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Eosinophilic alveolitis in BAL after lung transplantation

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Abstract Lung transplantation has become a therapeutic option for patients with end stage lung disease. However, outcome after transplantation is complicated by episodes of rejection and infections. Bronchoalveolar lavage is a valuable tool in monitoring patients after transplantation, since it allows the detection of pathogens. A marker specifically indicating rejection from changes in BAL fluid has not been found yet. Especially changes in differential cell count, like lymphocytosis or an increase in polymorphnuclear granulocytes, are unspecific. The role of high eosinophil levels in BAL has not been elucidated yet.
We analyzed 25 BAL samples and clinical data of 4 patients who underwent lung transplantation and presented with recurrent episodes of eosinophilic alveolitis in BAL. All patients demonstrated a deterioration of clinical condition, lung function, and blood gas analysis during times of eosinophilia in BAL, compared to previous examinations. In all cases, eosinophilia in BAL was accompanied by rejection. All patients were finally treated with high doses of steroids, resulting in improvement of all parameters. Eosinophilia was not associated with significant changes in the IL-5 concentration in BAL or the pattern of IL-5 expression in BAL cells. In conclusion, eosinophilic alveolitis may indicate acute rejection in patients after lung transplantation, if other causes of eosinophilia are excluded.

Key words Lung Tx · Eosinophilic alveolitis · Alveolitis · BAL

Introduction

Lung transplantation has become a therapeutic option for end stage lung disease as surgical techniques and methods of immunosuppression have improved significantly over the past 15 years. However, transplantation success is limited by rejection episodes and severe infection. Differentiation between rejection and infection may, however, be difficult, as clinical appearance, with acute onset of dyspnea, fever, deterioration of gas exchange and pulmonary function, are not characteristic for either complication. Bronchoalveolar lavage (BAL) is essential to exclude infections, but no marker in peripheral blood or BAL has yet been found to establish the direct diagnosis of rejection [21]. Transbronchial biopsies are often effective to detect rejections, however, there may be histopathological evidence of rejection even in samples from asymptomatic patients, and perivascular infiltrates thought to be characteristic for rejection may appear similar during infections [22, 33]. Considering the risk-benefit relation, some centers have given up transbronchial biopsies as routine surveil-

lance of patients after lung transplantation. In this situation, careful analysis of cytological, microbiological, and clinical data becomes all the more important for the correct diagnosis and treatment of complications after lung transplantation. We report data demonstrating that some patients present with eosinophilic alveolitis in bronchoalveolar lavage fluid early after transplantation. After exclusion of infections typically causing eosinophilic alveolitis, such as aspergillus, this finding may be indicative for acute rejection episodes.

Materials and methods

Subjects

In this retrospective study, clinical data and differential cell counts from BAL samples of 37 lung transplant recipients, who had been treated at the University of Kiel until December 1996 and were available for follow-up investigation, were analyzed. Of the 37 recipients, 8 received a single lung, 22 a bilateral-, and 7 a heart-lung transplant. For indications of lung transplantation see Table 1. Observation time was at least 6 months. A total of 476 BAL samples was screened for eosinophilia. Patients developing eosinophilic alveolitis were selected for further retrospective analysis.

Table 1 Pre-Transplant Diagnosis

Disease	Number of transplantations
COPD	8
A1ATD	8
PPH	6
Heart vitium with Eisenmenger reaction	5
CF	4
IPF	2
Sarcoidosis	1
LAM	1
ARDS	1
Bronchiectasis	1

Abbreviations: *COPD* chronic constructive pulmonary disease, *A1ATD* alpha 1 antitrypsin deficiency, *PPH* primary pulmonary hypertension, *CF* cystic fibrosis, *IPF* idiopathic pulmonary fibrosis, *LAM* lymphangiomyomatosis, *ARDS* adult respiratory distress syndrome

