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Impact of Human Metapneumovirus and Human Cytomegalovirus Versus Other Respiratory Viruses on the Lower Respiratory Tract Infections of Lung Transplant Recipients

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Viral respiratory tract infections in lung transplant recipients may be severe. During three consecutive winter-spring seasons, 49 symptomatic lung transplant recipients with suspected respiratory viral infection, and 26 asymptomatic patients were investigated for presence of respiratory viruses either in 56 nasopharyngeal aspirate or 72 bronchoalveolar lavage samples taken at different times after transplantation. On the whole, 1 asymptomatic (3.4%) and 28 symptomatic (57.1%) patients were positive for human metapneumovirus (hMPV, 4 patients), influenza virus A (3 patients), and B (2 patients), respiratory syncytial virus (2 patients), human coronavirus (2 patients), human parainfluenza virus (2 patients), rhinovirus (5 patients), while 4 patients were coinfected by 2 respiratory viruses, and 5 were infected sequentially by 2 or more respiratory viruses. In bronchoalveolar lavage samples, hMPV predominated by far over the other viruses, being responsible for 60% of positive specimens, whereas other viruses were present in nasopharyngeal aspirates at a comparable rate. RT-PCR (detecting 43 positive samples/128 examined) was largely superior to monoclonal antibodies (detecting 17 positive samples only). In addition, HCMV was detected in association with a respiratory virus in 4/18 HCMV-positive patients, and was found at a high concentration ($>10^5$ DNA copies/ml) in 3/16 (18.7%) patients with HCMV-positive bronchoalveolar lavage samples and pneumonia. Coinfections and sequential infections by HCMV and respiratory viruses were significantly more frequent in patients with acute rejection and steroid treatment. In conclusion: (i) about 50% of respiratory tract infections of lung transplant recipients were associated with one or more respiratory viruses; (ii) hMPV largely predominates in bronchoalveolar lavage of symptomatic lung transplant recipients, thus suggesting a causative role in lower respiratory tract infections; (iii) RT-PCR appears to be the method of choice for detection of respiratory viruses in lung transplant recipients, (iv) a high HCMV load in bronchoalveolar lavage is a risk factor for viral pneumonia, suggesting some measure of intervention for the control of viral infection. ***J. Med. Virol. 78:408–416, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: human metapneumovirus; human cytomegalovirus; lung transplant recipients; respiratory tract viral infections; nasopharyngeal aspirates; bronchoalveolar lavage; reverse transcription-polymerase chain reaction (RT-PCR)

INTRODUCTION

A number of respiratory viruses, including human respiratory syncytial virus (hRSV), human parainfluenza viruses (hPIV) types 1–4, influenza viruses A and B, and human adenoviruses (hAdV), known to be common causes of community-acquired respiratory

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tract infections in both children and adults, are also recognized as important pathogens in both pediatric and adult lung transplant recipients [Wend et al., 1995; Bridges et al., 1998; Krinzman et al., 1998; Palmer et al., 1998; Garantziotis et al., 2001; Vilchez et al., 2001, 2002, 2003; Billings et al., 2002]. The overall incidence of infections caused by different respiratory viruses is reported to vary between 3 and 7 cases per 100 patients in cumulated cohorts of lung transplant recipients, reaching a global incidence of about 20% when considering hRSV, influenzaviruses A and B, hPIV, and hAdV infections altogether [Ohori et al., 1995; Matar et al., 1999; Vilchez et al., 2001, 2002; Billings et al., 2002]. Most of these studies were conducted using poorly sensitive methods for virus detection, while other viruses, such as human coronaviruses (hCoV), rhinoviruses or the recently identified human metapneumovirus (hMPV), were not investigated.

Respiratory viruses commonly cause mild respiratory infections in immunocompetent subjects, while they are often responsible for more severe infections of the lower respiratory tract in lung transplant recipients [Marx et al., 1999; Glezen et al., 2000; Hall, 2001]. Patients with this type of infections have been reported to be predisposed to high-grade bronchiolitis obliterans syndrome with a relative risk of 2.3 [Billings et al., 2002]. In this respect, a number of retrospective studies have been performed, which led to conflicting conclusions [Billings et al., 2002; McCurdy et al., 2003; Khalifah et al., 2004]. However, the number of viruses investigated, the sensitivity of methodologies used, the frequency of respiratory sample collection and the number of respiratory episodes per patient, are variables which have been only partially considered or entirely overlooked in different studies.

