

Research article

Blood and alveolar lymphocyte subsets in pulmonary cytomegalovirus infection after lung transplantation

François Stéphan^{*1}, Jean-François Bernaudin¹, Danielle Cesari¹, Anne Fajac¹, Dominique Grenet MD², Isabelle Caubarrere² and Marc Stern²

Address: ¹Laboratoire d'Histologie-Biologie Tumorale, AP-HP Hôpital Tenon, 4 rue de la Chine 75020 Paris, France and ²Service de Pneumologie, Hôpital Foch, 40 rue Worth, BP 36, 92151 Suresnes, France

E-mail: François Stéphan* - francois.stephan@hcuge.ch; Jean-François Bernaudin - jean-francois.bernaudin@tnn.ap-hop-paris.fr; Danielle Cesari - jean-francois.bernaudin@tnn.ap-hop-paris.fr; Anne Fajac - jean-francois.bernaudin@tnn.ap-hop-paris.fr; Dominique Grenet - jean-francois.bernaudin@tnn.ap-hop-paris.fr; Isabelle Caubarrere - jean-francois.bernaudin@tnn.ap-hop-paris.fr; Marc Stern - jean-francois.bernaudin@tnn.ap-hop-paris.fr

*Corresponding author

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Abstract

Background: Cytomegalovirus (CMV) pneumonitis has been shown to be associated with lymphocytic alveolitis after lung transplantation. In the present study, we investigated a series of bronchoalveolar (BAL) and blood samples, collected in the absence of rejection or acute infectious episodes. in order -1: to evaluate intra-alveolar cell population changes concomitant with CMV replication and -2: to reappraise the value of cell population analysis in the management of patients after lung transplantation.

Methods: We used flow cytometry to investigate modifications of lymphocyte subpopulations related to pulmonary cytomegalovirus infections in blood and BAL samples from a series of 13 lung transplant recipients. After exclusion of samples obtained during pulmonary rejection, bronchiolitis obliterans or acute bacterial infection, 48 blood and BAL samples were retained for analysis: 17 were CMV positive by shell-vial assay and 31 were CMV negative in blood and BAL.

Results: Our results demonstrate that pulmonary CMV infection is associated with a significant increase in the total lymphocyte population in BAL samples, but with minor modifications of the various lymphocyte subpopulations and a significantly higher absolute number of B lymphocytes in blood samples.

Conclusions: Cytomegalovirus pulmonary infection is accompanied by only minor changes in BAL lymphocyte subpopulations. The study of BAL lymphocyte subpopulations therefore appears to be of limited clinical value in the diagnosis of pulmonary CMV infection. However, increased blood B-lymphocytes seems to be a clinical feature associated with CMV infection.

Background

Cytomegalovirus (CMV) infections are the most frequent viral infections in lung transplant recipients [1]. Studies in man and the murine model of CMV have demonstrated that development of pathological effects is not exclusively related to viral replication in the lung, and a host immune response is required for induction of pneumonitis [2,3]. For instance, CMV pneumonitis has been shown to be associated with lymphocytic alveolitis after bone marrow transplantation [4] or lung transplantation [5]. In murine models of CMV pneumonitis, increased numbers of both CD4+ T helper and CD8+ suppressor/cytotoxic pulmonary lymphocytes have been observed [6]. Moreover, in non-HIV immunodepressed patients, it has been shown that CD8+ T lymphocytes play a particularly important role in host defense against cytomegalovirus infection [7–9] and cytotoxic CD8+ T lymphocytes are activated in situ during CMV pneumonia [10]. However, after lung transplantation, the cellular alveolar modifications related to CMV replication have not been clearly characterized, particularly compared to those associated with rejection [11–13]. Bronchoalveolar lavage (BAL) has been largely developed in the management of lung transplant recipients and in the investigation of alveolar cells, particularly in order to detect an opportunistic infection, such as CMV pneumonitis [1]. All patients transplanted in our institution are routinely evaluated by BAL for detection of opportunistic infection and routine analysis of the various subsets of intra-alveolar cells, particularly lymphocytes. In the present study, we investigated a series of BAL and blood samples collected in the absence of rejection or acute infectious episodes, in order -1: to evaluate intra-alveolar cell population changes concomitant with CMV replication and -2: to reappraise the value of cell population analysis in the management of patients after lung transplantation.