Immunosuppression

Immunosuppression was started intraoperatively with a single dose of 1 g methylprednisolone (Urbason®, Hoechst, Bad Soden) i.v., followed by 3 days of 500 mg and a 3 month oral taper from 0.5 mg/kg to a dose of 0.1 mg/kg per day methylprednisolone. All patients received 5 mg/kg body weight azathioprine (Imurek®, Glaxo Wellcome, Hamburg) intraoperatively. Beginning on day 2 after transplantation, azathioprine was given at a dose of 2 mg/kg, adjusted to maintain blood neutrophils just above 4000/μl. Immediately after transplantation, administration of cyclosporine A (Sandimmun®, Sandoz, Nürnberg) was initiated maintaining a blood level of 250–300 μg/l. Therapy for rejection episodes consisted in methylprednisolone pulse therapy (0.5–1 g/day i.v. for 3 days). In some cases of ongoing or recurrent rejection, cytolytic therapy with rabbit-antithymocyte globuline (Tecelac®, Biotest, Dreieichen) was administered for 4 days at 1.5–2.5 mg/day, followed by a steroid taper from 0.5 to 0.1 mg/kg, which was applied for 8 weeks.

Bronchoalveolar Lavage (BAL)

Bronchoscopy and BAL were performed weekly during the first 2 months, every 2 weeks for the next 4 months, and at months 8, 10 and 12 after transplantation. Additional BAL samples were obtained if patients showed clinical, lung-functional, or radiographic deterioration. Bronchoscopy was carried out using flexible fiberoptic bronchoscopes (Olympus, Hamburg). Patients received atropine 0.5 mg (Atropinsulfat®, Braun, Melsungen) and hydrocodon 7.5 mg s.c. (Dicodid®, Knoll, Ludwigshafen) half an hour before examination. Up to10 ml 1% oxybuprocainhydrochloride (Novesine®,Wander, Nürnberg) or 2% lidocainehydrochloride (Xylocain®, Astra, Hamburg) were used as local anaesthesia. Supplemental oxygen was administered at a rate of 2–6 l/min. BAL was performed in the middle lobe bronchus or in the bronchial segment affected by infiltrates. 200 ml of 0.9% sodium chloride solution were applied in 20 ml fractions, each fraction being aspirated by gentle suction and pooled in polypropylene vessels for further examination.

Analysis of BAL fluid

BAL fluid was transported to the laboratory within 1 h for immediate processing. For cellular analysis, BAL fluid was filtered through gauze and adjusted to a concentration of 1×10^6/ml. 5×10^4 cells were used for each of 12 cytospin slides. Slides were dried by air, fixation was achieved by 100% acetone. Slides were stained by the May-Grünwald-Giemsa procedure or processed for immunocytochemical analysis. 500 cells were counted for differential cell analysis using light microscopy. The number of macrophages, lymphocytes, neutrophils and eosinophils was expressed as a percentage of the total cell number.

Criteria for the diagnosis of rejection

Episodes of acute deterioration in lung function (mainly decrease in FEV1, MEF50), decrease in pO_2, or newly developed infiltrates in the chest x-ray, were considered as rejection if no pathogen was detected in the BAL, and if treatment with corticosteroids proved effective. Transbronchial biopsies were not taken routinely.

Detection of infections

BAL was examined routinely for the presence of bacteria, virus or fungus. Methods included gram-staining and conventional culture techniques as well as special culture, immunocytochemistry, serologic testing, or PCR. In this way, detection procedures were carried out for general bacteria, mycobacteria, legionella, chlamydia, mycoplasma, pneumocystis carinii, candida, aspergillus, cryptococcus and viruses (paramyxovirus, parainfuenza, HSV, EBV, RSV, adenovirus and CMV).

Detection of interleukin 5 (IL-5) in BAL cells

Cytospin slides for immunocytochemistry were available from 21 patients. The slides were incubated with a rabbit anti-human IL-5

antibody (product no 1722–01, Genzyme, Cambridge) at a dilution of 1:100 for 30 min at room temperature. After washing, a second antibody (mouse anti-rabbit IgG, product no M0737, Dako; Hamburg) and a third antibody (anti-mouse IgG, product no Z0259, Dako; Hamburg) were applied, each at a dilution of 1:50 for 30 min. Finally alkaline phosphatase anti alkaline phosphatase staining procedure was performed (Dako). Identification of cell type was achieved by microscopical inspection. Stained cells were counted and expressed as a percentage of the total count of each cell type.