In addition, the pathogenic role of human cytomegalovirus (HCMV) infection in bronchoalveolar lavage remains to be defined [Bailey et al., 1995; Riise et al., 2000; Westall et al., 2004] as well as its relationship with bronchiolitis obliterans [Kroshus et al., 1997; Husain et al., 1999; Khalifah et al., 2004; Tamm et al., 2004].

In the present study, nasopharyngeal aspirates and bronchoalveolar lavage samples were examined from the entire cohort of lung transplant recipients followed at our institution in the presence or absence of acute respiratory symptoms, showing that: (i) respiratory viruses are major pathogens in symptomatic patients; (ii) hMPV predominates in positive bronchoalveolar lavage samples; (iii) PCR-based methods are largely superior to direct fluorescent antibody staining and cell cultures for respiratory virus detection; (iv) a high HCMV load represents a risk factor for viral pneumonia.

MATERIALS AND METHODS

Patients

From November 2001 through May 2004, a prospective surveillance study of upper and lower respiratory tract viral infections conducted at our institution in lung transplant recipients involved 49 patients with acute respiratory symptoms and 26 patients asymptomatic or affected by non-respiratory clinical symptoms, such as fever alone or rejection. Follow-up was restricted to winter-spring seasons (November through May) 2001–2002, 2002–2003, and 2003–2004. Viral tests were requested at different times from transplantation and included patients in the immediate post-transplant period as well as patients within 5 years after transplantation.

Patients were classified as symptomatic in the presence of at least one of the following: rhinorrhea, sore throat, cough, sputum, dyspnea, reduced lung function, chest X-rays abnormalities. On the whole, 29 patients had lower, and 20 patients upper respiratory tract infections. Nasopharyngeal aspirates ($n = 56$) were taken from patients complaining from symptoms of the upper respiratory tract without reduced lung function at the onset of acute infection and, when appropriate, during follow-up. Bronchoalveolar lavage samples ($n = 46$) were taken, whenever possible, in the presence of lower airways involvement. In addition, bronchoalveolar lavage samples ($n = 26$) were obtained from asymptomatic patients as a routine follow-up sampling during surveillance bronchoscopies. Bronchoalveolar lavage specimens were examined routinely for the presence of viral, bacterial, and fungal agents. Radiographic and functional data were examined in parallel. Finally, 28 patients (15 symptomatic and 13 asymptomatic) were tested 2–5 times during the same season because of repeated episodes of respiratory tract infection or repeated surveillance bronchoscopies. In this group, positive patients underwent follow-up sampling, whenever feasible, until a negative sample was obtained.

Patients were maintained on triple-drug immunosuppression with corticosteroids, azathioprine, and cyclosporine. In some patients presenting persistent acute rejection or progressive loss of graft function, cyclosporine was replaced by tacrolimus, and/or azathioprine by mycophenolate mophetil. In the absence of graft rejection, cyclosporine and steroids were progressively reduced within 6 months from surgery. The immunosuppression regimen was not modified substantially during episodes of acute respiratory infections.

Viral coinfections were defined as respiratory infections resulting in the simultaneous identification of more than one virus from the same sample. Sequential viral infections included subsequent infection of the same subject by at least one virus different from that detected upon the first episode of respiratory infection during the same season. Detection of the same virus for several weeks was considered evidence of protracted infection.

Specimens

On the whole, 75 lung transplant recipients were examined during the 3-year period for a total of 128 specimens (72 bronchoalveolar lavage samples, and 56 nasopharyngeal aspirates). Upon collection and following homogenisation, secretions from nasopharyngeal

aspirates were divided into four aliquots to be used for: direct fluorescent antibody staining of respiratory cells; inoculation onto cell cultures for virus isolation; molecular assays; and storage of back-up samples. Two diagnostic approaches were used for virus identification in respiratory samples. The immunological approach was aimed at virus identification following staining with monoclonal antibodies of both cells from nasopharyngeal aspirates and inoculated cell cultures. The molecular approach was based on use of PCR-based assays aimed at amplifying nucleic acids from respiratory samples. Viruses detected by monoclonal antibodies were influenza viruses A (H1N1 and H3N2) and B, hPIV 1–4, hRSV, and hAdVs. In addition, hCoV groups I (229E-like) and II (OC43-like), hMPV types A and B, and rhinoviruses were detected by RT-PCR only. The hCoVs were grouped by sequencing, while hMPVs were typed by sequencing and phylogenetic analysis [Rovida et al., 2005].

