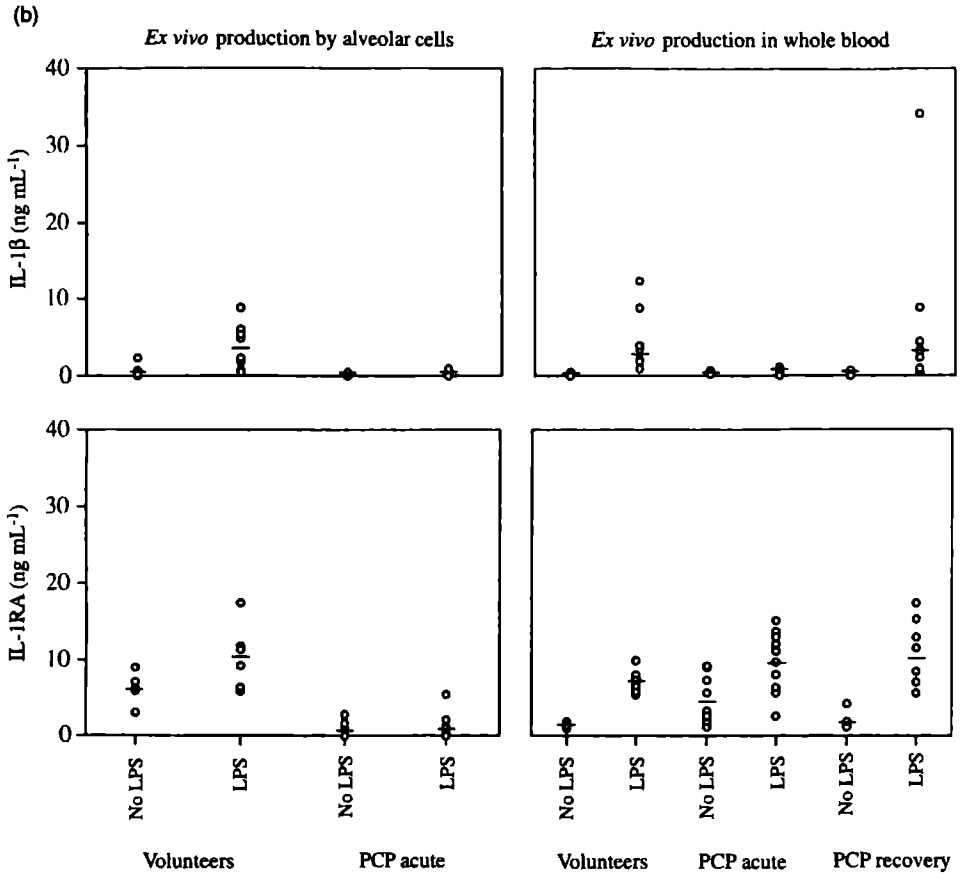


Figure 2. Continued

The LPS-stimulated ex-vivo production of the pro-inflammatory cytokines by the alveolar cells was suppressed in the acute phase of PCP (Fig.2 A and B).

We also found a significant suppression of the unstimulated and LPS-stimulated ex-vivo production of IL-1RA by alveolar cells, compared to the production in healthy volunteers ($p<0.005$, Fig.2B). BAL was not repeated during follow-up.

The same pattern as with the alveolar cells was seen for the ex-vivo production of the pro-inflammatory 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 hours after the start of high dose (80 mg) prednisone; in both samples the pro-inflammatory cytokine production was strongly suppressed (240 pg/ml and 260 pg/ml for IL-1ß, 300 pg/ml and 580 pg/ml for TNF-α before the high dose prednisone versus 100 pg/ml and 320 pg/ml for IL-1ß, 220 pg/ml and 320 pg/ml for TNF-α after the high dose).

ANOVA 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 pro-inflammatory 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-α, IL-1ß 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 counterregulation of pro-inflammatory 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 pro-inflammatory cytokines in blood cells was suppressed in the acute phase and improved during convalescence, while 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 pro-inflammatory 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 pro-inflammatory 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 downregulation of pro-inflammatory cytokine production of cells in the circulation would not necessarily imply a similar downregulation at the level of the tissues and at the site of infection. However, in this study we find such downregulation of pro-inflammatory cytokines also at the level of the alveolar macrophages, pointing to 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, while unstimulated blood cells did not produce detectable amounts of this cytokine inhibitor. These findings are in agreement with observations of Moore et al, who found that alveolar macrophages express steady-state levels of IL-1RA mRNA under unstimulated culture conditions, while peripheral blood cells did so only in response to LPS or adherent IgG [27-28]. 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 downregulation 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-α production capacity of the alveolar cells, TNF-α is present in the BAL fluid of 60% of the patients with PCP. The cellular source

of this TNF-α is currently unclear but unlikely to be derived from alveolar macrophages, since these cells are refractory in terms of TNF-α production.
What accounts for the downregulation of pro-inflammatory cytokine production with relatively preserved IL-1RA production in the course of an infection needs further research. IL-4, a T-lymphocyte derived cytokine known to block the production of IL-1ß, IL-6 and TNF-α 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 Tumor Growth Factor-ß 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 cyclosporine A decrease pro-inflammatory cytokine production [33-35]. With regard to IL-1RA production, Santos et al. have reported a minimal influence of hydrocortisone on endotoxin stimulated IL-1RA production in volunteers [36]. In our study however, the pattern of cytokine production did not appear to be influenced by prednisone dose (ranging from 0-80mg in the acute phase and from 0-20mg 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 6 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-α 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 pro-inflammatory cytokines, in the blood as well as at the site of infection. During convalescence the blood cells return to a balanced pro-inflammatory status.
IL-1RA production in acute PCP was downregulated in alveolar cells and preserved in blood cells.

Acknowledgements

We like to thank Dr.G.R.Adolph (Boehringer, Austria), for his gift of TNF-α, Dr.P.Graber (Glaxo, Switzerland) for his gift of IL-1ß, Dr.P.Ghiara (Sclavo, Italy) for the anti-IL-1ß antibodies, Dr.R.C.Thompson (Synergen, USA) for his gift of IL-1RA, Dr.C.A.Dinarello for the anti-IL-1RA antibodies.

REFERENCES

1. Walzer PD. Immunopathogenesis of Pneumocystis carinii infection. J.Lab.Clin Med.1991;118:206-216.
2. Harmsen AG, Stanckiewicz M. Requirement for CD4+ cells in resistance to Pneumocystis carinii pneumonia in mice. J.Exp.Med.1990;172:937-945.
3. Shellito J, Suzara VV, Blumenfeld W, Beck JM, Steger HJ, Ermak TH. A new model of Pneumocystis carinii infection in mice selectively depleted of helper T lymphocytes J.Clin.Invest.1990;85:1686-1693.
4. Kolls JK, Beck JM, Nelson S, Summer WR and Shellito J. Alveolar macrophage release of Tumor Necrosis Factor during murine Pneumocystis carinii Pneumocystis AM J Respir Cell Mol Biol. 1993;-8:370-376.
5. Von Behren LA, Pesanti EL. Uptake and degradation of Pneumocystis carinii by macrophages in vitro. Am Rev Respir Dis, 1978: 118;1051-1059.
6. Pesanti EL, Tomicic T, and Donta ST. Binding of ^{125}I-labelled tumor necrosis factor to Pneumocystis carinii and an insoluble cell wall fraction. J Protozool 1991,38.28S-29S.
7. Donta ST and Pesanti EL. Tumor necrosis factor-α binds to specific receptors on Pneumocystis carinii. Clin Res 1990,38:352A (Abstract).
8. Pesanti EL. Interaction of cytokines and alveolar cells with Pneumocystis carinii in vitro. JID 1991;163:611-616.
9. Chen W, Havell EA, Gigliotti F and Harmsen AG. Interleukin-6 production in a murine model of Pneumocystis carinii Pneumonia: Relation to resistance and inflammatory response. Infect. Immun 1993;61:97-102.
10. Chen W, Havell EA, Moldawer LL, et al Interleukin-1: An important mediator of host resistance against Pneumocystis carinii. J.Exp.Med 1992;176·713-718.
11. Chen W, Havell EA, and Harmsen AG. Importance of endogenous tumor necrosis factor alpha and gamma interferon in host resistance against Pneumocystis carinii infection. Infect.Immun.1992;60:-1279-1284
12. Theus SA, Linke MJ, Andrews RP and Walzer PD.Proliferative and cytokine responses to a major surface glycoprotein of Pneumocystis carinii. Infect.Immun 1993;61·4703-4709.
13. Hoffmans OA, Standing JE and Limper AH. Pneumocystis carinii stimulates tumor necrosis factor-α release from alveolar macrophages through a ß-glucan-mediated mechanism J.Immunol. 1993;150:

3932-3940.

14. Tamburrini E, De Luca A, Ventura G et al. Pneumocystis carinii stimulates in vitro production of tumor necrosis factor-α by human macrophages. Med Microbiol Immunol.1991;180;15-20.
15. Krishnan VL, Meager A, Mitchell DM and Pinching AJ Alveolar macrophages in AIDS patients:increased spontaneous tumour necrosis factor-alpha production in Pneumocystis carinii pneumonia. Clin.exp.Immunol.1990;80.156-160.
16. Kandil O, Fisman JA, Koziel H, Pinkston P, Rose RM, Remold HG. Human immunodeficiency virus type 1 infection of human macrophages modulates the cytokine response to Pneumocystis carinii. Infect.Immun.1994;62.644-650 17.Churukian CL, Schenk EA Staining Pneumocystis carinii and fungi in unfixed specimens with ammoniacal silver using a microwave oven. The J Histotechnol 1988;11:19-21.
18. Nerad JL, Griffith K, van der Meer JWM, Endres S, Keusch GT, Dinarello CA, Cannon JG. Whole blood assay of cytokine production applicable to field settings (abstract) Cytokine 1989,1:139
19. Van der Ven-Jongekrijg J, Demacker PMN, Van der Meer JWM A simple method for measuring cytokine production in vitro (abstract)Cytokine 1991,3 495
20. Lisi PJ, Chu CW, Koch GA, Endres S, Lonneman G, Dinarello CA Development and use of radio immunoassay for human interleukin-1ß Lymphokine Res 1987,6·229-44
21. Van der Meer JWM, Endres S, Lonneman G, et al Concentrations of immunoreactive human tumor necrosis factor alpha produced by human mononuclear cells in vitro J Leucoc Biol 1988;43:16-23.
22. Brockhaus M, Bar-Khayim, Gurwicz S, Frensdorf A, Haran N Plasma levels of tumor necrosis factor soluble receptors in chronic renal failure Kidney Int 1992,42 663-7
23. Van Deuren M, van der Ven-Jongekrijg J, Demacker PNM, Bartelink AKM, van Dalen R, Sauerwein R, Gallati H, Vannice JL, van der Meer JWM Differential expression of proinflammatory cytokines and their inhibitors during the course of meningococcal infections. JID 1994,169 157-61.
24. Keuter M, Dharmana E, Hussein Gasem M, Van der Ven-Jongekrijg J, Djokomoeljanto R, Dolmans WMV, Demacker P, Sauerwein R, Gallati H, Van der Meer JWM Patterns of proinflammatory cytokines and inhibitors during typhoid fever JID 1994,169 1306-1311
25. Simpson SQ, Modi HN, Balk RA, Bone RC, Casey LC. Reduced alveolar macrophage production of tumor necrosis factor during sepsis in mice and men Crit Care Med 1991,19 1060-1066.
26. Luger A, Graf H, Schwarz HP, Stummvoll HK, Luger TA Decreased serum interleukin 1 activity and monocyte interleukin 1 production in patients with fatal sepsis Crit Care Med 1986, 14:458-61.
27. Kline JN, Monick MM, Hunninghake GW. IL-1 receptor antagonist is regulated differently in human alveolar macrophages than in monocytes.J Appl Physiol 1992,72.1687-1692.
28. Moore SA, Strieter RM, Rolfe MW, Standiford TJ, Burdick MD, Kunkel SL Expression and regulation of human alveolar macrophage-derived interleukin-1 receptor antagonist. Am.J.Respir. Cell Mol Biol 1992,6·569-575.
29. Galve-de Rochemonteix B, Nicod LP, Chicheportiche R, Lacraz S, Baumberger C, Dayer JM. Regulation of interleukin-1ra, interleukin-1α, and interleukin-1ß production by humann alveolar macrophages with phorbol myristate acetate, lipopolysaccharide and interleukin-4. Am.J.Respir.Cell Mol Biol. 1993, 8.160-168.

30. Vannier E, Miller LC, Dinarello CA. Coordinated antiinflammatory effects of interleukin 4:interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist. Proc Natl Acad Sci USA 1992;89:4076-80.
31. Wahl SM, Costa GL, Corcoran M, Wahl LM, Berger AE. Transforming Growt Factor-ß mediates IL-1- dependent induction of IL-1 Receptor Antagonist J.Immunol.1993;150:3553-3560.
32. De Waal Malefijt R, Abrams J, Bennet B, Figdor CG, de Vries JE. Interleukin-10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991;174:1209-20.
33. Shieh JH, Peterson RHF, Moore MAS, Cytokines and dexamethason modulation of IL-1 receptors on human neutrophils in vitro J.Immunol 1993,150:3515-3524.
34. Huang ZB, Eden E. Effect of corticosteroids in IL1ß and TNFα release by alveolar macrophages from patients with AIDS and Pneumocystis carinii Pneumonia Chest 1993;104:751-55.
35. Peces R, Urra JM, Gorostidi M, Lopez-Larrea C. Role of maintenance immunosuppression and methylprednisone in OKT3-induced cytokine release. Transplant Proc 1992;24:2596-2599.
36. Santos AS, Scheltinga MR, Lynch E, Brown EF, Lawton P, Chambers E, Browning J, Dinarello CA, Wolff SM, Wilmore DW. Elaboration of interleukin 1-receptor antagonist is not attenuated by glucocorticoids after endotoxemia. Arch Surg 1993;128:138-144.