Interleukin-5 in BAL supernatant

Interleukin-5 was detected using an enzyme immunoassay with no crossreactivity to IL-3, IL-8 or GM-CSF (product no.1983, Immunotech, Hamburg). Assays were performed as indicated by the instruction manual. Briefly, an anti-IL-5 monoclonal antibody-coated microtiter plate was incubated with 50 µl of sample. In a second immunological step, 50 µl of biotinylated monoclonal antibody and a streptavidin-horseradish peroxidase conjugate were added. Using TMB as substrate, absorbance was taken at 450 nm. IL-5 concentrations were calculated from a standard curve obtained in the same assay procedure as the sample. The sensitivity of this assay was 1 pg/ml sample.

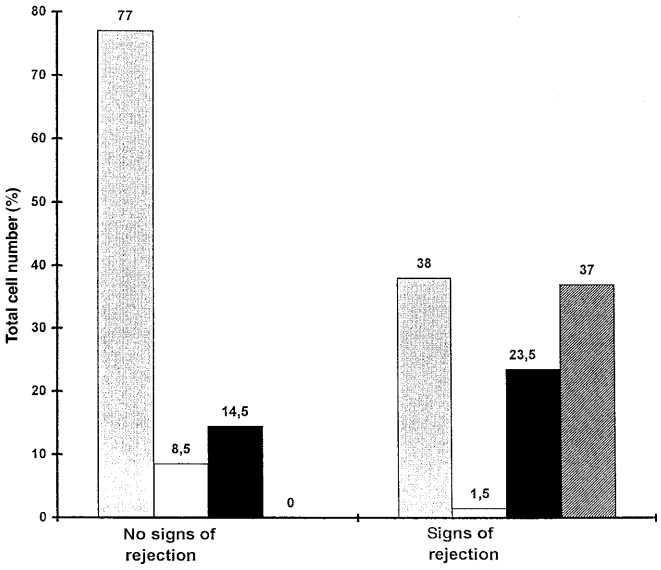

Fig. 1 Differential cell count in bronchoalveolar lavage fluids during episodes with and without signs of rejection, from patients after lung transplantation. ▨ Alveolar macrophages; □ lymphocytes; ■ granulocytes, ▨ eosinophils

Results

Differential Cell Count in BAL

Four out of 37 patients who were examined after lung transplantation demonstrated episodes of eosinophilic alveolitis. 25 BAL samples were obtained from these patients at different time points. There were 12 BAL samples with increase of eosinophils ranging from 5% to more than 60% of the total cell number. 5 BAL samples demonstrated eosinophilia of 25% or more. An increase in the number of eosinophils was accompanied by a raised number of neutrophils (Fig. 1). All patients fulfilled our criteria for indicating acute rejection. In most cases, treatment with steroids during episodes of eosinophilic alveolitis resulted in normalization of the clinical status, significant improvement of lung function, or reduction of infiltrates on chest x-ray. If control BAL samples were obtained after treatment, the number of eosinophils was found to be normal or significantly reduced.

The course of patient A (transplantation for cystic fibrosis of the lung) is shown in Fig. 2. Two of his BAL samples showing eosinophilic alveolitis (on 14–07, not included in the Fig., and on 23–07) were not followed by treatment. Lavages obtained 2 resp. 7 days later demonstrated no significant change in differential cell count. At these points of time, steroid treatments were initiated, leading to improvement in lung function and blood gas analysis. He suffered from recurrent reactivation of cytomegalovirus (CMV) infections after treatment with steroids, showing an increase in CMV IE-antigen positive lymphocytes in peripheral blood samples. CMV reactivation was not associated with elevated