Immunological Method

A pool of monoclonal antibodies to influenza viruses A and B, hPIV 1–4, hRSV, and hAdV, as well as individual monoclonal antibodies to the same viruses (Chemicon International, Inc., Temecula, CA) were used for virus identification by direct fluorescent antibody staining of both respiratory cells from nasopharyngeal aspirates and trypsinized cells from shell vial or conventional cell cultures [Zavattoni et al., 2003; Rovida et al., 2005].

PCR-Based Assays

RT-PCR assays were optimized to detect at least 10 input plasmid copies. Primers for hCoV (groups I and II) were selected from a published protocol [Poutanen et al., 2003] as well as primers for rhinoviruses [Steininger et al., 2001], whereas primers for hMPV types A and B (genes N and F) were designed originally from GenBank published virus sequences [Sarasini et al., 2005, in press]. Amplification products were cloned in PCR2.1 plasmid vector (TA Cloning Kit, Invitrogen, Carlsbad, CA) to prepare quantitative standards [Rovida et al., 2005]. Nuclisens® Iso Kit (BioMèrieux, Lyon, France) was used to extract nucleic acids, while RT and PCR reactions were mostly performed in real time, as reported [Rovida et al., 2005]. PCR products were examined on 3% agarose gel.

A new real-time PCR technique for HCMV DNA quantitation in clinical samples was developed. Briefly, using the ABI PRISM™ Primer Express™ software (Applied Biosystems, Foster City, CA), the following primer pair was selected for amplification of an IE1 fragment: CMVSMF 5′-TGGACGCTGTGTGGCG-3′; CMVSMR, 5′-GCCGACCCGAGCCACTAT-3′. In addition, a TaqMan® probe labeled at its 5′-end with 6-FAM fluorochrome and as its 3′-end with a MGBNFQ quencher probe was also selected: CMVSM 5′-TATCCCGAGAAAGGG-3′. Oligonucleotides were synthesized, purified and labeled by Applied Biosystems UK, Warrington, Cheshire, UK. Real-time PCR was performed by using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with standard reagents (TaqMan® Universal Master Mix, Applied Biosystems UK). HCMV DNA was coamplified with heterologous DNA (TaqMan® Exogenous Internal Positive Control, VIC™, Warrington, Cheshire, UK) using specific primers and probe to verify the absence of PCR inhibitors, and in parallel with serial amounts of a plasmid carrying the HCMV target sequence (external quantification standards). Following activation of Taq polymerase for 10 min at 95°C, reactions were cycled 50 times at 95°C for 15 sec and at 60°C for 60 sec. A standard curve was constructed automatically by the ABI PRISM 7000 instrument based on the signals from the external quantitation standards, while quantitation of HCMV DNA in clinical samples was obtained by interpolation.

HCMV-Specific T-Cell Immune Response Determination

A method developed recently based on stimulation of patient peripheral blood mononuclear cells by HCMV-infected autologous immature dendritic cells was used to determine HCMV-specific interferon-γ-producing $CD4^+$ and $CD8^+$ T-cells by cytokine flow cytometry [Gerna et al., 2005; Lozza et al., 2005]. Cut-offs to discriminate between positive and negative $CD4^+$ and $CD8^+$ HCMV-specific T cells were 0.4 cells/μl blood or 0.05% for both T-cell subpopulations.

Preemptive therapy of HCMV infections in lung transplant recipients was guided by the antigenemia assay [Gerna et al., 1992] as reported [Gerna et al., 2003].

Statistical Analysis

Comparisons of distribution frequencies of different parameters of the study was performed by using the Pearson chi-square test.