Chapter 6

CYTOKINE PROFILES IN BRONCHO-ALVEOLAR LAVAGE FLUID AND BLOOD IN HIV-SEROPOSITIVE PATIENTS WITH PNEUMOCYSTIS CARINII PNEUMONIA

Roos M. Perenboom

Robert W. Sauerwein

Pieter Beckers

Anita C.H.W. van Schijndel

Reindert P. van Steenwijk

Jan C.C. Borleffs

Rob van Leusen

Jos W.M. van der Meer

European Journal of Clinical Investigation, in press

ABSTRACT

Objective: To investigate cytokine profiles in Pneumocystis carinii pneumonia (PCP) in HIV-seropositive (HIVpos) patients and to correlate cytokine levels with disease activity.

Design: Prospective multicenter clinical study.

Methods: Concentrations of interleukin-1ß (IL-1ß), tumor necrosis-α (TNF-α), interleukin-6 (IL-6), interleukin-1-receptor antagonist (IL-1RA) and TNF soluble receptors were measured in broncho-alveolar lavage fluid (BAL) and blood of 23 HIVpos patients with PCP and compared with the concentrations in healthy HIV-seronegative controls and asymptomatic HIVpos persons. We also examined ex-vivo production by alveolar cells and in whole blood cultures.

Results: IL-1 was increased in BAL of HIVpos patients with PCP (117, range <20-1010pg/ml) compared to healthy controls (<20pg/ml, p= 0.0001). In plasma of the HIVpos patients higher concentrations of IL-1RA were found during acute PCP compared to levels after recovery (1.3, range <0.8-17.0 versus <0.8, range <0.8-2.0ng/ml, p=0.01). Ex-vivo production of pro-inflammatory cytokines was suppressed in whole blood cultures during PCP, whereas IL-1RA production by alveolar cells as well as in whole blood cultures was increased. Corticosteroids did not affect cytokine concentrations in BAL or blood, nor did they suppress the production in alveolar cells. In whole blood cultures however, LPS-stimulated production was significantly suppressed for IL-1 (0.3, range 0.1-7.4 versus 5.7, range 0.7-30.3ng/ml, p= 0.009) and for IL-6 (0.18 range 0.03-2.96 versus 2.06, range 0.16-4.95ng/ml, p=0.01). Correlations between inflammatory mediators and disease activity were not found.

Conclusion: In PCP pro-inflammatory cytokines are predominant in BAL, whereas anti-inflammatory mediators are more prominent in blood.

INTRODUCTION

Pneumocystis carinii pneumonia (PCP) is the most frequently occurring severe opportunistic infection in patients infected with the human immmunodeficiency 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 directly interact with the Pneumocystis carinii (P.carinii) [4-7]. Cytokines

are likely to be involved as soluble mediators in the host defense against P.carinii 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-α) by alveolar macrophages [8-13]. In-vitro studies suggest that P.carinii possesses binding sites for TNF-α and that this cytokine is capable of killing P.carinii [14-15]. In mice, TNF-α and interleukin-1ß (IL-1ß) are important for the clearance of P.carinii, while interleukin-6 (IL-6) regulates pulmonary inflammation and antibody response during resolution [16-18]. Thus, similar to a variety of other infections IL-1ß, IL-6, and TNF-α seem to play a role in the pathogenesis of and protection against PCP.

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

MATERIAL AND METHODS

Patients

Twenty three adult HIV-positive (HIVpos) patients with PCP, who underwent BAL according to a standardized protocol (see below) at the 4 participating centres were included in the study between October 1993 and November 1994. The diagnosis PCP was based on demonstration of P.carinii organisms on Silver and/or Giemsa stained BAL specimens [19].

Blood samples for measurements of circulating and ex-vivo produced cytokines and inhibitors were taken at the time of the BAL and after full recovery.

Respiration rate and arterial oxygen pressure (PO2) on admission were used as parameters for severity of infection.

Controls

Eight healthy non-smoking subjects, who volunteered to undergo BAL were used as controls [20]. None of them used medication or had evidence of lung disease or a history of recent respiratory tract infection. Prior to the BAL, a blood sample was taken for measurement of cytokines and white blood cell count (WBC).

Blood was obtained for cytokine measurements from twenty-five asymptomatic ambulant

HIVpos individuals with varying CD4 counts who visited the HIV-clinic of the University Hospital Nijmegen. They were the asymptomatic HIVpos control group.

Broncho-alveolar 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 subsegmental bronchus serving the area of greatest radiologic abnormality. In the controls the lavage was done in the right middle lobe. BAL was performed by injecting and aspirating 6-8 aliquots of 20 ml sterile saline. 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 following BAL, 30 μl was taken for cell count and cell viability was assessed by trypan blue dye exclusion. The remaining fluid was centrifuged at 500g for 15 minutes. The supernatant was removed and one percent bovine serum albumin (Sigma, St Louis, USA) was added. Then the fluid was filter sterilized, aliquoted and frozen at -80°C until assay.

The pellet was resuspended in Dulbecco's modified Eagle's medium at a concentration of 0.5 X 10^6 viable macrophages per ml; 100 μl was taken for preparing cytocentrifuged Giemsa stained preparations and half of the remaining fluid was incubated in 4 ml closed polystyrene tubes (Greiner, Alphen, The Netherlands) without any addition (unstimulated culture) while the other half was incubated with lipopolysaccharide (LPS, E Coli serotype 055;B5, Sigma) at a final concentration of 10 μg/ml. All incubations were done at 37° C for 24 hours. Following incubation the tubes were centrifuged at 1200g for 10 minutes and the supernatant and cell pellets separately frozen at -80°C until cytokine analysis.

Processing whole blood

Venous blood was drawn from patients and controls for cytokine assessment in whole blood as described before [21]. Briefly, venous blood was collected aseptically into three 5-ml-draw sterile vacuum tubes, containing lyophilized 7.2 mg EDTA and 250 μl aprotinin (Trasylol 2500 KIU, Bayer, Leverkusen, Germany; final concentration 625 KIE/ml).

One tube was immediately centrifuged at 1250g for 10 minutes. Plasma was transferred to

a polypropylene tube (Eppendorf, Sarstedt, Nümbrecht, Germany) and centrifuged at 15000g for 1 minute to remove platelets. Platelet-poor plasma was pipetted into another sterile polypropylene tube and frozen at minus -80 °C until cytokine analysis.
In one of the two remaining tubes 50 μl LPS was added (final concentration 10 μg/ml) to stimulate cytokine production. This tube and the third tube were then incubated at 37 °C for 24 hours. After incubation the tubes were centrifuged and the plasma frozen at -80°C as described above.

Cytokine assays

Plasma and BAL samples were analyzed by specific radioimmunoassays for Il-1ß , IL-1 receptor antagonist (IL-1RA) and TNF-α [22,23]. An Enzyme Linked Binding assay (Hoffman-la Roche, Basel, Switserland) was used for soluble receptors of TNF (sTNF-R;P55, P75), and an ELISA as described before for IL-6 (Inotherapy, Besançon, France) [24].
The detection limits of the various assays were: IL-1ß, 20 pg/ml; IL-1RA, 800 pg/ml; TNF-α, 20 pg/ml; sTNF-R:P55, 80 pg/ml, P75, 300 pg/ml; and IL-6, 20pg/ml.

Statistical analysis

Values are reported as medians and ranges. Differences between groups were analyzed 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 Ethical Committee and informed consent was obtained from all patients and healthy controls.

RESULTS

Clinical characteristics

Clinical characteristics of patients and controls are shown in table 1. PCP prophylaxis was only used by 4 patients (17%), all of them used monthly pentamidin. Mean duration of symptoms was 5 ($\pm$5) weeks. Mean arterial oxygen pressure was 8.6 ($\pm$ 1.5) kilo Pascal. Eight (35%) patients had Cytomegalovirus-early-antigen in BAL fluid. Two (9%)

patients needed a respirator and 2 (9%) died. Four of the 23 HIVpos patients with PCP (17%) had concomitant infections (two had cerebral toxoplasmosis, one cryptococcal pneumonia and one salmonella septicaemia).

Table 1. Clinical characteristics of patients and controls

	HIVpos PCP	HIVpos asymptomatic	healthy controls
Total number	23	25	8
male (%)	23 (100)	20 (80)	2 (25)
age in years (SEM)	35.8 (6.0)	37.4 (8.8)	23.3 (1.6)
smokers (%)	9 (39)	12 (48)	0 (0)
CD4 cells/mm^3 (SEM)	97 (133)	207 (226)	ND
corticosteroids (%)	8 (35)	0	0
body temperature (SEM)	38.4°C (1.0)	<37.5°C	<37.5°C
WBC x 10.9/L (SEM)	5.6 (0.5)	4.2 (0.4)	5.0 (0.5)
% neutrophils (SEM)	76 (3)	57 (2.2)	57 (3.6)
% monocytes (SEM)	6 (5)	8 (1)	4 (2)
% neutrophils in BAL (SEM)	15 (2.7)	ND	2 (0.6)
% macrophages in BAL (SEM)	68 (4.4)	ND	95 (1.1)

Legends : HIVpos= HIV seropositive, PCP=Pneumocystis carinii pneumonia, SEM = standard error of the means, ND = not done, BAL= broncho-alveolar lavage fluid.

Cytokine concentrations in BAL fluid and in plasma

BAL - BAL was performed in the acute phase of PCP and in healthy controls. Concentrations of all three pro-inflammatory cytokines were generally higher in the acute phase of PCP compared to levels in healthy controls, but significance was only obtained for IL-1ß ($p=0.0001$, Fig.1A). Although concentrations of IL-1RA were high in 3 HIVpos patients (>10 ng/ml), the difference with healthy controls did not reach statistical significance (Fig.1B). Both types of soluble TNF-receptors were low in BAL fluid.

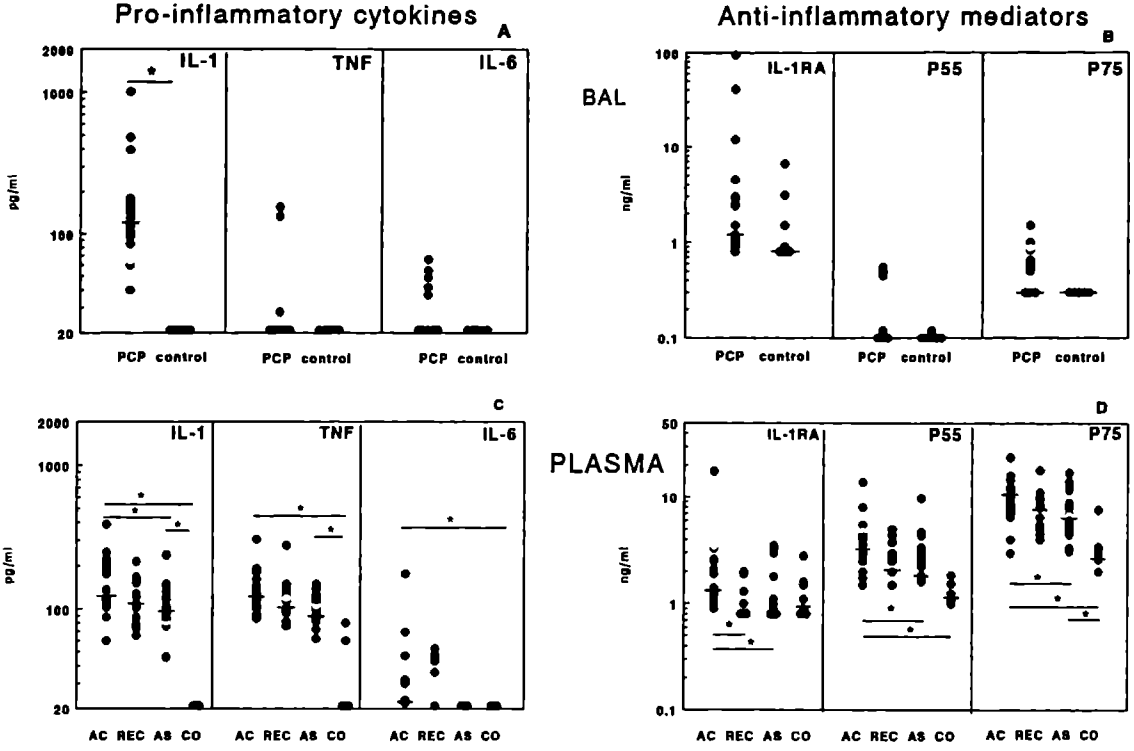


Fig 1 Concentrations of cytokines in BAL (Fig 1A and 1B) and plasma (Fig 1C and 1D) in HIVpos patients with PCP during the acute phase (AC) and recovery (REC), in asympytomatic HIVpos subjects (AS) and in healthy controls (CO) Bars indicate the medians The asterisks indicate statistical significance ($P \leq 0.05$, Wilcoxon rank sums test with Kruskal-Wallis Chi-square approximation)

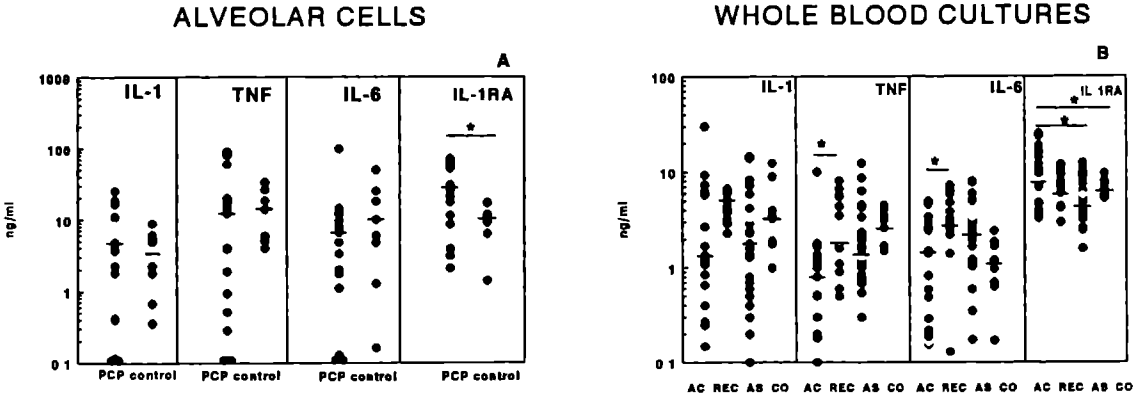


Fig 2 LPS-stimulated ex-vivo cytokine production in BAL (Fig2A) and blood (Fig 2B) in HIVpos patients with PCP during the acute phase (AC) and recovery (REC), in asymptomatic HIVpos subjects (AS) and in healthy controls (CO) Bars indicate the medians The asterisks indicate statistical significance ($P \leq 0.05$, Wilcoxon rank sums test with Kruskal-Wallis Chi-square approximation)

Plasma - With exception of IL-1RA ($p=0.01$), plasma concentrations of the pro-inflammatory cytokines and anti-inflammatory mediators did not differ between the acute and recovery phase in HIVpos patients with PCP (fig.1D).

