Local antibodies against *Pneumocystis carinii* in bronchoalveolar lavage fluid

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**ABSTRACT:** *Pneumocystis carinii* pneumonia (PCP) is a frequent cause of pneumonia among human immunodeficiency virus (HIV)-infected patients. Little is known, however, about the role played by humoral immunity to control the infection. This study was undertaken to elucidate the role played by local antibodies.

Bronchoalveolar lavage (BAL) fluids from 18 acquired immune deficiency syndrome (AIDS) patients with PCP, 20 HIV-antibody positive patients without PCP, and 20 lung cancer patients were examined for antibodies against *P. carinii* by the indirect immunofluorescence method. The ratio of albumin concentration in BAL fluid to serum was used to standardize the lavage fluids.

Immunoglobulin G (IgG) antibodies against *P. carinii* occurred less frequently, and immunoglobulin M (IgM) antibodies more frequently, in PCP patients than in other groups. Immunoglobulin A (IgA) antibodies against pneumocysts were found with the same frequency in all three groups, although the median titre was lower among HIV-antibody positive patients without PCP, compared with the other groups. When indexed (antibody titre in BAL fluid × albumin concentration in serum/antibody titre in serum × albumin concentration in BAL fluid) to express locally produced antibodies, IgG indices were significantly higher in HIV-infected patients without PCP, whereas IgM and IgA indices were significantly higher in PCP patients.

These findings suggest that the local IgG response is impaired in patients with PCP, whereas the local IgA and, to some extent, the IgM responses are preserved.


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*Pneumocystis carinii* can cause severe pneumonia in immunocompromised patients, including those with acquired immune deficiency syndrome (AIDS) [1]. The main strategy used by the immune system to control this infection is still largely unknown; but cellular immunity is often considered to be of major importance, as the infection is found in patients with T-cell defects [1]. However, humoral immunity may also be an important part of the main line of defence against the parasite, as *P. carinii* pneumonia (PCP) is also found among patients with congenital hypogammaglobulinaemia [2, 3]. Furthermore, passive immunoprophylaxis with monoclonal antibodies has been shown to significantly reduce the number of *P. carinii* in lungs of immunosuppressed animals [4].

Antibodies against *P. carinii* have predominantly been investigated in serum, and, although the antibody level in the respiratory tract may be more important, the occurrence of *P. carinii* antibodies in the bronchopulmonary system is less extensively studied [5, 6].

In a previous study [7], we found low serum immunoglobulin G and M (IgG and IgM), but high immunoglobulin A (IgA)-antibody titres against *P. carinii* in AIDS patients compared with human immunodeficiency virus (HIV)-positive and HIV-negative controls. However, these results may not necessarily reflect the antibody levels in the lungs, which may be more important for the defence against PCP. Therefore, we have investigated the levels of antibodies against *P. carinii* in bronchoalveolar lavage (BAL) fluid from AIDS patients with PCP (Group I), HIV-antibody positive patients without PCP (Group II), and lung cancer patients (Group III).

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**Material and methods**

**Patients**

BAL fluid and serum from a total of 58 patients were examined for specific antibodies against *Pneumocystis carinii*. The patients comprised three groups:

**Group I.** This group comprised 18 male patients with AIDS and acute PCP, aged 23–53 yrs (mean 39 yrs). Diagnosis was based on direct demonstration of *P. carinii* in the BAL fluid examined in this study. No other micro-organism was isolated, and granulocytes formed 1% (median value) of cells in the lavage fluids (range...
between the groups. The amount of saline installed and recovered did not vary
bodies, Kaposi's sarcoma, and other malignancies. The
thermore, examined for the presence of viral inclusion
in order to reveal bacteria and fungi. Biopsies were, fur-
fluorescence assay, and stained with haematoxylin-eosin
ids were examined for
stain), and mycobacteria (Ziehl-Neelsen). All lavage flu-
were examined for the presence of
in vivo bound immunoglobulin on P. carinii found in the lavage fluid.