RESULTS

Rate of Respiratory Viral Infections in Lung Transplant Recipients

During the three consecutive winter-spring seasons examined, the overall incidence of lung transplant recipients positive for respiratory viruses was 29/75 (38.7%) patients. However, if the patient population was divided into two subgroups according to the presence or absence of respiratory symptoms, the overall incidence of patients positive for respiratory viruses was 28/49 (57.1%) for symptomatic patients and only 1/26 (3.8%) for control (asymptomatic) lung transplant recipients (Fig. 1A). In detail, 18/29 (62.1%) patients with lower respiratory tract infections, and 10/20 (50.0%) patients with upper respiratory tract infections were positive for respiratory viruses. The seasonal proportion of symptomatic patients positive for respiratory viruses ranged from 47.8% (11/23 patients) in 2003–2004 to 58.3% (7/12 patients) in 2002–2003 and 71.4% (10/14 patients) in 2001–2002 (Fig. 1A).

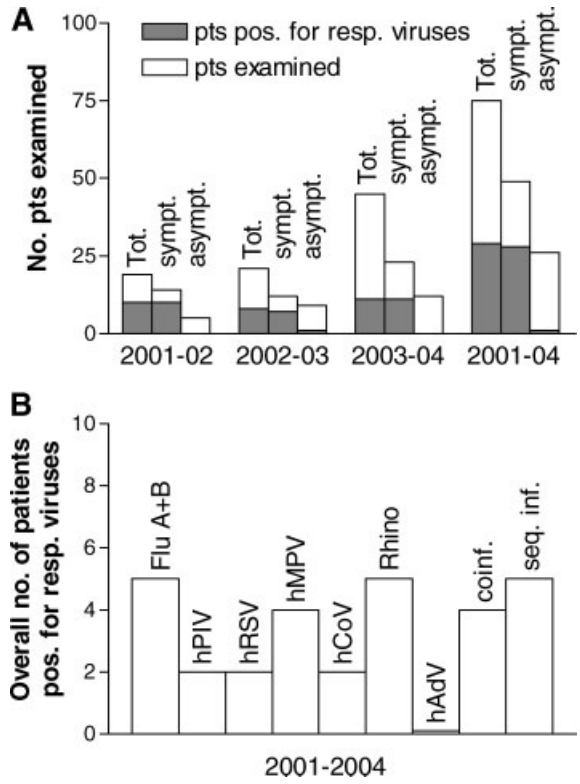

Fig. 1. **A**: Number of patients positive for respiratory viruses over the total number of patients examined among symptomatic and asymptomatic lung transplant recipients during the three winter-spring seasons examined. **B**: Overall number of lung transplant recipients positive for different respiratory viruses in the period 2001–2004.

As shown in Figure 1B, along the entire study period, three patients were infected by influenza virus A, two by influenza virus B, two by hPIV, two by hRSV, four by hMPV, five by rhinoviruses, and two by hCoV (OC43-like), while four patients had coinfections and five had sequential seasonal infections by respiratory viruses. In addition, HCMV was detected sporadically or intermittently in these patients (see below). As for the seasonal circulation of single respiratory viruses, including coinfections and sequential infections, hRSV was the most represented virus in 2001–2002 (four patients), while influenza virus A predominated in 2002–2003 (four patients), and rhinoviruses were the leading cause of respiratory infections in 2003–2004 (six patients).

As for the length of excretion, among the 28 patients examined more than once during the same season, only 3 showed persistent excretion of the same virus, that is, hRSV. The median duration of excretion was 14 (10–23) days. In this group of patients, new-onset clinical episodes were differentiated from continuation of previous episodes on the basis of appearance of a new virus.

Clinical Outcome

All patients with respiratory viral infection recovered from the acute episode either spontaneously or following administration of antiviral therapy. In particular, three patients with severe lower respiratory tract infection caused by influenza viruses were treated with zanamivir for 5 days with complete and rapid resolution of acute respiratory symptoms. In addition, four patients with severe acute hRSV infection were treated with ribavirin for 5–9 days with resolution of symptoms. However, while in one patient symptom resolution was associated with hRSV disappearance from bronchoalveolar lavage, in the other three patients hRSV persisted in the nasopharynx after resolution of symptoms. In 9/28 (32.1%) patients, acute respiratory infection was associated with an episode of acute or chronic rejection. No preferential association of rejection episodes with upper or lower respiratory tract infection was observed.