Methods

Patients and specimen collection

The lung transplantation protocol was approved by the institutional review board for human studies and informed consent was obtained from the subjects after detailed description of the procedure. According to French legislation, this study did not need Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (C.C.P.P.R.B.) approval.

Thirteen lung transplant recipients (4 females, 9 males) were studied, with a mean follow-up of 15 months (range: 1 to 42 months). The median age of the patients was 27 years (range: 18–56). The patient characteristics are reported in Table 1. The immunosuppressive regimen consisted of cyclosporin (whole blood level between 200 to 300 µg/L), azathioprine (1.5 – 2 mg/kg/d), and

steroids (started at d+5 at 0.2–0.3 mg/kg/d, up to 1 mg/kg/d at d+21 until the third month; doses were then tapered to 0.2 mg/kg at 6 months) with the additional perioperative infusion of antilymphocyte globulins (Thymoglobulines Mérieux 2.5 mg/kg/d for 5 days). Acute rejection was treated with IV pulses of methylprednisolone (15 mg/kg/d) for 3 consecutive days. Every episode of CMV infection was treated by intravenous ganciclovir, 5 mg/kg every 12 h for 14 days. Maintenance therapy (5 mg/kg/d) was initially provided for patients suffering from recurrent CMV infection.

Routine bronchoscopy with BAL and transbronchial biopsy (TBB) were performed at regular intervals after transplantation (monthly during the first 6 months, every 3 months until the end of the first year, then twice a year) to monitor for infection and rejection, and when clinically indicated. Peripheral blood samples were collected at the time of each bronchoscopy and at the attending physician's discretion. BAL fluids were analyzed for evidence of infection, differential cell counts, and lymphocyte marker studies, when possible. Peripheral blood lymphocyte markers were analyzed simultaneously.

Nine of the 13 patients studied developed a total of 36 CMV infections in blood or BAL or both. Four patients never developed CMV infections.

One hundred and twenty-two blood samples (median: 8 per patient, range: 1–17 per patient) and 107 BAL samples (median: 7 per patient, range: 1–17 per patient) were collected over the study period for CMV culture. Fifty nine BAL samples were excluded for the study owing to the presence of concomitant pulmonary rejection (n = 36), bronchiolitis obliterans (n = 11), bacterial pneumonia (n = 1), other viral infection (n = 1), or insufficient recovery rate of cells from BAL to allow differential cell counts and lymphocytes marker studies (n = 10). Forty-eight blood and BAL samples from 12 of the 13 patients were analyzed, as the 13th patient, who always suffered from concomitant pulmonary rejection, was excluded from the study.

Viral cultures

Rapid CMV culture by the shell vial technique was used for BAL fluid and blood, as previously described [14].

Definition of CMV infection

CMV infection was defined as the detection of CMV in blood or BAL fluid by culture [15]. CMV pneumonitis was defined as CMV infection and the presence of characteristic cytomegalic inclusion-bearing cells on cytologic examination of BAL specimens or histopathologic examination of transbronchial biopsy specimens [15].