HIVpos patients with PCP had higher circulating concentrations of IL-1ß ($p=0.002$, Fig.1C), IL-1RA and P75 ($p=0.01$, Fig.1D) than asymptomatic HIVpos patients.

Compared to healthy controls, HIVpos patients with PCP had significantly higher concentrations of pro-inflammatory 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 pro-inflammatory cytokines by alveolar cells of HIVpos patients did not differ from the production of healthy controls (Fig.2A), whereas LPS-stimulated production of IL-1RA was significantly higher ($p=0.01$, Fig.2A). Similarly, unstimulated ex-vivo production showed higher production of IL-1RA in HIVpos patients with PCP, median 12.5, range 1.6-95 ng/ml versus median 6.5, range 0.8-9.7 ng/ml in healthy controls, $p<0.05$.

Whole blood cultures - LPS-stimulated production of IL-6 and TNF-α was suppressed in the acute phase of PCP, and restored during recovery (Fig.2B.). Although the same trend was seen for production of IL-1ß, 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 to asymptomatic HIVpos persons, pro-inflammatory cytokine production was slightly lower in the acute fase of PCP and higher during recovery. Compared to healthy controls, LPS-stimulated production of TNF-α in HIVpos 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 pro-inflammatory cytokines was higher in HIVpos persons as compared to HIVneg controls and not influenced by the presence of PCP. Median concentrations in all HIVpos persons were 110 pg IL-1ß per ml (range <20-640 pg/ml) and 102 pg TNF-α per ml (range 60-282 pg/ml), while concentrations in healthy controls were <20 pg IL-1ß per ml (range <20-280 pg/ml), and 80 pg TNF-α per ml (range 60-120 pg/ml).

Table 2. Effect of corticosteroids on (anti-)inflammatory mediators. Plasma concentrations and LPS-stimulated production of (anti-)inflammatory mediators were measured in 2 HIVpos patients with PCP before administration of 40 mg prednisone (C-) and 24 hours after the first dose (C+).
Data show ng/ml.

	Plasma concentration		LPS-stimulated ex-vivo production	
	C-	C+	C-	C+
Patient A				
IL-1ß	0.12	0.11	31.8	0.80
IL-6	<0.02	<0.02	3.36	0.38
TNF-α	0.11	0.11	3.39	1.20
IL-1RA	1.0	<0.8	25.3	9.3
sTNF-R, p55	3.0	2.5	<detection limit	<detection limit
sTNF-R, p75	7.5	7.25	<detection limit	<detection limit
patient B				
IL-1ß	0.10	0.13	0.44	0.12
Il-6	0.01	0.02	0.16	0.15
TNF-α	0.09	0.10	0.18	0.13
IL-1RA	1.2	1.4	4.6	3.0
sTNF-R, p55	4.75	3.5	<detection limit	<detection limit
sTNF-R, p75	11.5	15.0	<detection limit	<detection limit

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

Corticosteroids (40-60mg prednisone) were given in 8 patients at 2-24 hours 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). However, LPS-stimulated ex-vivo production in whole blood showed significantly reduced IL-1ß (median

300, range 100-7400 pg/ml) and IL-6 (median 180, range 30-2960 pg/ml) production in corticosteroid-treated patients as compared to untreated patients (IL-1ß mean 5700, range 700-30300 pg/ml, p= 0.009 and IL-6 mean 2060, range 160-4950 ng/ml, p=0.01); there was no difference in TNF-α and IL-1RA synthesis. In 2 patients circulating concentrations of cytokines were not different before and within 24 hours after steroids administration. However, profound effects were seen on whole blood cytokine production in one patient, while in the other patient the production was already reduced before the administration of corticosteroids (Table 2).

Cytokine concentrations and production of the 4 HIVpos patients with PCP and another opportunistic infection (cerebral toxoplasmosis in two, cryptococcal pneumonia in one and salmonella septicaemia in one) were similar to those without concomitant infections. The same holds for the presence or absence of Cytomegalovirus in BAL fluid (data not shown).

DISCUSSION

HIVpos patients with PCP showed higher concentrations of the pro-inflammatory cytokine IL-1ß in BAL fluid than healthy controls. In the circulation, higher concentrations of the anti-inflammatory cytokine IL-1RA were found than during recovery.

Pro-inflammatory cytokines have previously been shown to be increased in BAL fluid of HIVneg patients with PCP [20]. TNF-α was significantly increased in BAL fluid of HIVneg renal transplant patients, while in this study IL-1ß is high in BAL fluid of HIVpos patients. These differences may be related to different underlying immune defects or disease stages. Anti-inflammatory mediators in BAL fluid of HIVneg and HIVpos patients with PCP show low concentrations of s-TNF receptors, while IL-1RA is significantly increased in the HIVneg patients and marginally increased in HIVpos patients. These data do not corroborate findings by Millar et al. who reported increased production of TNF-α by alveolar macrophages in HIVpos patients with PCP [25]. Differences in cell populations (adherent cells versus unselected BAL cells in our study), incubation time (4 versus 24 hours) and assays (immunoassay versus RIA) might be responsible for these discrepancies.

In the circulation concentrations of these anti-inflammatory mediators were higher in the acute phase of PCP than during recovery in both HIVneg and HIVpos patients. It is

tempting to hypothesize that the high concentration of anti-inflammatory mediators in the circulation ensures that the effect of the pro-inflammatory cytokines remains limited to the focus of infection. The host is subsequently protected against the deleterious systemic effects of pro-inflammatory 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-α and IL-1ß play a role in the acute phase of PCP [12,13,17,18].

For cytokine patterns in the lung, we had to compare HIVpos patients with healthy controls because a BAL procedure was not considered eligible during recovery or in asymptomatic HIVpos 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 some caution because of the use of different controls. Nevertheless our principle conclusion of divergence of local and systemic cytokine profile is supported by similar data in HIVneg patients with PCP [20] and a rat model [Perenboom et al, submitted].

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. who showed a significant correlation between the chemotactic cytokine IL-8 and severity of infection, as determined by PO2 and BAL neutrophilia [26,27]. TNF-α and IL-1ß, 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-1ß and TNF-α [28]. The high circulating concentrations of pro-inflammatory cytokines in HIVpos patients with PCP should be interpreted against the background of cytokine abnormalities in HIV. We could confirm that HIV infection per se results in high plasma concentrations of pro-inflammatory cytokines and s-TNFR's, and also in increased unstimulated pro-inflammatory cytokine production [29-31]. PCP did not further increase these plasma concentrations and unstimulated production. Plasma IL-1ß concentration in the acute phase of PCP in HIVpos patients was not significantly increased as compared to recovery, but was significantly higher than in asymptomatic HIVpos persons, which might be explained by the more severe HIV infection in the PCP patients; mean CD4 count 97 versus 207 in the asymptomatic persons (Table 1).

In a recent study, Huang et al. found diminished LPS-stimulated production of IL-1ß and TNF-α 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 of pro-inflammatory cytokine production [32]. In our study, however, production of pro-inflammatory cytokines by alveolar cells in HIVpos patients with PCP, was not reduced, even in patients who received high dose corticosteroids 2-24 hours before BAL. It is therefore not likely that the beneficial effect is due to suppression of local production of pro-inflammatory cytokine, unless one assumes that increased pro-inflammatory 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 [Perenboom et al, submitted].

Both HIVneg patients and HIVpos patients with PCP treated with steroids, showed suppressed production of pro-inflammatory cytokines in whole blood cultures in the acute phase. In contrast, pro-inflammatory cytokine production was not suppressed in the HIVpos patients with PCP, who did not receive steroids. Production in the latter group did neither differ from that in recovery, nor from that in asymptomatic HIVpos individuals. The high dosages of corticosteroids were only given to patients with PO2 < 8 kPa [33]. Thus, severe infection could also be responsible for suppression of pro-inflammatory cytokine production [28,34,35]. In 3 of 4 patients with PCP (2 HIVpos of the present series and 2 HIVneg [20]), blood was taken before and after high dose corticosteroids; in these patients we found that cytokine production was already suppressed before administration of steroids. Since all four patients had severe PCP with low PO2, severe infection rather than corticosteroids seems to lead to downregulation of systemic pro-inflammatory cytokine production.

Acknowledgments:

We thank Dr.P.Reiss from NATEC and Prof.Dr.S.Danner from the Department of Internal Medicine, Academic Medical Center, Amsterdam for their help in facilitating the start of this study, Dr. J. Wijdenes (Inotherapie, Besançon. France) for the anti-IL-6 antibodies, Dr.H.Gallati (Hoffmann-La Roche, Basel. Switserland) for the sTNF-R ELIBA. We greatly appreciated the statistical support from H.J.J.van Lier M.Sc. from the Department of Medical Statistics, University Nijmegen.

REFERENCES

1. Harmsen AG, Stanckiewicz M. Requirement for CD4+ cells in resistance to Pneumocystis carinii pneumonia in mice. J.Exp Med.1990;172:937-945.
2. Shellito J, Suzara VV, Blumenfeld W, Beck JM, Steger HJ, Ermak TH. A new model of Pneumocystis carinii infection in mice selectively depleted of helper T lymphocytes. J.Clin.Invest. 1990;85:1686-1693.
3. Phair J, Munoz A, detels R. The risk of Pneumocystis carinii pneumonia among men infected with human immunodeficiency virus type I. N Engl J Med 1990,332 161-165.
4. Von Behren LA, Pesanti EL. Uptake and degradation of Pneumocystis carinii by macrophages in vitro. Am Rev Respir Dis. 1978;118:1051-1059.
5. Masur H, Jones TC. The interaction in vitro of Pneumocystsis carinii with macrophages and L cells. J Exp Med 1978;147:157-170.
6. Forte M, Rahelu M, Stubberfield C et al. Interaction of human macrophages with Pneumocystis carinii. Int J Exp Pathol 1991;72.589-598.
7. Hidalgo HA, Helmke RJ, German VF, Mangos JA. Pneumocystis carinii induces an oxidative burst in alveolar macrophages. Infect Immun 1992,60 1-7.
8. Pesanti EL. Interaction of cytokines and alveolar cells with Pneumocystis carinii in vitro J Infect Dis 1991;163·611-616
9. Kolls JK, Beck JM, Nelson S, Summer WR and Shellito J Alveolar macrophage release of Tumor Necrosis Factor during murine Pneumocystis carinii Pneumocystis AM J Respir Cell Mol Biol. 1993,-8:370-376.
10. Tamburrini E, De Luca A, Ventura G et al. Pneumocystis carinii stimulates in vitro production of tumor necrosis factor-α by human macrophages. Med Microbiol Immunol. 1991,180.15-20.
11. Theus SA, Linke MJ, Andrews RP and Walzer PD. Proliferative and cytokine responses to a major surface glycoprotein of Pneumocystis carinii. Infect.Immun. 1993;61.4703-4709.
12. Krishnan VL, Meager A, Mitchell DM and Pinching AJ. Alveolar macrophages in AIDS patients:increased spontaneous tumour necrosis factor-alpha production in Pneumocystis carinii pneumonia. Clin.Exp Immunol 1990,80.156-160.
13. Kandil O, Fishman JA, Koziel H, Pinkston P, Rose RM, Reinold HG. Human immunodeficiency virus type 1 infection of human macrophages modulates the cytokine response to Pneumocystis carinii. Infect.Immun. 1994;62:644-650
14. Pesanti EL, Tomicic T, and Donta ST. Binding of ^{125}I-labelled tumor necrosis factor to Pneumocystis carinii and an insoluble cell wall fraction J Protozool 1991;38 28S-29S (Abstract).
15. Donta ST and Pesanti EL. Tumor necrosis factor-α binds to specific receptors on Pneumocystis carinii. Clin Res 1990;38.352A (Abstract).
16. Chen W, Havell EA, Gigliotti F and Harmsen AG. Interleukin-6 production in a murine model of Pneumocystis carinii Pneumonia: Relation to resistance and inflammatory response Infect. Immun 1993;61.97-102.
17. Chen W, Havell EA, Moldawer LL, et al. Interleukin-1 An important mediator of host resistance

against Pneumocystis carinii. J.Exp.Med 1992;176:713-718.