Rate of Respiratory Virus Detection in Upper (Nasopharyngeal Aspirates) and Lower (Bronchoalveolar Lavage) Respiratory Tract Infections

The overall incidence of bronchoalveolar lavage samples positive for respiratory viruses (25.0%; 18/72 samples) was significantly ($P = 0.002$) lower than that of nasopharyngeal aspirates (53.6%; 30/56 samples). However, the difference was not significant ($P = 0.16$), if only bronchoalveolar lavage samples from symptomatic patients were considered (18/46 samples, 39.1%). During the three consecutive winter-spring seasons, the aliquot of bronchoalveolar lavage samples positive for respiratory viruses ranged from 15.0% (6/40 samples) in 2003–2004 to 33.3% (5/15 samples) in 2001–2002, and to 41.2% (7/17 samples) in 2002–2003, whereas the aliquot of nasopharyngeal aspirates ranged from 38.5% (10/26 samples) in 2003–2004 to 71.4% (10/14 samples) in 2001–2002, and to 62.5% (10/16 samples) in 2002–2003 (Fig. 2A).

As for the distribution of different respiratory viruses, hMPV had a significantly higher incidence in bronchoalveolar lavage samples than in nasopharyngeal aspirates ($P = 0.008$), while the other respiratory viruses were represented at a comparable rate in upper and lower respiratory tract (Fig. 2B). In bronchoalveolar lavage respiratory virus infection was associated to a bacterial or fungal infection in only three lung transplant recipients (one patient was coinfected by hMPV and *A. fumigatus*, one patient by hMPV and *P. aeruginosa*, and one patient by influenza virus A and *P. aeruginosa*). Findings relevant to HCMV are reported below.

Immunological Versus Molecular Methods for Virus Detection in Respiratory Specimens

On the whole, of 128 samples examined, 43 (33.5%) were positive for respiratory viruses by RT-PCR, whereas only 17 (13.2%) were detected by monoclonal antibodies either in nasopharyngeal aspirates or cell cultures or both (Table I). In detail, the proportion of positive samples detected by monoclonal antibodies was very low in 2003–2004 (2/16, 12.5%), whereas it was greater than 50% in the previous two seasons (8/14,

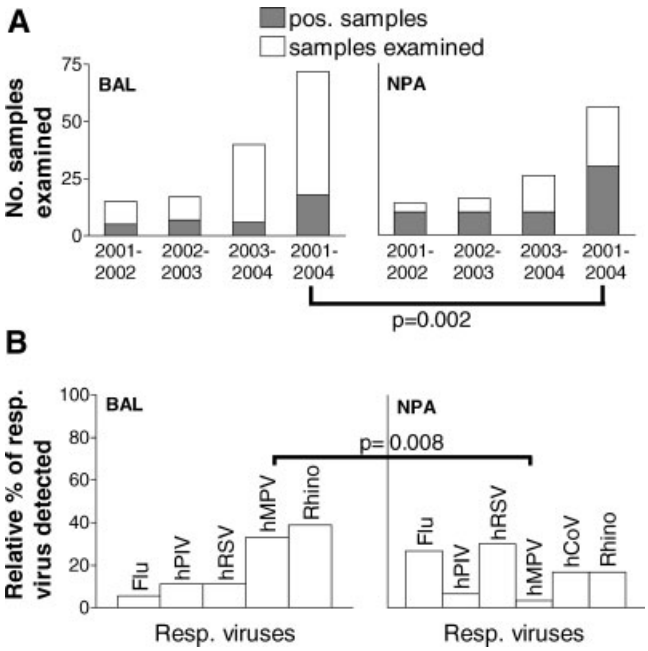

Fig. 2. **A**: Number of bronchoalveolar lavage and nasopharyngeal aspirate samples positive for respiratory viruses over number of the relevant samples examined during the three winter-spring seasons 2001–2004. **B**: Relative frequency of distribution of different respiratory viruses in bronchoalveolar lavage and nasopharyngeal aspirates from lung transplant recipients.

57.1% in 2002–2003; and 7/13, 53.8% in 2001–2002). No specimen detected by monoclonal antibodies was missed by RT-PCR, whereas as many as 26/43 (60.5%) RT-PCR-positive samples were missed by monoclonal antibodies ($P < 0.001$).

Rate of HCMV Infections in the Respiratory Tract of Lung Transplant Recipients

HCMV was detected in respiratory secretions of 18/75 (24.0%) lung transplant recipients. HCMV infection was associated with another respiratory viral pathogen in four patients, and was present as a single infectious agent in 14/18 (77.8%) patients. As for the localization of HCMV infection, virus was detected in 16/72 (22.2%) bronchoalveolar lavage, and in 6/56 nasopharyngeal aspirate (10.7%) samples. The seasonal incidence did not vary greatly, ranging from 24.1% (7/29 samples) in 2001–2002 to 15.2% (5/33 samples) in 2002–2003 and 2003–2004 (10/66 samples).