Table 1: Characteristics of lung transplant recipients entering the present study

Patient	Age (yr)	Sex	Disease	CMV status*		CMV infection episodes † (n)	Samples studied		Follow-up (mo)	Steroid dose (mg/kg)**
				D	R		BAL CMV + (n)	BAL CMV - (n)		
1	25	M	Idiopathic pulmonary fibrosis	+	+	1	1	0	1	0.7 (-)
2	53	M	Paracicatricial emphysema	+	+	2	1	2	17	0.2 (0.2-0.5)
3	56	F	Lymphangiomyomatosis	+	+	8	4 ‡	6	19	0.35 (0.2-1.5)
4	18	F	Cystic fibrosis	+	-	5	2	2	11	0.2 (0.2-0.9)
5	48	M	Idiopathic pulmonary fibrosis	-	+	4	2	2	5	0.9 (0.3-1.5)
6	20	M	Cystic fibrosis	-	+	4	2	1	5	0.8 (0.8-1)
7	42	M	Panlobular emphysema	-	+	4	2	2	15	0.25 (0.2-0.9)
8	37	F	Primary pulmonary hypertension	-	+	6	3	2	16	0.2 (0.2-1.5)
9	27	M	Cystic fibrosis	-	-	0	0	2	7	0.3 (0.2-0.4)
10	42	M	Centrolobular emphysema	-	-	0	0	1	24	0.2 (-)
11	24	F	Cystic fibrosis	-	-	0	0	5	30	0.2 (0.2-1)
12	23	M	Cystic fibrosis	-	-	0	0	6	42	0.2 (0.2-0.2)

* CMV status at time of transplantation: D, donor; R, recipient; GVH, graft-versus-host. † Total number of CMV infection episodes in the patient during the follow-up; ‡ One sample was taken into account as BAL became CMV positive 8 days later; ** expressed as median (min-max)

Lymphocyte immunotyping by flow cytometry

Lymphocyte subsets in BAL and blood were evaluated by multiparameter analysis of leukocytes by flow cytometry using a Coulter Profile II cytometer (Coulter Cytometry Products; Coultronics France SA, Margency, France). Following gentle mixing, 100 µl of each whole blood specimen or 0.5 × 10⁶ BAL cells were incubated with 10 µl of monoclonal antibody at room temperature for 15 min before lysis using the Coulter Q-Prep procedure. The following monoclonal antibodies – all conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (RD1) – (Coulter Cyto-Stat/Coulter clone; Coultronics France SA, Margency, France) were used: T11-RD1/B4-FITC (anti human CD2/CD19), anti human CD3 (IgG1)-FITC, T4-RD1/T8-FITC (anti human CD4/CD8), T8-FITC/S6F1-RD1 (anti human CD8/CD11a), NKH-1-RD1

(anti human CD56), TiGammaA.1-FITC (anti human Ti-GammaA). The cells were analyzed on forward-angle light scatter and 90° light scatter using a lymphocyte gate.

We focused our attention on surface adhesion molecules, particularly LFA-1, since the absence of cell surface LFA-1 has been proposed as a mechanism of escape from immunosurveillance [16]. The S6F1 (CD11a) lymphocyte surface antigen reacts with the LFA-1 antigen and is expressed on approximately 10% of unfractionated T cells, 15% of CD4+ cells and 50% of CD8+ cells. [17]. The S6F1 antibody can distinguish killer effector (CD8+/S6F1+) cells from the precursor of killer effector (CD8+/S6F1-) cells among CD8+ T lymphocytes [17]. NKH-1 (CD56) defining a natural killer cell antigen, is expressed on a

subpopulation of peripheral blood large granular lymphocytes demonstrating natural killer activity [18]. Anti-NKH-1 was found to react with approximately 12% of circulating lymphocytes and more than 90% of circulating NKH-1+ cells are large granular lymphocytes [18].

Anti human TiGammaA recognizes an antigenic determinant carried by a T Cell Receptor (TCR) γ chain. This antibody delineates a unique subset accounting for approximately 3% of human circulating lymphocytes [19]. The TiGammaA epitope is expressed on approximately two-thirds of the circulating CD3+ TCR- α/β -fraction [19].

As a sufficient recovery rate of cells from BAL and a lymphocyte percentage > 3–5% are required to allow reliable flow cytometry processing, immunotyping of alveolar lymphocytes could only be performed on 22 BAL samples (11 CMV+ samples and 11 CMV-samples).