18. Chen W, Havell EA, and Harmsen AG. Importance of endogenous tumor necrosis factor alpha and gamma interferon in host resistance against Pneumocystis carinii infection. Infect.Immun. 1992;60:-1279-1284.
19. Churukian CL, Schenk EA. Staining Pneumocystis carinii and fungi in unfixed specimens with ammoniacal silver using a microwave oven. J Histotechnol 1988;11:19-21.
20. Perenboom RM, Schijndel van JCHW, Beckers P, Sauerwein R, Hamersvelt van HW, Festen J, Gallati H, Meer van der JHW. Cytokine profiles in BAL and blood in HIV-seronegative patients with Pneumocystis carinii pneumonia. Eur J Clin Invest 1996;26:159-166.
21. Van Deuren M, Van der Ven-Jongekrijg J, Keuter M, Demacker PNM, Van der Meer JWM. Cytokine production in whole blood cultures. J Int Fed Clin Chem 1993;5:216-221
22. Lisi PJ, Chu CW, Koch GA, Endres S, Lonneman G, Dinarello CA. Development and use of radio immunoassay for human interleukin-1ß. Lymphokine Res 1987;6.229-44
23. Nerad JL, Griffith K, van der Meer JWM et al. Interleukin-1ß (IL-1ß), IL-1 receptor antagonist and TNF-α production in whole blood. J Leucoc Biol 1992;52: 687-692.
24. Wijdenes J, Clement C, Klein B, Morel-Fourrier, Vita N, Ferrera P, Peters A. Human recombinant dimeric IL-6 binds to its receptor as detected by anti-IL-6 monoclonal antibodies. Mol Immunol 1991;28:1183-1192.
25. Millar AB, Miller RF, Foley NM, Meager A, Semple SJ, Rook GA. Production of tumor necrosis factor-α by blood and lung mononuclear phagocytes from patients with human immmunodeficiency virus-related lung desease. Am J Respir Cell Mol Biol 1991,5(2) 144-148
26. Benfield TL, Vestbo J, Junge J, Nielsen TL, Jensen AMB, Lundgren JD. Prognostic value of interleukin-8 in AIDS-associated Pneumocystis carinii pneumonia. Am J Respir Crit Care Med 1995;151:1058-1062.
27. Benfield TL, van Steenwijk R, Nielsen TL, Dichters JR, Lipschik GY, Jensen BN, Junge J, Shelhamers JH, Lundgren JD. Interleukin-8 and eisosanoid production in the lung during moderate to severe Pneumocystis carinii pneumonia in AIDS: a role of interleukin-8 in the pathogenesis of P.carinii pneumonia. Respiratory Medicine 1995;89:285-290.
28. Van Deuren M, van der Ven-Jongekrijg J, Demacker PNM, Bartelink AKM, van Dalen R, Sauerwein R, Gallati H, Vannice JL, van der Meer JWM. Differential expression of proinflammatory cytokines and their inhibitors during the course of meningococcal infections. JID 1994;169:157-161.
29. Godfried M, Poll van der T, Jansen J et al. Soluble receptors for tumour necrosis factor a putative marker for disease progression in HIV infection. AIDS 1993,7:33-36.
30. Zangerle R, Gallati H, Sarcletti M, Wachter H, Fuchs D. Tumour necrosis fator alpha and tumour necrosis factor receptors in individuals with human immuno deficiency virus infection. Immunology letters 1994;41:229-234.
31. Breen EC, Rezai AR, Kajima K et al. Infection with HIV is associated with elevated IL-6 levels and production. J Immunol 1990:144:480-484.
32. Huang ZB, Eden E. Effect of corticosteroids on IL-1ß and TNF-α release by alveolar macrophages from patients with AIDS and PCP. Chest 1993;104:751-755.

33. Bozette SA, Sattler FR, Chiu J et al. A controlled trial of early adjunctive treatment with corticosteroids for Pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. N Engl J Med 1990;323:1451-1457.

34. Keuter M, Dharmana E, Hussein Gasem M et al. Patterns of proinflammatory cytokines and inhibitors during typhoid fever. J Infect Dis 1994;169.1306-1311.

35. Simpson SQ, Modi HN, Balk RA, Bone RC, Casey LC. Reduced alveolar macrophage production of tumor necrosis factor during sepsis in mice and men. Crit Care Med 1991;19:1060-1066.

MODULATION OF TNF IN EXPERIMENTAL PNEUMOCYSTIS CARINII PNEUMONIA

Roos M.Perenboom

Pieter Beckers

Jos W.M.van der Meer

Anita C.H.W.van Schijndel

Wim J.G.Oyen

Robert W.Sauerwein.

Submitted

ABSTRACT

We investigated the possible therapeutic role of TNF and a polyclonal murine anti-TNF-antibody in experimental PCP, in which the growth of the micro organisms as well as the magnitude of the host response could be monitored. P.carinii load was measured in the P.carinii density score. The inflammatory response was quantified by means of the lung/body weight ratio and the lung/muscle ratio in ^{111} Indium biodistribution, both proven in an earlier study to correlate with other inflammation parameters.
In experimental Pneumocystis carinii pneumonia, intratracheal TNF in the first weeks after infection in addition to suboptimal cotrimoxazole was not synergistic in eradication of Pneumocystis. Treatment with intratracheal anti-TNF in the 5th and 6th week after infection combined with standard dose cotrimoxazole did not reduce inflammation.

INTRODUCTION

Pneumocystis carinii pneumonia (PCP) is a common infectious complication in patients infected with the human immunodeficiency virus (HIV).
Tumor necrosis factor-α (TNF-α) seems to play a role in host defense against PCP. Increased concentrations of TNF-α have been detected in broncho-alveolar lavage (BAL) fluid of patients with PCP [10]. In mice, TNF-α was found necessary early in the infection to clear P.carinii (PC) [8]. In-vitro experiments suggest that TNF-α could kill P.carinii directly [12]. Despite these arguments for a favourable role in PCP, TNF-α is also known to be deleterious in serious infections. Endothelial damage and fibrosis may ensue from its action and such effects could occur in PCP.
Thus, TNF-α may be important in the beginning of infection to orchestrate microbicidal activity against P.carinii, whereas TNF-induced inflammation could result in respiratory failure and systemic symptoms at a later stage of disease. To test this hypothesis we investigated, in the rat, the effects of intratracheally administered TNF-α or α-TNF antibodies (α-TNF-abs) at different time points during PCP in combination with cotrimoxazole.

MATERIAL AND METHODS

Four to six weeks old, female Spraque-Dawley rats, were immunosuppressed with weekly

subcutaneous injections of 25 mg hydrocortisone and 8% protein-restricted diet. PCP was induced by close cohabitation with P.carinii infected rats. Co-infections were excluded by regular microbiological screening.

Two days before the animals were sacrificed, ^{111}Indium-IgG (^{111}In-IgG) was given intravenously as described [11]. Uptake of ^{111}In-IgG was measured in the right upper lung lobe and compared to normal calf muscle to calculate lung to muscle (L/M) ratios. PC density was assessed in Giemsa and silver stained impression smears and expressed in the logarithmic PC density score [3].

Post mortem broncho-alveolar lavage (BAL) was performed. BAL fluid and blood were collected and incubated with and without lipopolysaccharide (LPS) for 24 hours, as described before [11]. Supernatant and cell pellets were frozen at -80°C until analysis. The left upper lung lobe was homogenized, centrifuged and the supernatant frozen until analysis.

TNF-α was measured in plasma, BAL and lung homogenate samples by L929 cytotoxicity bio-assay (detection limit 20 pg/ml)[1,2]. Trimethoprim-sulfamethoxazole (Roche, The Netherlands) was administered by gavage in different dosages once daily during week 5 and 6 after start of the immunosuppression. Recombinant-murine-TNF-α was donated by Dr.G.Adolf, Ernst-Boehringer Institut, Vienna, Austria. Specific activity was 1.2×10^7 U/mg in LM cells; endotoxin concentration was <51 pg/ml.

Polyclonal α-TNF-antibodies were raised in a rabbit by intradermal administration of 50 μg recombinant-murine-TNF-α (rec-mTNF-α) in Freund's complete adjuvant and boosting with Freund's incomplete adjuvant. The rabbit antiserum was able to neutralize rat TNF-α in the L929 bio-assay. The amount of rec-mTNF-α that caused lysis of 50% of the L929 cells was defined as one unit of rec-mTNF-α and corresponded to 70 pg/ml. The amount of rabbit antiserum neutralizing 1 unit rec-mTNF-α was defined as one neutralizing unit (NU). The rabbit antiserum thus tested, contained 3×10^5 NU/ml. In-vivo, intraperitoneal administration of 1.2×10^6 NU α-TNF-abs in rats, was able to completely neutralize LPS-induced plasma TNF-α concentrations of 135-173 ng/ml.

As control, a monoclonal rat-anti-mouse-TNF-antibody (Moabs), V1Q (a gift from Dr.P.H.Krammer, Heidelberg, FRG) showed no neutralizing activity in the L929 bio-assay.

TNF-α and α-TNF-abs were administered intratracheally under ether anaesthesia as described [7]. In short, a modified spinal needle was inserted between the vocal folds, 200 μl

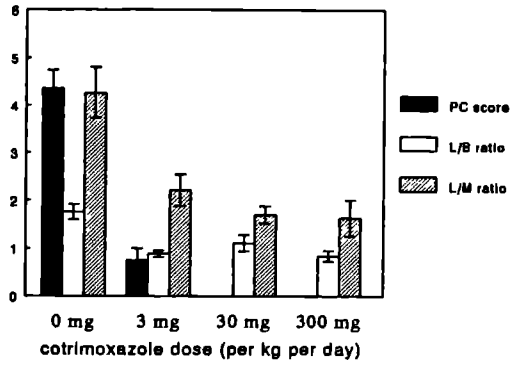


Fig.1

PC score and inflammatory parameters in experimental PCP in rats, treated with a 2 weeks course of cotrimoxazole. PC score = Pneumocystis carinii density score on Giemsa stained impression smears of the lungs. L/B ratio = Lung to body weight ratio. L/M ratio = ^{111}-Indium biodistribution lung to muscle ratio. Each column represents the mean and SEM of 5-7 rats. PC score, L/B ratio and L/M ratio were significantly reduced in all cotrimoxazole treated rats as compared to the untreated rats (Mann-Whitney test, $p<0.05$).

solution was instilled, whereafter 2 ml air was injected with some force to increase dispersion of the solution in the lungs.

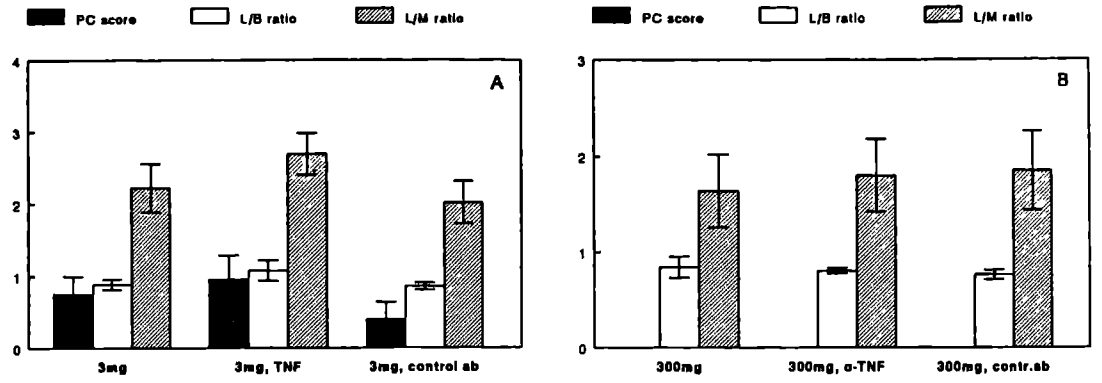
Statistics - Data are given in means and standard error of the means (SEM). Differences between groups were analysed by the non-parametric Mann-Whitney test.

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

RESULTS

As shown in fig.1, treatment with both 300 and 30mg/kg cotrimoxazole eradicated all cysts and significantly lowered L/B and L/M ratios, while 3mg/kg significantly reduced the number of cysts. Combination of suboptimal cotrimoxazole (3mg/kg) with 100ng rec-mTNF-α intratracheally once weekly during week 1-3 in the early phase of PCP did not change the number of PC cysts (fig.2a).

Pilot experiments had shown that 300mg/kg cotrimoxazole increased signs of inflammation, as indicated by increased L/B ratios after one week of treatment (data not shown). Addition of α-TNF-abs, $6x10^4$ NU, sufficient to neutralize at least 7500 pg/ml rat



PC score and inflammatory parameters in experimental PCP in rats, treated with either a 2 weeks course of cotrimoxazole 3 mg/kg per day in combination with intratracheal administration of TNF early in the infection (fig.2A) or with cotrimoxazole 300mg/kg per day and α-TNF-abs intratracheally late in the infection (fig.2B). Control rats were given an antibody without neutralizing activity (contr.ab). PC score = Pneumocystis carinii density score on Giemsa stained impression smears of the lungs. L/B ratio = Lung to body weight ratio. L/M ratio = ^{111}Indium biodistribution lung to muscle ratio. Each column represents the mean and SEM of 3-5 rats. PC score, L/B ratio and L/M ratio did not differ between groups treated with the same dose cotrimoxazole with or without intratracheal TNF or α-TNF-abs (Mann-Whitney test).