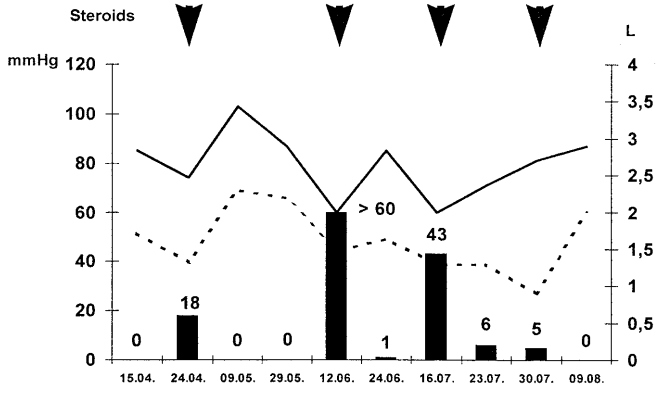

Fig. 2 FEV1, PO_2 and relative number of eosinophils in bronchoalveolar lavage fluids from patient A at different timepoints after lung transplantation (transplantation was on 10–03–96). Dates of starting a steroid intervention are indicated by *arrowheads*. ■ Eosinophils in BAL (%) — pO2 (mmHg) - - - FEV1 (L)

counts of eosinophils in BAL, compared to times of dormant CMV status. With this patient, 2 episodes of eosinophilic alveolitis (> 60% on 12–06 and 46% on 14–07, resp.) were accompanied by blood eosinophilia (9%). None of the other episodes of eosinophilia in BAL (including all 4 patients) showed blood eosinophilia of more than 6%.

Clinical and laboratory data of patient B (transplantation for pulmonary hypertension) are presented in Fig. 3. Steroid therapy was initiated (on 25–03), because signs of rejection appeared. At that time, the count of

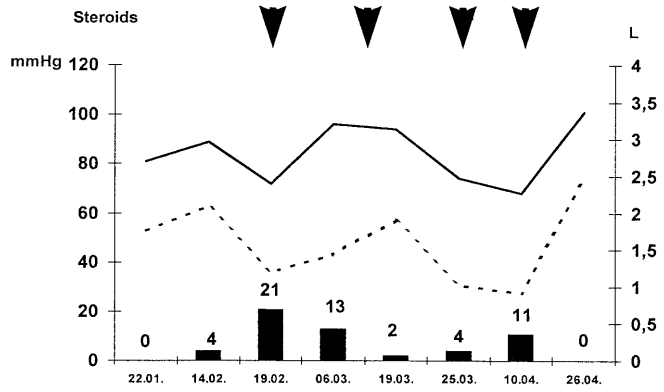

Fig. 3 FEV1, PO_2 and relative number of eosinophils in bronchoalveolar lavage fluids from patient B at different timepoints after lung transplantation (transplantation was on 28–12–95). Dates of starting a steroid intervention are indicated by *arrowheads*. ■ Eosinophils in BAL (%) — pO2 (mmHg) - - - FEV1 (L)

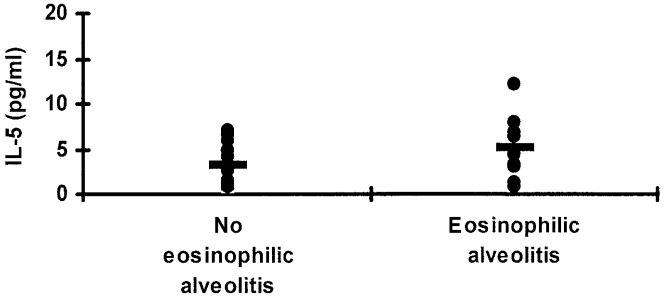

Fig. 4 Interleukin 5 levels in bronchoalveolar lavage fluids from patients after lung transplntation during episodes without eosionophilic alveolitis and episodes of eosinophilic alveolitis

eosinophils was 4%. Despite treatment however, the percentage of eosinophils increased to 11%. Complete resolution was achieved by a further application of high dose steroids for 3 days followed by a taper. With this patient, another episode of slightly eosinophilic alveolitis occurred half a year later. At that time, transbronchial biopsies were obtained that showed signs of rejection.