Statistical analysis

Results are expressed as mean \pm SEM. Comparisons of quantitative data were performed using a Mann-Whitney U test for unpaired data or a Wilcoxon's signed-rank test for paired data. A P value < 0.05 was considered significant.

Results

Study population

Seventeen of the 48 blood and BAL samples were CMV positive by culture (referred to as CMV+ samples): 12 in BAL, 4 in blood and BAL, and 1 in blood only. However, this last sample was taken into account because BAL became CMV positive 8 days later. Thirty-one samples were negative in blood and BAL (referred to as CMV-samples). Four \pm 0.7 blood and BAL samples were obtained per patient (range 1 to 10) between 1 to 42 months of follow-up after transplantation; 6 months \pm 1 (range 1 to 15) for CMV+ samples versus 11 months \pm 2 (range 1 to 42) for CMV-samples ($P < 0.05$). Seven samples were taken during antiviral treatment (2 CMV+ samples and 5 CMV-samples). A statistical analysis was unable to show that antiviral treatment influences the cell counts. Four of the patients with CMV infection had characteristic cytomegalic inclusion-bearing cells on cytologic examination of BAL or histopathologic examination of transbronchial biopsy specimens, and were therefore considered to present CMV pneumonitis [15].

Differential cell counts in BAL and blood samples

Results of differential cell counts in BAL are reported in Table 2. An increased percentage of alveolar lymphocytes was observed in CMV+ samples compared to CMV-samples (14.8 ± 3.4 versus $5.3 \pm 0.6\%$, $P < 0.001$). For the 4 episodes of CMV pneumonia, the percentage of

alveolar lymphocytes was $11 \pm 3\%$, and number of alveolar lymphocytes was $40 \pm 9 \times 10^3/\text{mL}$. The blood WBC count was 6.68 ± 0.7 and $6.97 \pm 0.7 \times 10^9/\text{L}$ (NS), and the absolute lymphocyte count was 0.60 ± 0.07 and $0.59 \pm 0.08 \times 10^9/\text{L}$ (NS) in CMV+ and CMV-samples, respectively.

Table 2: Differential cell counts in BAL from CMV+ and CMV-episodes (mean \pm SEM).

	CMV+	CMV-	P value
Total cell count ($10^3/\text{ml}$)	422 ± 64	383 ± 45	NS
Lymphocytes (%)	14.8 ± 3.4	5.3 ± 0.6	< 0.001
Alveolar macrophages (%)	76.5 ± 5.2	87.9 ± 2.6	0.03
Neutrophils (%)	8.7 ± 3.3	6.6 ± 2.6	NS
Siderophages* (%)	37 ± 8	47 ± 6	NS

*: Siderophages, characterized by deep green-blue staining after Perl's reaction, were expressed as the percentage of the total macrophage population. NS: non significant ($p > 0.1$)

Lymphocyte subset counts in blood and BAL

As shown in Table 3, CMV+ blood samples had a significantly higher B lymphocyte count (CD19+ cells); while CMV-samples tended to have higher natural killer lymphocyte (CD56+ cells) and TiGammaA+ lymphocyte counts. No difference was observed for CD8+ or CD4+ lymphocytes, expressed as percentage or in absolute numbers. CD11a was expressed on approximately 50% of CD8+ T lymphocytes in CMV+ and CMV-samples. The absolute number of CD8+ T lymphocytes in BAL was significantly higher in CMV+ samples (Table 3). Twenty percent of CD8+ T lymphocytes expressed CD11a.

CD4/CD8 and CD8/CD3 ratios in BAL versus blood

CMV+ samples had a lower CD4/CD8 ratio in BAL than in blood ($p = 0.008$), and a higher CD8/CD3 ratio in BAL than in blood ($p = 0.002$). In contrast, no significant difference was found between BAL and blood for either CD4/CD8 or CD8/CD3 ratios in CMV-samples. Comparisons of CD4/CD8 and CD8/CD3 ratios in BAL and blood were not statistically different between CMV+ and CMV-samples.