TNF-α, intratracheally twice weekly to 300mg/kg cotrimoxazole daily in week 5 and 6, did not affect L/B and L/M ratio as compared to cotrimoxazole with control Mabs (fig.2b). Bio-active TNF-α was measured in week 6, in BAL fluid, lung homogenates and plasma in rats treated with 300mg/kg cotrimoxazole monotherapy, 3mg/kg cotrimoxazole combined with intratracheal TNF-α or 300mg/kg cotrimoxazole and intratracheal TNF antiserum. In all groups there was no significant difference between TNF-α concentrations (63-100 pg/ml) in BAL and lung homogenates, while TNF-α levels in plasma were undetectable. In all groups LPS-stimulated TNF-α production by alveolar cells was >2000 pg/ml, while production in whole blood cultures was under the detection limits.

DISCUSSION

Protection by exogenous recombinant TNF-α has been demonstrated in-vitro and in-vivo in a number of infection models, even in pulmonary infection. Blanchard et al demonstrated a protective effect of TNF-α in experimental Legionella pneumophilia infection [5]. Beck et al reported benefit of intratracheal IFN-gamma in PCP in mice suggesting that part of this was due to priming macrophages for TNF-α release [4].

Beneficial effects of α-TNF-abs in adjunct to antimicrobial therapy were reported in sepsis [14]. In humans with PCP, hypoxia often increases after initiation of therapy and corticosteroids are used to prevent the initial decline in oxygenation and improve prognosis [6]. Drug-induced death of P.carinii may enhance the inflammatory responses. This hypothesis is supported in this study by the slightly higher L/B ratio observed after one week of high dose cotrimoxazole treatment.

However, intratracheal administration of TNF-α in the first weeks of PCP in combination with suboptimal cotrimoxazole doses neither resulted in a decline of P.carinii density score, nor in lower L/B and L/M ratios.

The presence of bioactive TNF-α in BAL and lung homogenates after 2 weeks of cotrimoxazole (in any regimen) suggests that TNF-α is indeed induced by killed PC, since no TNF-α could be detected in such samples from untreated rats with PCP [unpublished observations]. Despite these observations, administration of α-TNF-abs together with standard dosages of cotrimoxazole did not affect inflammatory parameters. Therefore, TNF-α may either not play a role in our PCP model or local TNF activity was not adequately neutralized by our interventions. Further studies may be required to evaluate administration of TNF-α or α-TNF by aerosol rather than by instillation or by different dosages or frequency of administration.

Finally we could confirm findings by other authors that lower than usual doses of cotrimoxazole are remarkably effective in elimination or suppression of P.carinii [9,13].

Acknowledgements

The expert help of Gerry Grutters and Henny Eikholt of the Central Animal Laboratory, of Emiel Koenders of the Nuclear Medicine Department and of Tita Oettinger of the Department of Parasitology is greatfully acknowledged. We thank Maite Vogels and Ineke Verschueren for the rabbit anti-mouse-TNF-antiserum and 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 fruitfull diccussions during the regular meetings.

REFERENCES

1. Aarden LA, de Groot ER, Schaap DL, Lansdorp PM. 1987. Production of hybridoma growth factor by human monocytes. Eur J Immunol 17:1411-1416.

2. Aggarwal BB. 1985 Human lymphotoxin Methods Enzymol. 116:441-444
3. Bartlett MS, Queener SF, Jay MA, Durkin MM, Smith JW. 1987. Improved rat model for studying Pneumocystis carinii pneumonia. J Clin Microbiol 25: 480-484.
4. Beck JM, Liggit HD, Brunette EN, Fuchs HJ, Shellito JE, Debs RJ. 1991. Reduction in intensity of Pneumocystis carinii pneumonia in mice by aerosol administration of gamma interferon. Infect Immun 59:3859-3862.
5. Blanchard DK, Djeu JY, Klein Tw et al. 1988. Protective effect of tumor necrosis factor in experimental Legionella pneumophilia infections in mice via activation of PMN function. J Leukocyte Biol 43:429-435
6. Bozette SA, Sattler FR, Chiu J et al 1990 A controlled trial of early adjunctive treatment with corticosteroids for Pneumocystis carinii pneumonia in the acquired immuno deficiency syndrome. N Engl J Med 323.1451-1457.
7. Brain JD, Knudson DE, Sorokin SP, Davis MA. 1976. Pulmonary distribution of particles given by intratracheal instillation or by aerosol inhalation. Environmental Research 11·13-33
8. Chen W, Havell EA, and Harmsen AG. 1992 Importance of endogenous tumor necrosis factor alpha and gamma interferon in host resistance against Pneumocystis carinii infection Infect.Immun. 60·1279-1284
9. Hughes WT, Killmar JT, Oz HS. 1994. Relative potency of 10 drugs with anti-Pneumocystis carinii activity in an animal model. J Infect Dis 170 906-911
10. Perenboom RM, Schijndel van JCHW, Beckers P et al 1996 Cytokine profiles in BAL and blood in HIV seronegative patients with Pneumocystis carinii pneumonia Eur J Clin Invest 26.159-166.
11. Perenboom RM, Oyen WJG, Schijndel van JCHW, Beckers P, Corstens FHM, Meer van der JWM. 1995. Serial ^{111}In-labelled IgG biodistribution in rat Pneumocystis carinii pneumonia. A tool to monitor the course and severity of the infection. Eur J of Nuclear Medicine 122.1129-1132.
12. Pesanti EL 1991 Interaction of cytokines and alveolar cells with Pneumocystis carinii in-vitro. J Infect Dis 163:611-616.
13. Schneider MME, Nielsen TL, Nelsing S et al. 1995. Efficacy and toxicity of two doses of trimethoprim-sulfamethoxazol as primary prophylaxis against Pneumocystis carinii pneumonia in patients with human immunodeficiency virus. J Infect Dis 171.1632-1636.
14. Silva At, Bayston KF, Cohen J. 1990 Prophylactic and therapeutic effects of a monoclonal antibody to tumor necrosis factor-α in experimental gram-negative shock. J Infect Dis 162:421-427

Chapter 8

GENERAL DISCUSSION AND CONCLUSIONS

The objective of the study was to increase insight in the role of cytokines in the pathogenesis of PCP. Since in a focal infection such as pneumonia, local effects of cytokines are expected to be important and possible different from systemic effects, we compared cytokine patterns in various compartments. We did in-vitro experiments, followed by descriptive studies on cytokine patterns in different PCP models and finally explored the effects of cytokine modulation.

I. IN-VITRO EXPERIMENTS

We could not confirm earlier reports that P.carinii cysts are able to induce in-vitro production of cytokines in peripheral blood mononuclear cells and alveolar cells [1-2]. Our results however corroborate those of Helmke and Hidalgo, who also reported failure of in-vitro TNF-α production on exposure to P.carinii [3]. Differences in cell selection, P.carinii preparations and culture media might be partly responsible for these divergent results.

II. IN-VIVO CYTOKINE PROFILES

A. Local and systemic cytokine concentrations in human and rat PCP

Cytokines in BAL and lung homogenates - In BAL fluid of HIVpos and HIVneg patients with PCP, concentrations of immunoreactive pro-inflammatory cytokines are generally higher than in healthy controls, but only significant for IL-1ß in HIVpos patients (184 $\pm$ 47 pg/ml versus <20 pg/ml, $p=0.01$), and for TNF-α in HIVneg patients (551 $\pm$ 322 pg/ml versus <20 pg/ml, $p=0.02$). Cytokine ratios are shown in Fig.1A. Increased concentrations of pro-inflammatory cytokines are also found in rats with PCP: with increased concentration of IL-1ß nd IL-6 in respectively lung homogenates and BAL fluid (data not shown). Thus, at least one of the pro-inflammatory cytokines is present at the site of infection, although this is not always the same mediator. Why different pro-inflammatory cytokine are present in BAL in the various PCP models (HIVpos patients,

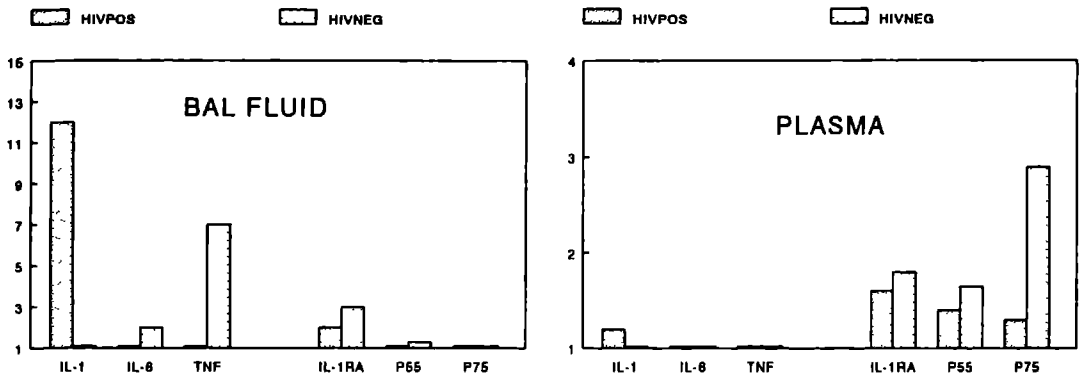


Fig.1 Cytokine ratios in BAL (1A) and plasma (1B) during the acute phase of PCP Ratios represent: $\frac{\text{median concentration of patients}}{\text{median concentration of controls}}$
Healthy HIVneg volunteers were controls for BAL concentrations in HIVneg and HIVpos patients (1A). Control for plasma data was own plasma from the patients after recovery (1B)

immunosuppressed HIVneg patients and steroid treated rats) needs further study. The explanation for these differences is most likely multifactorial and may reflect different underlying immune deficiencies or disease stages.

It is remarkable that TNF-α, which is most frequently mentioned in relation to HIV and P.carinii, is only increased in BAL in HIVneg patients. It is however in accordance with the results of Kandil et al.[4]. They show that HIV infection modulates the response to P.carinii; monocyte-derived macrophages in-vitro infected with HIV and co-incubated with P.carinii, produce less TNF-α and IL-1ß as compared to non-HIV infected cells. In our rat model the use of a bio-assay could account for the undetectable levels of TNF-α in BAL and lung homogenates.

In some of the HIVpos patients the increased concentration of IL-1ß is accompanied by a local increase of IL-1RA. High TNF-α levels in BAL fluid of HIVneg patients are not accompanied by increased concentrations of sTNF-receptors, IL-1RA concentrations are however significantly increased.

Plasma cytokines - Circulatory pro-inflammatory cytokine concentrations during the acute phase of PCP are not different from those after full recovery in both patients and rats (Fig.1B). However, in the acute phase of PCP, plasma concentrations of anti-inflammatory mediators IL-1RA ($p=0.01$) and sTNF receptors, especially P75 ($p=0.04$), are significantly increased in respectively HIVpos and HIVneg patients.

Thus in PCP, pro-inflammatory cytokines are more prominently present at the site of infection, while anti-inflammatory mediators are relatively higher in the circulation

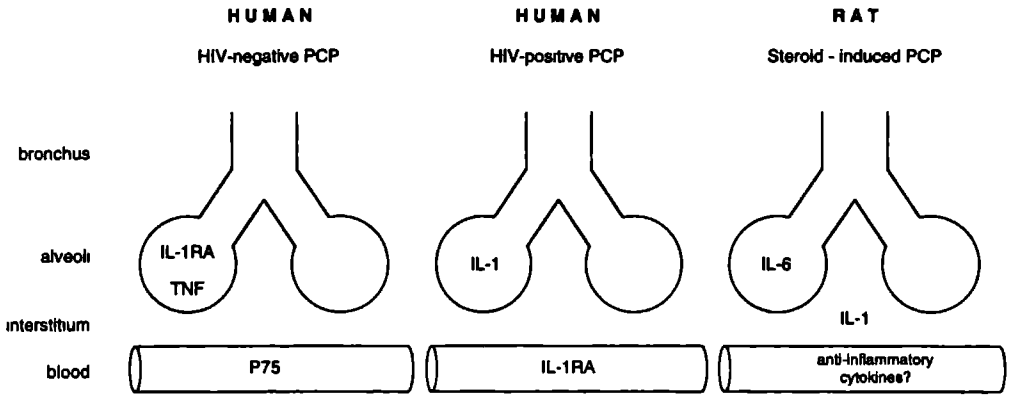


Fig.2 Compartmentalization of cytokine profiles in lungs (alveolar space and interstitium) and blood in patients and rats with Pneumocystis carinii pneumonia. The predominant cytokines at the various sites under the three conditions are schematically indicated It can be concluded that pro-inflammatory cytokines dominate at the focus of infection, while anti-inflammatory cytokines are more prominently present in the circulation.

(Fig.2). It is tempting to speculate that the high concentrations of anti-inflammatory mediators in the circulation ensure that the effect of the pro-inflammatory cytokines remains limited to the site of infection. The host is subsequently protected from the deleterious systemic effects of pro-inflammatory cytokines, including damage to the endothelium, shock and mortality. In the lungs, pro-inflammatory cytokines are considered important, at least at the start of the infection, to activate the host response systems in order to eliminate or directly kill P.carinii. The fact that in both patient groups the increase of one particular pro-inflammatory cytokine in BAL is accompanied by an increase of its specific inhibitor in plasma supports this hypothesis.