**Group II.** This group comprised 20 HIV-antibody posi-
tive male patients admitted with pulmonary symptoms, such as cough, dyspnoea and fever, because of which BAL was performed. The age varied from 18–62 yrs (mean 38 yrs). No micro-organisms (bacteria, virus, fungi or protozoa) could be demonstrated in the lavage fluids. Granulocytes formed 1% (median value) of cells in the lavage fluids (range 0–38%). Six patients had AIDS, the diagnosis of which was based on previous oesophageal candidiasis (1 patient), generalized mycobacteriosis (1 patient), and Kaposi's sarcoma (4 patients). The other 14 patients had either oral candidiasis or hairy leucoplaikia. None had previously suffered from PCP, or received prophylaxis against PCP. One patient developed PCP in a follow-up period of 6 months. CD4-cell counts varied between 0–300 cells·µl⁻¹.

**Group III.** This group comprised 15 male and 5 female patients with lung cancer. Their age varied between 38–77 yrs (mean 62 yrs). None of the patients were sus-
pected of having lung infection at the time of bronchoscopy and lavage. Granulocytes formed 3% (median value) of cells in the lavage fluids (range 0–74%). The HIV status was not known.

**Bronchoalveolar lavage**

Bronchoscopy was performed with the patient in general anaesthesia, using an Olympus BF IT 10 fibre optic bronchoscope as described previously [8]. After exam-
ination of the tracheobronchial tree, the tip of the fibrescope was wedged into a subsegmental bronchus of a radiogra-
phically abnormal region, or in the right middle lobe, if the chest X-ray or computerized tomography (CT)-scan revealed diffuse infiltrates, or if no infiltrates were seen. In lung cancer patients, lavage was limited to the lung that was not involved by the malignancy. Five to six aliquots of 20 ml, 0.9% sterile NaCl, were instilled and by gentle suction recovered into sterile vials; thereafter, biopsies with touch imprints were taken. All lavage flu-
ids from HIV-infected patients were cultured for bacte-
ria, mycobacteria, viruses and fungi. Imprints and biopsies were stained for P. carinii (Giemsas and Gomori silver stain), and mycobacteria (Ziehl-Neelsen). All lavage flu-
ids were examined for Legionella spp. by a direct immuno-
fluorescence assay, and stained with haematoxylin-eosin in order to reveal bacteria and fungi. Biopsies were, fur-
thermore, examined for the presence of viral inclusion bodies, Kaposi's sarcoma, and other malignancies. The amount of saline installed and recovered did not vary between the groups.

BAL fluids were centrifuged immediately after the bron-
choscopy, and the supernatants were stored at -70°C until use. A serum sample was obtained simultaneously and stored at -70°C.

**Protein determination**

Albumin concentrations of unconcentrated lavage flu-
ids were determined by nephelometry at the Department of Clinical Chemistry, Rigshospitalet, Copenhagen. BAL fluids were then concentrated to about 5% of the original volume by means of centrifugal microconcentrators (Amicon®) containing filters with a cut-off level of 30,000 kD. The centrifugation was carried out at 5,000×G at 2°C, and the fluids were stored at -70°C until use. The albumin concentration in serum and concentrated BAL fluids were measured by rocket immunoel-
rophoresis as described previously [9]. All lavage fluids were adjusted with phosphate-buffered saline (PBS) to the same ratio of albumin concentration in BAL, to albumin concentration in serum (0,0158).