Discussion

In the present study, BAL and blood lymphocyte subpopulations from lung transplant recipients with or without CMV pulmonary infection, but without graft rejection or bacterial infection, were compared. Lymphocytes were increased in bronchoalveolar fluid from patients with CMV pulmonary infection, but surprisingly only minor

Table 3: Lymphocyte counts and lymphocyte subsets counts in blood and BAL.

	CMV+ samples *	CMV-samples *	P value
Lymphocytes in blood † (/mm ³)	602 ± 75	590 ± 80	NS
CD2+ blood (/mm ³)	428 ± 64 [69 ± 4%]	480 ± 73 [77 ± 3%]	NS
CD3+ blood (/mm ³)	414 ± 64 [66 ± 4%]	421 ± 61 [74 ± 3%]	NS
CD4+ blood (/mm ³)	192 ± 39 [30 ± 3%]	188 ± 29 [33 ± 3%]	NS
CD8+ blood (/mm ³)	210 ± 32 [35 ± 3%]	209 ± 28 [37 ± 3%]	NS
CD19+ blood (/mm ³)	126 ± 23 [23 ± 3%]	74 ± 13 [16 ± 3%]	0.02
CD8/CD11a+ blood (/mm ³)	134 ± 26 [60 ± 5%]	105 ± 17 [48 ± 5%]	NS
NKH-1+ blood (/mm ³)	3 ± 0.8 [0.6 ± 0.15%]	7.3 ± 1.9 [1.3 ± 0.3%]	0.1
TiγAI + blood (/mm ³)	5.7 ± 1.5 [1.1 ± 0.3%]	25 ± 8 [3.4 ± 0.7%]	0.08
Lymphocytes in BAL ‡ (× 10 ³ /ml)	69.5 ± 19	27.4 ± 3.9	0.04
CD2+ BAL (× 10 ³ /ml)	64.3 ± 19 [90 ± 3%]	25.2 ± 3.7 [91 ± 1%]	0.1
CD3+ BAL (× 10 ³ /ml)	60.4 ± 18 [82 ± 3%]	22.1 ± 4.2 [81 ± 3]	0.1
CD4+ BAL (× 10 ³ /ml)	11.6 ± 5.6 [14 ± 2%]	4.2 ± 0.8 [18 ± 4%]	NS
CD8+ BAL (× 10 ³ /ml)	49.4 ± 13.2 [69 ± 4%]	17.6 ± 3.9 [60 ± 7%]	0.04
CD19+ BAL (× 10 ³ /ml)	0.06 ± 0.03 [0.2 ± 0.01]	0.1 ± 0.03 [0.5 ± 0.12]	NS
CD8/CD11a+ BAL (× 10 ³ /ml)	13 ± 7 [20 ± 10%]	2 ± 0.5 [18 ± 10%]	NS
NKH-1+ BAL (× 10 ³ /ml)	1 ± 0.5 [1.7 ± 0.9%]	0.3 ± 0.01 [1.3 ± 0.4%]	NS
TiγAI+ BAL (× 10 ³ /ml)	0.9 ± 0.4 [1.2 ± 0.3]	1.2 ± 0.7 [4.4 ± 3%]	NS

*: Respective percentages given in brackets †: Lymphocyte subset counts in blood were determined in 17 CMV+ samples and 31 CMV-samples. ‡: Lymphocyte subset counts in BAL were determined in only 11 CMV+ samples and 11 CMV-samples. NS: non significant ($p > 0.1$)

changes were observed in the distribution of the various subpopulations. In contrast, blood B-lymphocytes were significantly increased during CMV infection.