Details relevant to patients with coinfections and sequential infections, including both respiratory viruses and HCMV, are reported in Table II. On the whole, the incidence of multiple (simultaneous and sequential) seasonal infections was 6/8 (75%) in lung transplant recipients with acute rejection and antirejection steroid treatment, and 4/21 (19.0%) in patients with neither rejection nor steroid treatment. This difference was statistically significant ($P = 0.009$).

Quantitation of HCMV DNA in respiratory secretions showed that high amounts of viral DNA (>1,000 copies/10 μl) in the lower respiratory tract were detected only in 3/16 bronchoalveolar lavage samples from 3 lung transplant recipients (Table III). These 3 patients were part of the group of 21 patients with respiratory infections not caused by common respiratory viruses. All three patients were admitted to the Intensive Care Unit within 6 months after transplantation because of fever, dyspnoea, reduced lung function, and radiologic signs of pneumonia. In two patients, bacterial infections (*P. aeruginosa, S. aureus*) were associated with HCMV and treated with antibiotics. In patient #1, HCMV infection was both systemic and local, and HCMV load in blood reached a peak greater than 100 pp65-positive/2×10^5 leukocytes examined (cut-off for preemptive therapy), thus requiring initiation of antiviral treatment. In patients #2 and #3, HCMV infection was only local and virus was not detected in blood. Thus, patients were not treated, following guidelines adopted in our department. However, all three patients recovered within 2 months and long-term control of HCMV infection in both blood and bronchoalveolar lavage was attained. This was likely due to the fact that in patient #1, the high viral load detected in both blood and lungs 1 month after transplantation was initially controlled by antiviral treatment with ganciclovir prior to the subsequent development of an efficient T-cell immune response. On the other hand, in patients #2 and #3 HCMV load in lungs (prompted by steroid treatment due to acute rejection occurring more than 2 months after transplantation) was controlled by the HCMV-

TABLE I. Diagnostic Efficiency of Monoclonal Antibodies on Respiratory Cells From Nasopharyngeal Aspirates and Shell-Vial Cultures Versus PCR-Based Assays in Detecting Respiratory Viruses and Human Cytomegalovirus (HCMV) in Upper and Lower Respiratory Tract Secretions of Lung Transplant Recipients

	Respiratory samples examined		Number (%) of respiratory samples positive for						
			Respiratory viruses			HCMV			
Winter-spring season	Type	Total number	MAb	RT-PCR	Total	MAb	PCR	Total	Overall total positive samples
2001–2004	BAL	72	2 (3)	15 (21)	15 (21)	7	16 (22)	16 (22)	31 (43)
	NPA	56	15 (27)	28 (50)	28 (50)	0	6 (11)	6 (11)	34 (61)
	Total	128	17 (13)	43 (34)	43 (34)	7	22 (17)	22 (17)	65 (51)

NPA, nasopharyngeal aspirate; BAL, bronchoalveolar lavage; MAb, monoclonal antibody.

TABLE II. Time and Type of Coinfections and Sequential Respiratory Infections by Different Viruses Occurring in Lung Transplant Recipients During the three Consecutive Winter-Spring Seasons Examined