The high concentration of IL-1RA in BAL fluid of HIVneg and some HIVpos patients may be explained by the necessity to counteract also locally the possible deleterious

effects of the pro-inflammatory cytokines.

B. Ex-vivo cytokine production by alveolar cells and whole blood

Alveolar cells - In the HIVpos patients with PCP, LPS-stimulated production of pro-inflammatory cytokines is not affected, while production in HIVneg patients is reduced. In the rats, pro-inflammatory cytokine production slowly declines to profound suppression over a 6 weeks period; however at week 4 a higher response is found in PCP rats as compared to control steroid rats. Stimulated as well as spontaneous production of IL-1RA is increased in HIVpos patients and reduced in HIVneg patients with PCP.

Whole blood cultures - LPS-stimulated pro-inflammatory cytokine production in whole blood cultures is suppressed in the acute phase of PCP, in patients as well as rats. IL-1RA production is intact both in HIVpos as in HIVneg patients.

The data on in-vivo cytokine concentrations in BAL and blood and in-vitro cytokine production in whole blood are to some extent similar in all PCP patients and rats. PCP is therefore likely to be primarily responsible for these findings. However, ex-vivo production of cytokines by alveolar cells differs substantially between the PCP groups. This suggests that underlying immunoregulatory defects (HIV infection, steroids) play an important role in these differences (see below). The differential regulation of alveolar macrophages and peripheral blood cells shown in patients and rats, is in accordance with other studies. Differentiation of blood monocytes into alveolar macrophages changes their capacities to release pro-inflammatory cytokines and their inhibitors (5-8). In general, blood monocytes are higher producers of IL-1ß, while alveolar macrophages produce relatively more IL-1RA (5). Our results, especially in HIVpos patients are in line with these observations. Due to their localisation, alveolar macrophages are continuously exposed to external stimuli. It may be conceivable that they produce more readily IL-1RA to reduce potential damage by pro-inflammatory cytokines. This theory, however, does not hold for the TNF family. Nicod reports that blood monocytes are higher producers of the sTNF-receptor P75, while alveolar macrophages produce more TNF-α. We could confirm the higher TNF-α production by alveolar cells, but sTNF receptors could hardly be found in our whole blood cultures.

III. THE EFFECT OF CORTICOSTEROIDS AND HIV INFECTION ON CYTOKINE PROFILES

Corticosteroids - Eight out of 23 HIVpos patients received corticosteroids 2-24 hours before BAL. Concentrations of cytokines in BAL and blood of these 8 patients were the same as of those not treated with corticosteroids. Similarly, cytokine concentrations in BAL and blood of HIVneg patients were also not influenced by adjunctive corticosteroid therapy. In the animal model the same was found, i.e. in rats corticosteroid treatment did not affect cytokine concentrations in lung or blood. Thus, although corticosteroids are well known for major anti-inflammatory activity, by inhibiting NF-KB activity, we did not find an effect on cytokine concentrations in BAL or plasma in humans or rats infected with PCP. Similarly, corticosteroids did not immediately suppress pro-inflammatory cytokine production by alveolar cells in HIVpos patients and rats. Suppression of cytokine production by alveolar cells was however seen in the rat after 4-6 weeks of steroid administration. And in HIVneg patients with PCP, all of them on longstanding steroid treatment, cytokine production by alveolar cells was profoundly suppressed.

In whole blood cultures, in patients as well as in rats, steroids had an immediate significant suppressive effect on cytokine production. This differential effect of steroids on cytokine production in alveolar cells and blood is in accordance with the findings of Strieter et al, who showed in in-vitro experiments that the inhibitory effects of steroids on TNF-α production was more pronounced in peripheral blood monocytes than in alveolar macrophages [11].

Thus, administration of corticosteroids immediately reduces ex-vivo pro-inflammatory cytokine production in whole blood cultures, while production by alveolar cells became only apparent after some weeks. In-vivo concentrations of cytokines in BAL fluid and blood did not seem to be affected by corticosteroid administration.

HIV infection - HIV infection per se results in high plasma concentrations of pro-inflammatory cytokines and s-TNFR's and increased spontaneous pro-inflammatory ex-vivo cytokine production. These findings are in accordance with those of others [12-18]. PCP does not further increase plasma concentrations of pro-inflammatory cytokines. These findings are not in concert with the studies by Jones et al [19]; they report that

increased plasma concentrations of TNF-α in HIV infected persons were caused by opportunistic infections, such as PCP, and not by the HIV infection per se.

IV. CORRELATIONS BETWEEN CYTOKINE CONCENTRATIONS AND SEVERITY OF INFECTION

Cytokine concentrations neither correlate with disease activity in patients nor with inflammatory parameters in the rat model. This is in contrast with findings of Benfield et al who show a significant correlation between the levels of chemotactic IL-8 and severity of infection, as determined by PO2 and BAL neutrophilia [20,21]. The explanation for this discrepancy remains speculative. Possibly the time of sampling is important. It has been reported that the concentrations of IL-8 persist for longer than that of TNF-α and IL-1ß [22].

We could confirm that the clinical pictures of PCP in HIVpos and HIVneg patients show differences [23,24]. HIVpos patients show a more slowly developing clinical disease and are less ill than HIVneg patients. They generally had a longer duration of symptoms (mean 5 versus 2.8 days) and higher PO2 (8.6 kPa versus 8.2 kPa). The percentage of patients admitted to the intensive care unit as well as the mortality was lower in HIVpos patients than in HIVneg patients with PCP (9% versus 40% and 9% versus 30% respectively). Increased concentrations of TNF-α, with concomitantly unchanged concentrations of sTNF-receptors in BAL in the HIVneg patients is in agreement with this finding. In the HIVpos patients the inflammatory activity by increased concentration of IL-1ß in BAL may be more adequately neutralized by the presence of the high IL-1RA concentrations.

V. CYTOKINE INTERVENTIONS

Intratracheal TNF-α early in the infection in addition to suboptimal cotrimoxazole was not synergistic in eradication of P.carinii organisms and intratracheal administration of α-TNF-antibody later in the infection did not reduce inflammation.

It is well appreciated that only one carefully chosen experimental condition has been tested. As is often the case with negative results, we cannot exclude that other doses,

timing or frequency of administration of TNF-α and/or α-TNF-antibody and assesment of inflammation parameters on an earlier time point after start of therapy would have produced different results. So far, however, we have no evidence that modulation of TNF-α reactivity changes the course of a PCP in our rat model.

CONCLUSION

In conclusion, in the acute phase of PCP local cytokine profiles differ from those in the circulation: pro-inflammatory cytokines dominate in the lung and anti-inflammatory mediators in the circulation. In focal infections the local balance between pro- and anti-inflammatory cytokines influences severity of disease. Steroids have immediate profound effects on cytokine production in blood, and not in alveolar cells. Lower than usual doses cotrimoxazole can be effective in PCP. Intratracheal administration of TNF-α early or anti-TNF later in the infection does not influence P.carinii load or inflammation parameters in our test conditions.

REFERENCES

1. Pesanti EL. (1991) Interaction of cytokines and alveolar cells with Pneumocystis carinii in vitro. J Infect Dis 163, 611-616.
2. Tamburrini E, De Luca A, Ventura G et al. (1991) Pneumocystis carinii stimulates in vitro production of tumor necrosis factor-α by human macrophages Med Microbiol Immunol 180, 15-20.
3. Helmke R, Hidalgo H. (1992) Failure of Pneumocystis carinii to induce tumour necrosis factor and interleukin-6 in alveolar macrophages. FASEB J 6, A1614 (Abstract).
4. Kandil O, Fisman JA, Koziel H, Pinkston P, Rose RM, Remold HG. (1994) Human immunodeficiency virus type 1 infection of human macrophages modulates the cytokine response to Pneumocystis carinii. Infect Immun 62, 644-650.
5. Nicod LP, Galve-De-Rochemonteix B, Dayer JM (1994) Modulation of IL-1 receptor antagonist and TNF-soluble receptors produced by alveolar macrophages and blood monocytes. Annala New York Academie of Sciences, 725:323-330.
6. Wewers MD, Herzyk DJ. (1989) Alveolar macrophages differ from blood monocytes in human IL-1ß release. J Immunology 143:1635-1641
7. Kline JN, Monick MM, Hunninghake GW. (1992) IL-1 receptor antagonist release is regulated

differently in human alveolar macrophages than in monocytes. J Appl Phys 73(4) 1686-1692.

8. Iwamoto GK, Monick MM, Burmeister LF, Hunninghake GW. (1989) Interleukin 1 release by human alveolar macrophages and blood monocytes. Am J Physiol.256 (Cell Physiol 25), C1012-C1015.
9. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin Jr. AS (1995) Role of transcriptional activation of IKBÓ in mediation of immunosuppression by glucocorticoids Science, 270·283-285.
10. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M (1995) Immunosuppression by glucocorticoids: Inhibition of NF-KB activity through induction of IKB synthesis. Science, 270:286-289.
11. Strieter RM, Remick DG, Lynch JP et al. (1989) Differential regulation of tumor necrosis factor-α in human alveolar macrophages and peripheral blood monocytes a cellular and molecular analysis. Am J Respir Cell Mol Biol 1:57-63.
12. Chollet-Martin S, Simon F, Matheron S, Joseph CA, Elbim C, Gougerot-Pocidalo MA. (1994) Comparison of plasma cytokine levels in African patients with HIV-1 and HIV-2 infection. AIDS 8,879-884.
13 Godfried M, Poll van der T, Jansen J et al (1993) Soluble receptors for tumour necrosis factor a putative marker for disease progression in HIV infection. AIDS 7, 33-36.
14. Zangerle R, Gallati H, Sarcletti M, Wachter H, Fuchs D. (1994) Tumour necrosis fator alpha and tumour necrosis factor receptors in individuals with human immuno deficiency virus infection. Immunology letters 41, 229-234
15. Lafeuillade A, Poizot-Martin I, Quilichini R et al. (1991) Increased interleukin-6 production is associated with disease progression in HIV infection AIDS 5, 1139-1140
16. Noronha IL, Daniel V, Schimpf K, Opelz G. (1992) Soluble IL-2 receptor and tumour necrosis factor-α in plasma in haemophilia patients infected with HIV Clin Exp Immunol 87, 287-292.
17. Breen EC, Rezai AR, Kajima K et al (1990) Infection with HIV is associated with elevated IL-6 levels and production J Immunol 144, 480-484.
18. Hober D, Haque A, Wattre P, Beaucaire G, Mouton Y, Capron A. (1989) Production of tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) in patients with AIDS Enhanced level of TNF-α is related to a higher cytotoxic activity. Clin Exp Immunol 78, 329-333.
19. Jones PD, Shelley L, Wakefield D. (1992) Tumour necrosis factor-α in advanced HIV infection in the absence of AIDS related secondary infections. J Acquir Immune Defic Syndr 5, 1266-1271.
20. Benfield TL, Vestbo J, Junge J, Nielsen TL, Jensen AMB, Lundgren JD. (1995) Prognostic value of interleukin-8 in AIDS-associated Pneumocystis carinii pneumonia Am J Respir Crit Care Med 151, 1058-1062.
21 Benfield TL, van Steenwijk R, Nielsen TL, Dichters JR, Lipschik GY, Jensen BN, Junge J, Shelhamers JH, Lundgren JD. (1995) Interleukin-8 and eisosanoid production in the lung during moderate to severe Pneumocystis carinii pneumonia in AIDS a role of interleukin-8 in the pathogenesis of P carinii pneumonia Respiratory Medicine 89,285-290.

22. Strieter RM, Lukacs NW, Standiford TJ, Kunkel SL. (1993) Cytokines. 2. Cytokines and lung inflammation: mechanism of neutrophil recruitment to the lung. Thorax 48, 765-769.

23. Kovacs JA, Hiemenz JW, Macher AM et al (1984) Pneumocystis carinii pneumonia: a comparison between patients with the acquired immuno deficiency syndrome and patients with other immuno deficiencies. Ann Intern Med 100,663-671.

24. Limper AH, Offord KP, Smith ThF, Martin II WJ. (1989) Pneumocystis carinii pneumonia. Differences in lung parasite number in patients with and without AIDS. Am Rev Respir Dis 140, 1204-1209.

SUMMARY

In **Chapter one**, we briefly summarized the data from literature showing that pro-inflammatory cytokines are involved in PCP. We explained our objectives and gave the arguments why we decided to explore cytokine responses in the different compartments, i.e. lung and blood.

In **Chapter two**, a rat model was presented for establishment of PCP. A consistent and reproducible PCP model was developed in corticosteroid-treated rats. IgG scintigraphy and ^{111}Indium biodistribution are sensitive and quantitative methods to assess the severity of PCP. Combining data on lung/body weight ratio, P.carinii density score in Giemsa stained impression smears and ^{111}Indium-IgG biodistribution is a good method for monitoring course and severity of PCP.

In **Chapter three** we studied the capacity of Pneumocystis carinii to induce cytokine production in-vitro. In our system, Pneumocystis carinii were not able to stimulate in-vitro cytokine production by alveolar cells or peripheral blood cells.