A significant increase in alveolar lymphocytes, expressed either in absolute number or as a percentage, was observed in the BAL fluid sampled concurrently with CMV infection. Such an increase has already been reported in pulmonary CMV infection and during acute rejection [5,12,13]. The presence of lymphocytic alveolitis is therefore considered to be useless to differentiate rejection from CMV infection. An increase in pulmonary CD8+ T lymphocytes during CMV infection has previously been reported [13], and CD8+ cytotoxic lymphocyte counts were found to be increased during CMV pneumonia [10]. However, interestingly, we found that pulmonary CMV infection was associated with only minor modifications in the respective alveolar lymphocyte subpopulations limited to a slight increase in the CD8+T lymphocyte subpopulation. The change in the CD4/CD8 ratio reflects an increase in the CD8 lymphocyte subset rather than a decrease in T helper lymphocytes [13]. As demonstrated by the differences in the CD4/CD8 and CD8/CD3 lymphocyte ratios in blood and BAL during CMV infection, the higher CD8+ T lymphocyte count in BAL is highly suggestive of local pulmonary recruitment.

The cellular changes observed in our studies suggest a slight increase in cell-mediated immune effectors in the

lungs [2,3]. Pulmonary CMV infection primes the host immune system, either in a CMV-specific or non-specific fashion [20–23] and several arguments are in favor of a CMV-specific immune response of the recipient as it has been previously shown that CD8+ T lymphocytes play an important role in host defense against CMV infection [7–9]. Lymphocyte proliferation kinetic studies have also shown accumulation of primed CMV-specific lymphocytes within the lung allograft during CMV infection [22]. However, in the present study, no difference was observed in terms of the absolute number of activated cytotoxic (CD8+/CD11a+) lymphocytes. This could be a consequence of either pharmacological immunosuppression or CMV "per se" [24]. Alternatively, the cellular changes observed in the alveolar spaces may be related to a CMV-induced allereaction which could provide a non-virus-specific response [23]. It has been demonstrated that alveolar macrophages are activated during pulmonary CMV infection [10,12] and secrete cytokines such as IL-6, IL-1, and TNF-?? which could therefore promote the recruitment and maturation of CD8+ cytotoxic lymphocytes [10]. Moreover, endothelial cells can be infected with CMV [25] and may be the target of allereactive cytotoxic lymphocytes [26], which could subsequently migrate to alveolar spaces.

Gamma/delta T lymphocytes tend to be more numerous in the blood of patients without infection; however, their role and function during CMV infection are largely un-

known. Recently, it has been demonstrated that gamma/delta T cell expansion in kidney allograft recipients follows CMV infection and could possibly be an immunologic consequence of this infectious disorder [27].

Lastly, our finding of a poor inflammatory response to pulmonary CMV infection could be in agreement with the hypothesis that CMV pneumonia is not exclusively a consequence of an immunopathological process [28,29]. In the present study, the BAL samples collected in 4 patients during CMV pneumonia showed a particularly low percentage of alveolar lymphocytes.

In the present study, blood B lymphocytes were significantly increased during CMV infection. The effects of CMV infection on B-cell differentiation have not yet been extensively studied [30]. However, there is some evidence that CMV can directly activate B-cells in vitro [31,32]. This response to CMV infection seems to consist of polyclonal activation of B cells, as previously demonstrated [32] and confirmed by us after κ/λ staining of these B-lymphocytes (J-F Bernaudin, personal results), which could probably be responsible for anti-CMV antibody secretion [32,33]. Interestingly, such an event was not observed in the alveolar space, in which B lymphocytes were almost entirely absent. As lymphocyte immunotyping by flow cytometry is rapid and easy, monitoring of variations in blood B-lymphocyte counts could therefore be indicative of CMV infection.

Conclusion

An increase in blood B-lymphocytes appears to be a clinical feature of pulmonary CMV infection. In contrast, pulmonary CMV infection is accompanied by only minor changes in BAL lymphocyte subpopulations. In our experience, evaluating the main subsets of alveolar lymphocytes therefore appears to be of limited clinical value in the diagnosis of pulmonary CMV infection.

Competing interests

none declared

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