**Antigen**

Cysts of P. carinii were prepared, essentially as described by Walzer et al. [10]. Wistar male rats, weighing about
250 g, were immunosuppressed by adding dexametha-
sone 1 mg·l⁻¹, and tetracycline 1 mg·ml⁻¹ to the drinking water. For the first two weeks, normal rat chow was offered, and later this was substituted with a low protein diet. Signs of pneumonia usually developed after 6–8 weeks, and the rats were then sacrificed and the lungs aseptically removed. Imprints from each lung were stained by Giemsas and methenamine silver. Lungs with bacterial and fungal infections were discarded, and only those with moderate to severe P. carinii infection were used for cyst preparation. Lung tissue was cut into small pieces and squeezed through a nylon mesh into a PBS solution containing 10 mM of sodium citrate. After cen-
trifugation in a Beckman J2-21 centrifuge at 11,000×G for 15 min, the pellet was digested with 0.2% collagenase (Sigma type 7) and 0.2% hyaluronidase (Sigma type 6) and dissolved in 50 mM TRIS-buffer, pH 7.1, containing 0.05 mM CaCl₂ and 0.05 mM MgCl₂, for 60 min, at 37°C. After enzyme treatment, the suspension was centrifuged at 11,000×G for 20 min and washed twice in PBS. The solution of cysts was purified by density gra-
dient centrifugation with Percoll (Pharmacia) for 30 min at 31,000×g [11]. Layers with densities from 1.018–1.062 g·ml⁻¹ were found to contain a high amount of cysts. After washing several times in PBS at 11,000×g for 15 min, the number of P. carinii cysts was counted microscopi-
cally, diluted with PBS, and stored, in aliquots of 1 ml containing 10⁶ P. carinii cysts·ml⁻¹ at -70°C, until use.

**Indirect immunofluorescence**

Five µl of the P. carinii cyst suspension, diluted 1:10 with PBS, containing approximately 10,000 cysts were applied to gelatine coated slides, air dried, and fixed in
99% methanol for 15 min. In order to remove bound immunoglobulin and protein covering antigenic sites, the cysts were trypsinized at 37°C for 30 min with 0.2% trypsin (Sigma type XI) in PBS, a procedure which has previously been used without loss of antigenicity [12]. Sera were diluted in PBS and investigated in doubling dilutions starting at 1:5. Lavage fluids were examined undiluted and in doubling dilutions. Serum was incubated at room temperature, whereas lavage fluid was incubated at 5°C in order to avoid any degradation of antibodies. Incubation of serum at 5°C or room temperature was shown to give the same P. carinii-antibody titre. After incubation with serum or lavage fluid, the slides were washed for 30 min in PBS and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human immunoglobulin or anti-secretory piece was added (Dakopatts, Copenhagen). The FITC-conjugated rabbit anti-γ, anti-α, and anti-µ with F/P ratios of 2.3 were used at a dilution of 1:80, 1:20 and 1:40, respectively. All antibodies were specific for heavy chains according to the manufacturer. FITC-conjugated rabbit anti-secretory piece had a FITC to protein (F/P) ratio of 0.66 and was used at a dilution of 1:10. The optimal dilutions of conjugate were found by chessboard titrations. After washing for 30 min, the slides were mounted with 70% glycerol in PBS and examined under the fluorescence microscope using an epiluminiscence and a 100 W HBO mercury bulb. Preparations with fluorescence around the entire rim of the P. carinii were considered positive. A positive serum and a negative PBS control were included in all experiments. When secretory piece, the protein protecting IgA in secretions from proteolysis, was looked for, a positive lavage fluid was used. All slides were read blind.

The results were expressed both as adjusted titres and as antibody titre indices. All BAL fluids were concentrated and adjusted to the same ratio of albumin concentration in serum to albumin concentration in BAL, and antibody titres in concentrated BAL fluids are referred to as adjusted titres. Antibody index was calculated as titre in BAL × albumin concentration in serum/serum titres × albumin concentration in BAL. Samples with a corresponding serum titre of 0 were omitted from this calculation.

Direct immunofluorescence

BAL fluids with P. carinii were centrifuged for 15 min at 10,000 × g, and sediments were fixed in 99% methanol and incubated with FITC-conjugated mouse anti-P. carinii monoclonal antibodies, generously supplied by Genetic System Incorp.© and rhodamine-conjugated rabbit anti-γ, anti-α, or anti-µ antibodies (F/P ratio 0.40) (Dakopatts Copenhagen). Incubation was performed at 37°C for 30 min. All antibodies were specific for heavy chains according to the manufacturer. After incubation, slides were washed in PBS, mounted with 70% glycerol in PBS and examined under the fluorescence microscope using an excitation filter at 490–515 nm for FITC conjugates and an excitation filter of 545–590 nm for rhodamine-labelled antibodies.

Statistical methods

Albumin concentration of unconcentrated BAL and serum and the ratios of these concentrations in the three groups of patients were compared using the Kruskal-Wallis one-way analysis of variance. Frequencies of low titres and indices in the different groups were examined with a chi-squared test. The correlation between IgA and secretory IgA was examined using regression analysis.