In **Chapter four** we studied pro-inflammatory cytokine profiles in the rat PCP model. Cytokine profiles were assessed at 2-weekly intervals. IL-1ß was elevated in lung homogenates and IL-6 in BAL fluid, whereas IL-1ß, IL-6 and TNF-α were not increased in plasma. In rats with PCP elevated pro-inflammatory cytokines concentrations were restricted to the lung compartments.
Corticosteroids did not significantly affect 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 start of the steroid injections, while the production in whole blood cultures was immediately and completely suppressed.

In **Chapter five** the question of cytokine profiles in HIVneg patients with PCP was addressed. TNF-α was detectable in BAL fluid during the acute phase of PCP in HIVneg

patients. At the same time, plasma concentrations of pro-inflammatory cytokines were low, and of anti-inflammatory mediators were high. Thus, in HIVneg patients with PCP, concentration of the pro-inflammatory cytokine TNF-α is increased in BAL, while anti-inflammatory mediators dominate in blood. Ex-vivo production of pro-inflammatory cytokines was suppressed in blood as well as in alveolar cells during the acute phase of PCP, while production of IL-1RA was suppressed in alveolar cells but preserved in blood. Correlations between inflammatory mediators and disease activity were not found.

In **Chapter six** investigations were presented on cytokine profiles in HIVpos patients with PCP. We showed that IL-1ß was increased in BAL of HIVpos patients with PCP as compared to healthy controls. In plasma of HIVpos patients, higher concentrations of IL-1RA were found during acute PCP compared to levels after recovery. Ex-vivo production of pro-inflammatory cytokines was suppressed in whole blood cultures during PCP, whereas IL-1RA production by alveolar cells as well as in whole blood cultures was increased. Corticosteroids did not seem to affect cytokine concentrations in BAL or blood, nor did they seem to suppress production in alveolar cells. In whole blood cultures however, the LPS-stimulated production was significantly suppressed for IL-1ß and for IL-6. Correlations between inflammatory mediators and disease activity were not found.
We concluded that in PCP pro-inflammatory cytokines are predominantly present in BAL, whereas anti-inflammatory mediators are more prominent in blood.

In **Chapter seven** we examined effects of local administration of TNF-α and anti-TNF combined with cotrimoxazole in experimental Pneumocystis carinii pneumonia. Intratracheal TNF-α in the first weeks after infection in addition to suboptimal cotrimoxazole was not synergistic in eradication of Pneumocystis carinii. Treatment with intratracheal anti-TNF in the 5th and 6th week after infection combined with standard dose cotrimoxazole did not reduce inflammation.

In **Chapter eight** we summarized the common features of cytokine profiles in the different models and conclude that in PCP a predominance of pro-inflammatory cytokines is seen at the site of infection, while anti-inflammatory cytokines are more prominent in

the circulation. We hypothesized that the high concentrations of anti-inflammatory cytokines in the circulation ensure that the effect of the pro-inflammatory cytokines remains limited to the site of infection, thereby protecting the host from deleterious systemic effects of pro-inflammatory cytokines. The influence of steroids and HIV infection on the observed cytokine profiles, the absence of correlation between disease activity and cytokine patterns and the failure to influence disease activity by local TNF modulation were discussed.

SAMENVATTING

Pneumocystis carinii pneumonie (PCP) is een vaak voorkomende en ernstige opportunistische infectie in HIV-seropositieve (HIVpos) patienten. Ook HIV-seronegatieve (HIVneg) patienten, met name gebruikers van corticosteroiden, kunnen een ernstige PCP ontwikkelen. Aan de cytokinen interleukine-1ß (IL-1ß), interleukine-6 (IL-6) en tumor necrosis factor-α (TNF-α) wordt een belangrijke rol toegedacht in de pathogenese van bacteriële en parasitaire infecties. De precieze rol van deze cytokinen in de pathofysiologie en de symptomatologie van PCP is echter niet duidelijk.

Andere onderzoekers lieten zien dat Pneumocystis carinii (P.carinii) in staat is om de in-vitro productie van TNF-α door alveolaire macrophagen te stimuleren. TNF-α zou in staat zijn om P.carinii direct te doden. Chen en co-werkers toonden in een muizen PCP model aan dat TNF-α, IL-1ß en IL-6 vroeg in de infectie nodig waren om P.carinii uit te roeien. In meerdere proefdier- en patientenstudies werd aannemelijk gemaakt dat alveolaire macrophagen in PCP geactiveerd zijn en in staat zijn tot een verhoogde productie van TNF-α.

Daar cytokinen voornamelijk lokaal werkzaam zijn, is het te verwachten dat de cytokine patronen op de plaats van de ontsteking (in geval van PCP, in de long) verschillen van die in de circulatie. Men zou tevens kunnen veronderstellen dat in de long, binnen de verschillende compartimenten (alveolaire of interstitiele ruimten) het cytokine patroon niet hetzelfde is. Immers fenotype en functie van macrofagen, hoofdproducenten van TNF-α en IL-1ß, worden beinvloed door het micromilieu.

Vrijwel alle mensen en dieren ademen geregeld P.carinii organismen in. Een pneumonie ontstaat echter alleen als er sprake is van een immuunstoornis. Immuunstoornissen op zich hebben óók invloed op cytokine patronen. HIV infectie is geassocieerd met toegenomen pro-inflammatoire cytokine productie, terwijl corticosteroiden over het algemeen een pro-inflammatoire cytokine productie onderdrukken. Bij het interpreteren van cytokine patronen in PCP moet hiermee rekening gehouden worden. Wat wordt veroorzaakt door de PCP en wat door de onderliggende immuunstoornis?

STUDIE OPZET

Het doel van ons onderzoek was om meer inzicht te krijgen in de rol van cytokinen in PCP. De vraag of cytokine concentraties en productie in de verschillende compartimenten (long en bloed) van elkaar verschilden, stond daarbij centraal. Wij onderzochten daartoe in-vivo concentraties en ex-vivo productie van een aantal cytokinen in verschillende compartimenten in dieren en patienten met verschillende onderliggende immuunstoornissen.

In een ratten PCP model, waarbij PCP werd geinduceerd door corticosteroiden, werden immunoreactief IL-1ß en bioactief TNF-α en IL-6 bepaald. De cytokine concentraties werden gemeten in plasma, longspoelsel (broncho-alveolaire lavage, BAL) en longhomogenaten.

Twee groepen patienten met PCP werden onderzocht, HIV-seronegatieve patienten, die prednison gebruikten (voornamelijk niertransplantatie patienten) en 23 HIV-seropositieve patienten. De onderzochte cytokinen in patienten waren IL-1ß, TNF-α, IL-6, en de remmers van IL-1ß en TNF-α, respectievelijk IL-1 receptor antagonist (IL-1RA) en de oplosbare TNF receptoren (sTNF-R). Cytokine concentraties werden bepaald in plasma en in longspoelsel.

Daarnaast werd zowel in patienten als in ratten de ex-vivo productie door alveolaire cellen en in volbloedkweken gemeten. De ex-vivo productie is een maat voor het vermogen van cellen om cytokinen te produceren.

In het rattenmodel werd ook de kinetiek van de cytokinen bestudeerd. Meer inzicht in de rol van de onderliggende immuunstoornis (HIV infectie, corticosteroid gebruik) werd verkregen door vergelijking van cytokine patronen in de verschillende modellen, met verschillende controlegroepen en in een beperkt aantal patienten vlak voor en vlak na toediening van corticosteroiden.

Tenslotte werden enkele interventie-experimenten verricht met intratracheaal toegediend TNF-α en een polyklonaal anti-TNF antiserum in de hoop door lokale cytokine modulatie het beloop van de infectie te beinvloeden.

In **hoofdstuk 1**, de introductie, beschrijven we wat er tot nu toe bekend is over de rol van de door ons bestudeerde cytokinen in PCP. We leggen uit waarom we denken dat

onderzoek in verschillende compartimenten belangrijk is en beschrijven de studie opzet.

In **hoofdstuk 2** wordt het ratten model beschreven. We zijn erin geslaagd om in ratten een consistente en reproduceerbare PCP te induceren, door behandeling met corticosteroiden, een eiwitbeperkt dieet en nauw contact met door P.carinii geinfecteerde ratten. Het verloop van de PCP werd gevolgd door op vaste tijdstippen de ernst van de infectie en mate van gastheerafweer te bepalen. De ernst van de infectie werd bepaald door telling van het aantal P.carinii cysten in Giemsa gekleurde deppreparaten van een doorgesneden longkwab (P.carinii score). De mate van gastheerweerstand werd gekwantificeerd door een histologische ontstekingsscore van paraffine coupes van de long, de ratio long/lichaamsgewicht (L/B ratio), ^{111}Indium-IgG (^{111}In-IgG) biodistributie long/spier ratio, en het aantal en de differentiatie van ontstekings cellen in het longspoelsel. Al deze parameters correleerden met elkaar. ^{111}In-IgG long/spier (L/M) ratio bleek een goede parameter voor de mate van lokale ontstekingsreactie, de infectie werd vroeg gesignaleerd en was betrouwbaar te kwantificeren. In de navolgende experimenten kozen wij daarom voor P.carinii score, L/B ratio en L/M ratio als parameters om de ernst en het verloop van de PCP te volgen.

In **hoofdstuk 3** wordt de vraag behandeld of Pneumocysten in staat zijn om in-vitro cytokinen te stimuleren. In onze experimenten bleek dat, in tegenstelling tot wat anderen vonden, niet het geval. Verschillen in cel populaties (ongeselecteerde alveolaire cellen in ons systeem ten op zichte van adherente cellen in de andere studies), in kweekcondities of P.carinii preparaties verklaren mogelijk de verschillen in de uitkomsten.

In **hoofdstuk 4** beschrijven wij de cytokine patronen in het ratten PCP model.
In Sprague-Dawley ratten met door corticosteroiden geinduceerde PCP, werden verhoogde waarden gevonden voor IL-1ß in longhomogenaten en voor IL-6 in BAL vloeistof, terwijl de pro-inflammatoire cytokine concentraties in het bloed niet verhoogd waren. Er was ter plaatse van de ontsteking, in de long, dus sprake van een pro-inflammatoire cytokine status. Door vergelijking van cytokine profielen in met corticosteroiden behandelde ratten met en zonder PCP, zagen wij dat corticosteroiden nauwelijks effect hadden

op de in vivo gemeten cytokine concentraties (zowel lokaal als systemisch). Corticosteroiden remden echter wel de ex-vivo cytokine productie, een maat voor het vermogen van cellen om cytokinen te produceren. Er was daarbij een duidelijk verschil tussen de lokale en de systemische cytokine productie. De productie door alveolaire cellen werd geleidelijk in de loop van 6 weken geremd, terwijl de systemische productie (in bloedkweken) vrijwel meteen volledig werd geblokkeerd.

In **hoofdstuk 5** beschrijven wij de cytokine profielen in de HIVneg PCP patienten. Bij hen werd in de acute fase van PCP een verhoogde concentratie van TNF-α in BAL gevonden. Tegelijkertijd werd in de circulatie een verhoogde concentratie gemeten van anti-inflammatoire mediatoren. Na herstel van de PCP daalden de anti-inflammatoire cytokine concentraties in het bloed.
Tijdens de acute fase van PCP bleek de ex-vivo productie van pro-inflammatoire cytokinen (in bloed en alveolair celkweken) en die van IL-1RA door alveolaire cellen onderdrukt, terwijl de ex-vivo productie van IL-1RA in het bloed behouden bleef. Correlaties tussen inflammatoire mediatoren en ziekte-activiteit kon niet worden aangetoond. Ook in dit model zagen wij dus een pro-inflammatoire cytokine status in de long tijdens de acute fase van PCP en dit ging gepaard met een duidelijke dominantie van de anti-inflammatoire mediatoren in de circulatie.

De cytokine profielen in de HIVpos patienten met PCP worden beschreven in **hoofdstuk 6**. Bij deze patienten vonden wij verhoogde concentraties van IL-1ß in BAL en verhoogde concentraties IL-1RA in de circulatie tijdens de acute fase van PCP. Tezelfdertijd was de ex vivo productie van pro-inflammatoire cytokinen onderdrukt in bloedkweken, terwijl de IL-1RA productie juist was verhoogd (zowel in alveolair cellen als in bloedkweken). Cytokine concentraties in BAL en bloed leken, net als in het rattenmodel, niet te worden beinvloed door gebruik van corticosteroiden. De LPS-gestimuleerde ex vivo productie van Il-1 en IL-6 in bloedkweken werd, conform de bevindingen in het ratten model, echter wel duidelijk onderdrukt door het gebruik van corticosteroiden. Ook in de HIVpos patienten werden geen correlaties gevonden tussen cytokine concentraties en ziekte-activiteit.
Onze eindconclusie was dat in PCP bij HIVpos patienten pro-inflammatoire cytokine

overheersen in de BAL, terwijl de anti-inflammatoire mediatoren overheersen in de circulatie.

In **hoofdstuk 7** onderzochten wij de effecten van lokaal toegediend TNF-α en anti-TNF in combinatie met cotrimoxazol in het ratten PCP model. Intratracheaal TNF-α toegediend in het begin van de infectie bleek samen met een suboptimale dosis cotrimoxazol niet alle P.carinii cysten te kunnen uitroeien. En intratracheaal toegediend anti-TNF in combinatie met hoge doses cotrimoxazol later in de infectie verminderde de inflammatoire gastheerreacties niet.