In patient C, (transplantation for emphysema) one episode of eosinophilic alveolitis occured that was treated with steroids. This treatment resulted in a reduced but nevertheless elevated count of eosinophils. Additionally, high-dose methylprednisolone treatment followed by a taper was administered for 3 days, resultingin normalization of BAL differential cell count.

In patient D, (transplantation for postinfectious respiratory distress syndrome) very high counts of eosinophils in BAL (> 63%) were detected during a period of clinical deterioration. Treatment with steroids improved her status significantly, however, close follow-up lavage results were not available.

Expression of interleukin-5 in BAL cells

Twenty-one of the 25 BAL samples of patients with intermittent eosinophilic alveolitis were analyzed for interleukin-5 (IL-5) expression. In all samples IL-5 was detected immunocytochemically on macrophages (40%–96% of macrophages). Expression on lymphocytes was found in 4 BAL samples and ranged between 2%–13% of total lymphocyte count. There was no correlation between the number of lymphocytes expressing IL-5 and the number of eosinophils in BAL. In 14 BAL samples polymorphnuclear granulocytes (PMN) expressed IL-5 (2%–23%). PMN expressing IL-5 were detected only if total number of PMN was increased. Elevation of eosinophils was not correlated to the number of PMN expressing IL-5. There was no immunocytochemical detection of IL-5 in eosinophils.

Interleukin-5 in BAL supernatant

Concentration of interleukin-5 (IL-5) was assayed in BAL supernatant using ELISA technique. IL-5 in native BAL fluid was determined at levels between less than 1 pg/ml and 12,2 pg/ml. In BAL samples without eosinophilic alveolitis the median IL-5 content in unconcentrated fluid was 3.2 pg/ml. Lavages with an increased number of eosinophils showed IL-5 levels slightly higher at a median of 5.4 pg/ml (Fig. 4). The difference was statistically not significant (U-Test of Wilcoxon, Mann, Whitney).

Discussion

Eosinophilic granulocytes are involved in the course of many diseases, mainly allergic, infectious, hematological, and in collagen disorders. Pulmonary diseases characterized by increased numbers of eosinophils in BAL and tissue are asthma, idiopathic pulmonary fibrosis, acute and chronic eosinophilic pneumonia, Churg-Strauss syndrome, filarial and fungal infections, and drug reactions [1, 6, 10, 17]. Eosinophils participate in the immunomodulatory cell network. They are capable of both responding to and producing cytokines. They were identified as potent proinflammatory cells, producing reactive oxygen species and releasing toxic granule proteins, such as eosinophil cationic protein and major basic protein [28]. In the lung, eosinophils are able to degrade connective tissues and cause severe epithelial and microvascular injury. Reduced ciliary motility and respiratory epithelial necrosis, as described for asthmatic patients, enhance the risk of infections [3, 27].

Tissue eosinophilia is involved in allograft rejection after renal, hepatic, pancreatic and cardiac transplanta-

tion [34]. In renal- and hepatic allografts, episodes of rejection were observed that were associated with peripheral blood eosinophilia [2, 11]. Our results indicate that eosinophils may play a significant role in lung allograft rejection, too. These findings are in keeping with prior animal and human studies. In a rat-lung allograft transplantation model, BAL eosinophilia was found to be a marker of rejection [14]. Histological examination of transbronchial biopsies of patients suffering from rejection showed eosinophils in less than half of the cases, but the number of cells was reduced after treatment with steroids [7]. In transbronchial biopsies of patients presenting with acute cellular rejection early after lung transplantation, 22 % of the samples displayed eosinophilic infiltrates if rejection was classified as mild. In cases of severe rejection, all samples had eosinophils indicating a correlation between the extent of tissue damage and the number of eosinophils. All cases with intense infiltrates of eosinophils (more than 50 % of infiltrating cells) observed in transbronchial biopsies within one month after transplantation, were associated with rejections. However, there was only one case described exhibiting BAL eosinophilia [32]. Riise et al. reported data demonstrating activation of eosinophils during rejection assessed by eosinophil cationic protein in BAL, but there was only one case of BAL eosinophilia of more than 10 % [18]. Results of our study indicate that after lung transplantation some patients show severe eosinophilic alveolitis during episodes regarded as rejection. To our knowledge, this is the first report about recurrent BAL eosinophilia in lung transplant recipients.