Results

Albumin content

The albumin content of unconcentrated lavage fluid and serum differed among the three groups of patients. Among PCP patients (Group I), albumin concentration in BAL fluid ranged between 18.6–185.6 mg·l⁻¹ with a mean value of 56.6 mg·l⁻¹; and in serum the concentration range was 15.3–34.6 g·l⁻¹ with a mean value of 25.2 g·l⁻¹. In HIV-antibody positive patients without PCP (Group II), the albumin concentration in BAL fluid ranged between 32.4–305.7 mg·l⁻¹ with a mean value of 56.6 mg·l⁻¹; and in serum the concentration range was 22.4–38.8 g·l⁻¹ with a mean value of 32.6 g·l⁻¹. In HIV-antibody positive patients without PCP (Group II), the albumin concentration in BAL fluid ranged between 70.3–213.2 mg·l⁻¹ with a mean value of 112.8 mg·l⁻¹; and the concentration of serum albumin ranged between 25.3–54.8 g·l⁻¹ with a mean value of 41.8 g·l⁻¹. The ratio of albumin concentration in serum to albumin concentration in BAL fluid did not differ significantly between the groups (p>0.10, Kruskal Wallis one way analysis of variance).

Immunoglobulin titres

BAL fluids from all patients were concentrated and adjusted to give the same ratio of albumin concentration in BAL fluid to albumin concentration in serum. Antibody titres found in BAL fluid treated in this way are referred to as adjusted antibody titres. Figure 1 shows the adjusted antibody titres against P. carinii in BAL fluid. In patients with PCP (Group I) the median IgG-antibody titre was 0, whereas both HIV-antibody positive patients without PCP (Group II) and lung cancer patients (Group III), but 1 among HIV-infected patients without PCP (65%), and in 13 of 20 lung cancer patients (65%). This difference was on the borderline of statistical significance (p<0.10, chi-squared test).

The median IgA-titre was 2 in PCP patients (Group I) and lung cancer patients (Group III), but 1 among HIV-antibody positive patients without PCP (Group II) (fig. 1b). IgA-antibodies against P. carinii occurred in BAL fluid in 12 of 18 patients with PCP (65%), in 13 of 20 HIV-infected patients without PCP (65%), and in 13 of...
20 lung cancer patients (65%) (ns). Secretory piece median titre was 1 in PCP patients (Group I), and 0 in the other groups (fig. 1c). Secretory piece was detected in BAL in 11 of 17 PCP patients (65%), in 7 of 20 HIV-antibody positive controls (35%), and in 7 of 20 lung cancer patients (35%) (ns).

In order to investigate the relationship between IgA and secretory piece, three samples of BAL fluid were absorbed with anti-IgA. After absorption with anti-IgA, neither IgA-antibodies nor secretory-piece could be detected in any of the BAL samples, indicating that anti-IgA had removed both specific IgA-antibodies and secretory piece. The correlation between IgA-antibody titres and secretory piece titres in BAL fluids from the three groups of patients are shown in figure 2. In PCP patients (Group I) and in HIV-infected patients without PCP (Group II) a correlation between IgA-antibodies and secretory piece was found (PCP patients, p<0.05; HIV-infected patients without PCP, p<0.001; analysis of correlation). In 14 lung cancer patients (Group III) with IgA-antibody titres between 1 and 8, secretory piece could only be demonstrated in seven patients; whereas, seven other patients had undetectable levels of secretory piece in BAL fluid. One patient with no IgA-P. carinii-antibody in bronchial secretions had secretory piece at the titre of 1. No statistically significant correlation between IgA-P. carinii-antibodies and secretory piece could be found in lung cancer patients.

The median titre of IgM-antibodies was between 0 and 1 among PCP patients (Group I) and 0 in the other two groups (fig. 1d). Among PCP patients, 9 of 17 (53%) had titres between 1 and 8, whereas only 3 of 19 (16%) and 1 of 20 patients (5%) in the groups of HIV-antibody positive patients without PCP and lung cancer patients, respectively, had titres above 0. This difference was statistically significant. (p<0.05, chi-squared test).