In **hoofdstuk 8**, de algemene discussie en conclusie, vatten wij de gemeenschappelijke kenmerken van de cytokine profielen in de verschillende modellen samen. Wij concluderen dat in PCP een pro-inflammatoire cytokine overwicht wordt gezien in de long, terwijl op dat moment in de circulatie de anti-inflammatoire mediatoren domineren. Het lijkt er dus op dat de hoge concentratie anti-inflammatoire cytokinen in de circulatie de actie van de pro-inflammatoire cytokinen beperkt tot de plaats waar die actie nodig is, de plaats van infectie. De gastheer zou zo beschermd worden tegen de schadelijke effecten van de pro-inflammatoire cytokinen die, wanneer ze in de circulatie in hoge concentratie aanwezig zijn, ernstige schade (endotheel schade, shock, mortaliteit) kunnen veroorzaken. Ter plekke van de ontsteking lijken de pro-inflammatoire cytokinen, zeker in het begin van de infectie, juist nodig om de afweersystemen van de gastheer te activeren ten einde de P.carinii te bestrijden. Ook ter plekke van de ontsteking moet waarschijnlijk in het beloop van de ziekte, de balans pro- versus anti-inflammatoire cytokinen weer worden hersteld om te voorkomen dat de lokale ontstekingsreactie, opgeroepen en onderhouden door pro-inflammatoire cytokinen, tè veel schade aanricht (oedeem, fibrose). De kinetiek van verandering en herstel van die balans pro/anti-inflammatoire cytokinen moet, ter bescherming van de gastheer, lokaal anders verlopen dan in de circulatie. Gegevens over cytokine patronen in andere infecties (meningococceninfecties, buiktyfus), bestudeerd door onze groep wijzen in dezelfde richting en steunen deze hypothese.
De invloed van corticosteroiden en HIV infectie op de waargenomen cytokine profielen, het ontbreken van correlaties tussen ziekte-activiteit en cytokine patronen en het negatieve

resultaat van de interventie-experimenten wordt besproken.

DANKWOORD

Solowerk was dit onderzoek beslist niet. Velen hebben een bijdrage geleverd. Enkelen wil ik met name noemen. Allereerst mijn co-promotoren Dr.R.W.Sauerwein en Dr.P.Beckers. Robert en Pieter, tijdens de vele besprekingen die we met elkaar hadden, hebben jullie mij geleerd wat nodig was om dit onderzoek uit te voeren, door te zetten en af te ronden. Jullie maakten altijd wel tijd voor me vrij en het was nog gezellig ook. Ik kon weleens wanhopig worden als ik mijn stukken voorzien van commentaar van je terug kreeg Robert, maar het uiteindelijk resultaat was wel oneindig veel beter. Dan mijn promotor Prof. Dr. J.W.M. van der Meer. Jos, je gaf me de gelegenheid om, na vele jaren intensief klinisch werk, mijn tanden nu eens stuk te bijten op basaal onderzoek. Het is me niet meegevallen, maar ik heb er veel van geleerd. Ik bewonderde het tempo waarin je mij, ondanks je overvolle agenda, de stukken gecorrigeerd en soms bijna herschreven terug gaf. Mijn analiste J.C.H.W. van Schijndel. Anita, zonder jou was het natuurlijk niets geworden. Met eindeloos geduld en grote precisie heb je alle bepalingen uitgevoerd, je raakte nooit verstoord als ik op het laatste moment nog wat wilde veranderen of toevoegen. Werken in het weekend, mij vervangen op het dierenlab, mee naar Ann Arbor, het was nooit een probleem. Leo, dank voor je hulp bij het opzetten en operationeel houden van het computerprogramma. I am grateful to Dr. Robert Strieter and Marie Burdick from University Michigan Medical Centre, Division of Pulmonary and Critical Care, Ann Arbor, USA, for the stimulating weeks I spent at their lab.

Mijn dank gaat ook uit naar Tita, Theo, Eric, Truus en Wilma van lab parasitologie, naar Johanna en Ineke van lab interne en naar Emile Koenders van nucleaire geneeskunde voor de verschillende klussen die zij voor me klaarden. Gerry Grutters van het Centraal Dierenlaboratorium was fenomenaal en daarom onmisbaar bij de vele dierexperimenten. Henny, Geert, Leo en Ivette, mijn dank voor jullie goede en trouwe verzorging van de dieren en jullie hulp bij het 'verwerken' van de beestjes. Dr. W.J.G. Oyen. Wim, de 'nucleaire' stukken waren er zonder jouw hulp nooit gekomen, met name je statistische hulp was onmisbaar. De collega's van de andere AIDS centra (AMC, AZU, Rijnstate) ben ik zeer erkentelijk voor hun medewerking, zonder hun hulp had ik nooit voldoende HIV-positieve patienten met PCP in mijn studie kunnen opnemen. Wil, Rob, Annet en

Ton, kamergenoten en mede-proefschriftzwoegers, dank voor alle hulp bij computer en lab problemen, voor de gezelligheid en vriendschap. Grote dank gaat uit naar de patienten en de studenten-vrijwilligers die aan mijn studies meededen. Last but not least, het thuisfront. Pa, Ma, Don, An, Jack, Marietje, Frank, Meeuw, Jim, Sil, Yuur, Brian the lyon, Omi, Roweentje, Mereltje, Olieveke, Jana, Sierk en natuurlijk Frits, geweldig dat jullie er waren, wanneer ik jullie nodig had.

CURRICULUM VITAE

Na het behalen van het eindexamen gymnasium ß aan het Carolus Borromeus college in Helmond, studeerde Roos Perenboom geneeskunde aan de Katholieke Universiteit Nijmegen. Tijdens haar studie werkte zij in het kader van een wetenschappelijke stage een half jaar aan de Yale University, New Haven, Connecticut, USA. In de laatste fase van haar studie werkte zij als student-doctor, co-assistent 4 maanden in Biharamulo Hospital, Tanzania. In 1976 deed zij met succes artsexamen en volgde zij de NTA (Ned. tropencursus voor artsen). Ter voorbereiding op haar werk als tropenarts, werkte zij 6 maanden als assistent interne in het St.Lambertus ziekenhuis in Helmond en 18 maanden als assistent chirurgie en gynecologie/verloskunde in het Elisabeth Gasthuis in Arnhem. Van 1978 tot 1982 werkte zij als tropenarts in het Bukumbi Hospital in Tanzania. Na terugkomst in Nederland specialiseerde zij zich van 1982-1987 tot internist in het Academisch Ziekenhuis Nijmegen (AZN, Hoofd: Prof. dr. A van 't Laar). Van 1987 tot 1989 werkte zij in het Academisch Medisch Centrum, Amsterdam, op de AIDS afdeling en op de afdeling Tropische Geneeskunde, waarna zij van 1989 tot eind 1991 als universitair docent en internist verbonden was aan het Muhimbili Medical Center in Dar Es Salaam, Tanzania. Van 1992 tot 1996 werkte zij als internist-onderzoeker op de afdeling algemene interne geneeskunde van het AZN (hoofd: Prof. dr. JWM van der Meer). Het onderzoek in het AZN, onder leiding van Prof. dr. JWM van der Meer, resulteerde in dit proefschrift.
Zomer 1996 werkte zij als internist in het Rijnstate Ziekenhuis in Arnhem en vanaf 1 oktober 1996 is zij als internist verbonden aan de afdeling interne geneeskunde van het Academisch Ziekenhuis van de Vrije Universiteit, Amsterdam.

PUBLICATIONS

G.P. Verburg , C.I. Perre, R.M. Perenboom. De Ziekte van Mondor. Ned Tijdschr Geneesk 1989 ;41: 2035-2037

R.M. Perenboom, M.C.M. Rijk, G.H.J.M. Rikken, J.H.M. van Tongeren. Het ziektebeloop bij colitis ulcerosa. Ned Tijdschr Geneeskd 1990 ;134 :438-443

R.M. Perenboom, P. Reiss, S.A. Danner, J.W. van 't Wout. Zidovudinetherapie bij 141 patienten met symptomen van HIV infectie ;een multi-centra-onderzoek. Ned Tijdsch Geneeskd 1990;134 :120-125

F.W. Nielsen-Abbring, R.M. Perenboom, R.J.A.M van der Hulst. Quinine-Induced Hearing Loss. Otolaryngylogy 1990;52:65-68

R.M. Perenboom. Veilig op reis: de gezonde reiziger. Tijdschrift voor Huisarts Geneeskunde, 1994;11:265-273

R.J.J. Koopman, R.M. Perenboom. Ziek terug: Huidafwijkingen. Tijdschrift voor Huisarts Geneeskunde, 1994;11:651-654

Kitinya J.N., Richter C., Perenboom R.M., Chande H., Mtoni I.M. Influence of HIV status on pathological changes in tuberculous pleuritis. Tubercle and Lung Disease. 1994;75:195-198

C. Richter, R.M. Perenboom, I. Mtoni, J. Kitinya, H. Chande, A.B.M. Swai, R.R. Kazema, L.M. Chuwa. Clinical features of HIV positive and negative patients with tuberculous pleural effusion in Dar Es Salaam, Tanzania. Chest, 1994;106:1471-1475.

C. Richter, R.M. Perenboom, A.B.M. Swai, J.Kitinya, I.Mtoni, H.Chande, R.R. Kazema, L.M. Chuwa, D.H. Mwakyusa, S.Y. Maselle. Diagnosis of tuberculosis in patients with pleural effusion in an area of HIV-infection and limited diagnostic facilities.

Tropical and Geographical Medicine, 1994;46:293-298

C.Richter, M.J.W. Koelemay, A.B.M.Swai, R.M.Perenboom, D.H. Mwakyusa, J.Oosting. Predictive markers of survival in HIV-seropositive and HIV-seronegative Tanzanian patients with extra-pulmonary tuberculosis. Tubercle and Lung Disease, 1995;76:510-517

R.M. Perenboom, C. Richter, A.B.M. Swai, J. Kitinya, I. Mtoni, H. Chande, R.R. Kazema, L.M. Chuwa, D.H. Mwakyusa, S.Y. Maselle. Diagnosis of tuberculous lymphadenitis in an area of HIV-infection and limited diagnostic facilities. Tropical and Geographical Medicine, 1994;46:288-293

R.M. Perenboom, C. Richter, A.B.M. Swai, J. Kitinya, I. Mtoni, H. Chande, R.R. Kazema, L.M. Chuwa, D.H. Mwakyusa, S.Y. Maselle. Clinical features of HIV positive and HIV-negative patients with TB lymphadenitis in Dar es Salaam. Tubercle and Lung Disease, 1995;76:401-406.

R.M. Perenboom, W.J.G. Oyen, P. Beckers, A.C.H.W. van Schijndel, F.H.M. Corstens, J.W.M. van der Meer. Serial 111In-labelld IgG biodistribution in rat pneumocystis carinii pneumonia: a tool to monitor the course and severity of the infection. European Eur.J.of Nuclear Medicine, 1995;22:1129-1132.

R.M.Perenboom, A.C.H.W.van Schijndel, P.Beckers, R.Sauerwein, H.W.van Hamersvelt, J.Festen, H.Gallati, J.W.M. van der Meer. Cytokine profiles in BAL and Blood in HIV-seronegative patients with Pneumocystis carinii pneumonia. European Journal of Clinical Investigation, 1996;26:159-166.

Perenboom RM, Beckers P, van der Meer JWM, van Schijndel JCHW, Oyen WJG, Corstens FHM, Sauerwein RW. Pro-inflammatory cytokines in lung and blood during steroid-induced Pneumocystis carinii pneumonia in rats. J Leucocyte Biology, in press

Oyen W, Boerman OC, Storm GS, van Bloois L, Koenders EB, Claessens RAMJ, Perenboom RM, Crommelin DJA, van der Meer JWM, Corstens FHM. Tc-99m labeled stealth(r) liposomes to detect infection and inflammation. J. of Nucl Med, 1996;37:1392-1397.

Stellingen

1. In Pneumocystis carinii pneumonia, pro-inflammatory cytokines are more prominently present at the site of infection, whereas anti-inflammatory mediators dominate in the circulation.

This thesis

2. The beneficial effect of steroids on the clinical course of Pneumocystis carinii pneumonia is not due to suppression of pro-inflammatory cytokine production by alveolar cells.

This thesis

3. Both steroids and severe infection can induce downregulation of systemic pro-inflammatory cytokine production.

This thesis

4. Het interpreteren van cytokine profielen in Pneumocystis carinii pneumonie lijkt soms op het oplossen van een vergelijking met teveel onbekenden.

5. ^{111}Indium-IgG biodistributie is een goede, gemakkelijk te kwantificeren parameter voor de mate van lokale ontsteking in Pneumocystis carinii pneumonie.

Dit proefschrift

6. P.carinii onderzoek wordt sterk belemmerd door het feit dat wij Pneumocysten niet kunnen kweken en het transmissie stadium niet kennen.

7. Als je iets niet weet, dan geeft dat te denken.

8. De vibratie van de stilte reflecteert de essentie van de muziek.

9. Geld steken in wervingscampagnes ten behoeve van de eigen universiteit is een door de politiek afgedwongen oneigenlijk gebruik van onderwijsgelden.

10. Het door organisatiebureaus laten oplossen van precaire bestuursproblemen is vooral een modieuze manier van geld weggooien.

11. Een zorgzame overheid hoort niet luchtig te doen over luimige zaken.

12. Onze welvaart doet de wereld stinken.

13. Academic Departments of Tropical Medicine should be where they belong, in the tropics themselves.
The Lancet 1996;347;629

14. De kreet "one world, wanhoop" getuigt van meer werkelijkheidszin dan het motto "one world, one hope", het motief van de wereld AIDS conferentie in Vancouver.

2 december 1996 Roos Perenboom

Omslagontwerp en -illustraties : Marie-Louise Perenboom-Hooymans