To validate our findings regarding eosinophils in BAL and the diagnosis of acute rejection, a number of prerequisites have to be fulfilled to exclude other mechanisms. Firstly, it should be a temporary finding. Secondly, simultaneous clinical signs of rejection should occur. Thirdly, active infective processes should be excluded. Fourthly, response to anti-rejection therapy must be proven [12]. All four criteria were fulfilled by our patients. It remains uncertain, however, why some patients demonstrate eosinophils in the BAL during episodes regarded as rejection after transplantation, and others do not. Most of our patients (33 out of 37) did not produce significant eosinophilia, although most of them showed episodes of rejection by the criteria mentioned above. There may be an association between the extent of rejection and the total number of eosinophils infiltrating affected tissue. Differences in adhesion of the eosinophils to the lung tissue may be involved as well. Blood eosinophilia was uncommon in our patients, with signs of rejection after lung transplantation suggesting the origin of the process to be the affected lung. Peripheral blood eosinophils may be normal during times of rejection, although a relative increase in eosinophil counts is considered a specific marker of rejection [26].

In our patients, eosinophilic alveolitis was accompanied by an increased number of neutrophils. Usually, in acute rejection lymphocytosis is found in up to 25 % of the cases. However, neutrophilic alveolitis is described in patients with acute rejection associated with pulmonary infiltrates on the chest x-ray [8].

Increased numbers of eosinophils may be found in patients with asthma. None of our patients and none of the donors was known to have asthma. Eosinophilic alveolitis has been described in angioinvasive aspergillosis after lung transplantation. In one of these cases there were features of acute eosinophilic pneumonia with no proof of aspergillus infection in the first biopsies, and aspergillus was found later [32]. In patients with asthmatic symptoms, increased IgE and eosinophilia in combination with recovery of aspergillus, allergic bronchopulmonary aspergillosis (ABPA) must be suspected. However, none of the 4 patients with BAL eosinophilia had signs of ABPA. Increased numbers of eosinophils after lung transplantation have been found in association with coxsackie A2 and pseudomonas aeruginosa infection as well [32]. Two of our patients had pseudomonas aeruginosa detectable in sputum or tracheal secretion. This pathogen was found consistently, with no association to periods of eosinophilia in BAL. Treatment with steroids would have been detrimental if pseudomonas were the pathogen causing decrease in clinical parameters of lung function. In one of the patients, corynebacterium jeikeium was detected during an episode of eosinophilic alveolitis, which however persisted after treatment with steroids, and resolution of eosinophilia and symptoms. Thus, we think the pathogen was not responsible for the eosinophilia.

Since ciprofloxin may be related to blood eosinophilia, this antibiotic needs to be considered responsible for BAL eosinophilia as well. During one period of eosinophilia in BAL, 1 of the patients received ciprofloxin at the same time. However, eosinophilia was restricted to the lung with no blood eosinophilia, which would be unusual for allergic drug reactions.

Differential diagnosis of eosinophilic alveolitis must include acute eosinophilic pneumonia. Criteria defining this disease are essentially the same as the criteria we used for the diagnosis of acute rejection with the exception of BAL eosinophilia. However, recurrent episodes of BAL eosinophilia – as we observed in 2 of our patients – are uncommon in acute eosinophilic pneumonia [24]. Previous reports indicate tissue eosinophilia and activation of eosinophilic markers during episodes of rejection [2, 11, 14, 18, 32, 34]. Summarizing our findings regarding acute eosinophilic pneumonia and eosinophilia during rejection, we suspect the eosinophilic alveolitis in lung transplant recipients to be a distinctive form of acute eosinophilic pneumonia indicative for rejection. In one of our cases of eosinophilic alveolitis, transbronchial biopsies were taken, showing signs of acute and

chronic rejection, supporting our assumption. However, since this practice was not a common approach when evaluating patients after lung transplantation, we cannot completely exclude different causes in the other cases of eosinophilia in BAL.