**Antibody index**

In order to estimate the amount of locally produced antibodies, an antibody index was used. This method has previously been used by others to assess locally produced antibodies in the central nervous system (CNS) in patients with Herpes simplex encephalitis [13]. Low indices were defined as values which included the majority of lung cancer patients, who were used as controls in this study. For IgG-antibodies, an index above 1 was found in 3 of 15 PCP patients (Group I) (20%) and in 5 of 19 lung cancer patients (Group III) (21%), whereas 12 of 19 HIV-antibody positive patients without PCP (Group II) (63%) had an index above this level (fig. 3). This difference was statistically significant (p<0.025, Chi-squared test). As for IgA-antibodies, both groups of HIV-infected patients had higher indices than lung cancer patients (Group III),
none of whom had an index above 15. Seven of 14 PCP patients (Group I) (50%) and 5 of 16 HIV-infected patients without PCP, and in 1 of 20 patients (5%) of lung cancer patients. This difference was statistically significant (p<0.001, Chi-squared test).

Pneumocytes isolated from four PCP patients, identified with FITC-labelled monoclonal antibodies directed against P. carinii trophozoites and cysts, were examined for the presence of IgG, IgA and IgM immunoglobulin on cyst surface with rhodamine-labelled antibodies directed against the three classes of immunoglobulin. IgA was

Fig. 2. – The correlation between IgA-antibody titres to P. carinii and secretory piece in BAL fluid. Titres were adjusted to the same ratio of albumin concentration in BAL fluid to albumin concentration in serum. Correlations in: a) Group I - AIDS patients with P. carinii; b) Group II - HIV-antibody positive patients without P. carinii pneumonia; and c) Group III - lung cancer patients, are shown. For abbreviations see legend to figure 1.

Fig. 3. – Antibody titre to Pneumocystis carinii in BAL fluid adjusted to the same ratio of albumin concentration in the lavage fluid to albumin concentration in serum divided by the corresponding serum titre. Titres from: Group I - AIDS patients with P. carinii pneumonia; Group II - HIV-antibody positive patients with other lung infections; and Group III lung cancer patients, are shown. a) IgG antibody index; b) IgA antibody index; c) IgM antibody index. For abbreviations see legend to figure 1.
found to cover pneumocysts in some fields, but was also found without any relationship to the parasite in other fields. A small amount of IgG could be found on pneumocysts, and some IgG was also seen without any relationship to parasites identified by FITC-labelled antibodies. IgM could not be found on the pneumocysts.

Discussion

Antibodies against *P. carinii* were found in BAL from PCP patients, HIV-antibody positive controls and lung cancer patients. To make a comparison meaningful, all fluids were standardized to the same degree of dilution, using the ratio of albumin concentration in BAL fluid to albumin concentration in serum. The albumin concentration has been used by others to standardize lavage fluids [14]. The ratio of albumin concentration of serum to albumin concentration of undiluted lavage fluids did not differ significantly between the groups, and an equal amount of saline was instilled and recovered in the three groups. Thus, the leakage of protein including immunoglobulins across the alveolar membrane seemed to be the same in the three groups. Others [15] have found more albumin in lavage fluid from PCP patients than from healthy controls. The reason for this discrepancy is not clear; but the duration of illness might be of importance, as this could result in greater damage to the alveolar membrane, and thus give rise to a higher albumin content of the lavage fluid. In addition, the serum albumin level might influence the concentration of albumin in BAL fluid. CHLEBOWSKI et al. [16] found a mean serum albumin concentration among AIDS patients of 29 g·l⁻¹, whilst HUANG et al. [17] found a mean serum albumin concentration of 39.5 g·l⁻¹. Thus, differences in serum albumin concentration might contribute to different reports on albumin content of BAL fluid. Our controls all had lung tumours, and a high percentage of these patients are known to be heavy smokers with some chronic inflammation of the bronchi as reflected in the high percentage of granulocytes seen in a few of these patients and a level of BAL albumin comparable with that of PCP patients. This may explain why the median percentage of granulocytes was actually lower in PCP patients, in contrast to results reported by others [15]. However, some of the PCP patients had high levels of granulocytes (33%). Antibodies have previously been suggested to be of importance in immunity against *P. carinii*, as infection with this microorganism occurs in patients with hypogammaglobulinaemia [3]. However, in a previous study [7] the serum titres of IgG-antibodies against *P. carinii* were only slightly lower among AIDS patients with PCP than in normal controls, whereas the serum IgA-antibody titres were elevated in these patients compared with controls. On the other hand, the antibody level in bronchial secretions might be more relevant in the defence against *P. carinii*. In order to assess the amount of locally produced antibodies against *P. carinii*, an antibody index was calculated. This procedure is analogous with the one used as a diagnostic measure in patients suspected of *Herpes simplex* encephalitis, where albumin concentrations in serum and cerebrospinal fluid (CSF) is measured to control for leakage across the blood-CSF barrier [13].

In this study, we have found that the IgG-antibody level expressed both as adjusted titres and as antibody index was lowest in BAL fluids from PCP patients. Thus, although the IgG-antibody titres in serum did not differ significantly, it cannot be precluded that IgG-antibodies in BAL fluid have a protective function against *P. carinii*. Adjusted IgA-antibody titres were in the same range in all groups of patients, but a correlation with the level of secretory piece in BAL fluids was found only in HIV-positive patients, both with and without PCP. This may suggest that the IgA-antibodies found in these patients were produced locally by the bronchoalveolar-lymphoid system. This is also supported by the high IgA-antibody index found in these patients. The high level of IgA-antibody indices against *P. carinii* in BAL fluids from HIV-positive patients, both with and without PCP, may suggest that the local IgA-antibody response is normal or almost normal in these patients, and HIV-antibody positive patients without detectable parasites seem to have recently been infected with *P. carinii*. The higher IgA indices among PCP patients are probably the result of a more intense stimulation of the immune system. Contrary to this, the IgA-antibody indices in lung cancer patients were low, and the IgA-antibody detected in BAL fluids was not correlated to secretory piece. None of these patients had PCP and the results suggest that secretory IgA-antibodies have no, or only limited, protective effect against *P. carinii*, whereas IgG-antibodies in BAL fluid may be of importance.

The low level of specific IgG-antibodies in BAL fluid from PCP patients is probably not caused by consumption by parasites, as only scarce amounts of IgG were found on parasites isolated from BAL fluids. These findings are in accordance with the result of one study [18], but not with others [19, 20]. However, it may be suggested that the production of specific IgG-antibodies is decreased in HIV-positive patients with PCP. IgG antibody indices were higher among HIV-infected patients without PCP than the other two groups, indicating that these patients had been exposed to the parasites and had been able to produce antibodies against the parasites contrary to PCP patients. Frequent contact with persons clinically or subclinically infected with pneumocysts might explain this. Cross-reactivity with other microorganisms or polyclonal activation of B-cells, which is known to occur in HIV-infected patients, cannot be ruled out.

IgM-antibodies against *P. carinii* in BAL fluid were found at higher levels in PCP patients than in other groups, suggesting that antibodies of this class have little or no protective effect against *P. carinii*. RANKIN et al. [6] examined lavage fluids from two PCP patients and found increased IgG-antibody level in one patient and increased IgM-antibody level in the other, and they suggested that local antibody production against this micro-organism may occur. In accordance with our results, BLUMENFELD et al. [5] detected *P. carinii* antibodies of the IgG, IgA, and IgM classes in AIDS patients with PCP, in HIV-positive patients without PCP, and in...
HIV-negative controls; however, no attempt to measure antibody titres was made, and it is difficult to compare the results of the two studies.

In conclusion, we have found impaired local production of IgG antibodies in the lungs of PCP patients, while the IgA and, to some extent, the IgM response seems to be preserved in these patients. Secretory piece was found to be significantly correlated to IgA in HIV-infected patients, but not in lung cancer patients. We suggest that IgG, but not IgA, may give protection against infection with *P. carinii* in the bronchopulmonary system. The mechanisms by which Ig-antibodies work are not elucidated.

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**References**