The cytokine IL-5 is known to be involved in the process of eosinophilic inflammation. IL-5 is able to promote differentiation, recruitment and activation of eosinophils [29]. Therefore, in patients who presented eosinophilic alveolitis recurrently, we analyzed BAL cells immunocytochemically for IL-5 presence and BAL fluid for IL-5 content by ELISA.

The dominant cells in BAL staining positive for IL-5 were macrophages. In addition, IL-5 was detected in neutrophils and T-lymphocytes, but there was no correlation to the number of eosinophils. No IL-5 was detected in eosinophilic granulocytes. Studies analyzing IL-5 expression in patients after lung transplantation demonstrated IL-5 mRNA in 40 %–50 % of the samples with a slight increase during episodes of rejection. Cell type specification and quantity of IL-5 expression were not examined [30].

In asthmatic patients, raised expression of IL-5 mRNA was detected in BAL and in bronchial mucosal cells, where T lymphocytes, especially the CD4 + Th2 subset, represented a major source of cytokine expression [5, 19]. The number of BAL cells expressing IL-5 mRNA was higher in asthmatic patients who were not treated with steroids, compared to patients who were [20]. IL-5 appeared to be locally produced, since systemic cytokine levels measured in the blood were low. In patients with asthma, local allergen challenge was performed inducing significant airway eosinophilia [4]. It was demonstrated that this eosinophilic inflammation was associated with local expression of interleukin 5 in eosinophils indicating an autocrine stimulation.

However, while IL-5 seems to be involved in asthma, hypereosinophilic syndrome, eosinophilic cystitis and in eosinophilic heart disease, some studies suggest, that IL-5 expression is not observed in all disorders associated with eosinophilic infiltration, such as Crohn's disease [9]. This might be true as well for eosinophilic inflammation during rejection episodes, since none of our patients demonstrated significant IL-5 levels in unconcentrated BAL. Even during episodes of significant eosinophilia, IL-5 was detectable at a concentration of only 13 pg/ml BAL fluid or lower, although there was a slight difference between episodes of eosinophilia and episodes without eosinophilia (median of 5.4 vs. 3.2 pg/ml). The small number of samples analyzed limits the findings with regards to IL-5 concentrations. The difference may become significant in a larger study population. In chronic eosinophilic pneumonitis, increased levels of IL-5 in BAL fluid were observed in affected lung segments, corresponding to the extent of pulmonary eosinophilia [13]. Similar results were obtained in vivo, where anti-IL-5 antibody inhibits infiltration of eosinophils in a mouse model [15], and in asthmatic patients. In symptomatic asthmatic patients with numbers of eosinophils higher than 1×10^6/ml, IL-5 concentration in BAL was elevated to 274 pg/ml, whereas in patients with asymptomatic asthma, or with eosinophilic cell counts lower than 1×10^6, IL-5 was below 13 pg/ml [23]. IL-5 in the BAL fluid of our patients was low even in the presence of high numbers of eosinophils. We therefore hypothesize, that eosinophilic inflammation during rejection episodes may be induced by cytokines different from IL-5, but known to be involved in eosinophilic inflammation, such as IL-3, GM-CSF and IL-8 [5, 25]. In-vitro studies demonstrated saline induced migration of eosinophils into peritoneum mediated by LTB4 that was released by resident mast cells and macrophages [5]. This process appears to be mediated either by IL-5 inducing a specific eosinophilic migration, or by IL-8 inducing a mixed migration of eosinophils and neutrophils [16]. BAL differential cell count from our study population showed significant amounts of neutrophils, suggesting the possibility of an IL-8 mediated process.

In summary, our observations indicate that after lung transplantation some patients may develop eosinophilic alveolitis similar to acute eosinophilic pneumonia, which may be considered as an acute rejection episode if pathogens causing eosinophilia are excluded. This process does not seem to be mediated by IL-5. We suggest further studies including transbronchial biopsies for the grading of rejection and exclusion of differential diagnoses to confirm our observation.

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