Season	Patient #, age (years)	Date of Tx	Type and date of sample collection	Coinfections and sequential infections	Time post-Tx	Clinical data
2001–2002	1195, 63	September 16, 1998	NPA, January 18, 2002	hPIV-3	40–43 months	Fever, hypoxemia
			NPA, March 11, 2002	hRSV + HCMV		Bronchitis, Hypoxemia, fever
			NPA, April 3, 2002	hRSV		
	1719, 41	October 4, 1999	NPA, January 28, 2002	hCoV (OC43)	27–30 months	Bronchitis
			NPA, March 21, 2002	HCMV		Asymptom
			BAL, April 26, 2002	Rhino + HCMV		Pharingitis, bronchitis
	5053, 36	January 31, 2000	NPA, March 19, 2002	FluA (H3N2) + hRSV	26 months	Rhinitis, fever
	2289, 39	March 26, 2000	BAL, Feburary 6, 2002	hPIV + hRSV	23 months	Rhinitis, bronchitis
2002–2003	4043, 48	July 30, 2001	NPA, Feburary 18, 2003	FluA (H3N2)	19–20 months	Fever, bronchitis hypoxemia
			NPA, February 26, 2003	FluA (H3N2)		Ac. reject. (ST)
			NPA, March 7, 2003	hRSV + hMPV		Fever
			NPA, March 11, 2003	hRSV + HCMV		Fever
			NPA, March 17, 2003	hRSV		Fever
	20491, 41	October 4, 1999	BAL, December 3, 2002	hMPV + Rhino	34 months	Fever, bronchitis, hypoxemia, <FEV, ac. reject. (ST)
	21418, 61	October 17, 2002	BAL, December 16, 2002	hRSV + Rhino	2 months	Fever, bronchitis, pneumonia, hypoxemia, ac. reject. (ST)
2003–2004	22330, 33	October 30, 2003	BAL, January 9, 2004	HCMV	3 months	Asymptomatic
			NPA, Feburary 10, 2004	FluA (H3N2)	4 months	Rhinitis, bronchitis, pneumonia, <FEV, ac. reject. (ST)
			BAL, April 5, 2004	hMPV (B)	6 months	Bronchitis, <FEV
			BAL, May 5, 2004	Rhino	7 months	Rhinitis
	543, 62	February 24, 2003	BAL, January 12, 2004	hMPV (A) + HCMV	11 months	Rhinitis, ac. reject. (ST)
	585, 57	September 17, 2003	BAL, January 13, 2004	Rhino	4 months	Rhinitis, fever, ac. reject. (ST)
			NPA, February 3, 2004	hCoV (229E)	5 months	Rhinitis, fever
			NPA, February 24, 2004	hCoV (229E)	6 months	Asymptomatic

NPA, nasopharyngeal aspirate; BAL, bronchoalveolar lavage; FluA, influenza virus A; hPIV, human parainfluenza virus; hRSV, human respiratory syncytial virus; hMPV, human metapneumovirus; hCoV, human coronavirus; Rhino, rhinovirus; HCMV, human cytomegalovirus; ST, steroid therapy; FEV, forced expiratory volume.

TABLE III. Outcome of HCMV Infection in Blood and Bronchoalveolar Lavage (BAL) From three Lung Transplant Recipients

Patient #, years	Time post-Tx (days)	HCMV load in		Antiviral treatment	HCMV-specific T-cells[a]		Clinical events	Outcome of HCMV infection in lungs
		Blood (Ag)	BAL (DNA copies/ml)		$CD4^+$	$CD8^+$		
1, 59	32	120	5.8×10^6	GCV	−	−	Pneumonia	
	60	Neg	Neg	Discontinued	−	+	Resolved	Resolution
2, 46	195	Neg	2.2×10^5	No	+	+	Ac. reject., steroid treatment, pneumonia	
	210	Neg	Neg	No	+	+	Resolved	Resolution
3, 32	72	Neg	5.0×10^5	No	+	+	Ac. reject., steroid treatment, pneumonia	
	93	Neg	$<1.0 \times 10^3$	No	+	+	Resolved	Resolution

Ag, antigenemia, that is, number of pp65-positive leukocytes/2×10^5 leukocytes examined.
[a]−, <0.4 T-cells/μl blood; +, >0.4 T-cells/μl blood.

specific $CD4^+$ and $CD8^+$ immune responses, which were reconstituted 3–6 months after surgery (Table III). In two patients, bronchiolitis obliterans syndrome emerged 6–12 month after the reported episodes of pneumonia.

DISCUSSION

Major conclusions emerging from this study are: (i) respiratory viruses were detected in the majority of lung transplant recipients with respiratory symptoms; (ii) coinfections and sequential infections were detected in a fairly high proportion of symptomatic patients (9/28, 32.1% for respiratory viruses, and 10/28 including HCMV); (iii) the rate of respiratory virus detection was comparable in both bronchoalveolar lavage and nasopharyngeal aspirates from symptomatic patients; (iv) however, hMPV infections were significantly higher in bronchoalveolar lavage samples compared to nasopharyngeal aspirates; (v) respiratory viruses were detected less frequently by monoclonal antibodies compared to RT-PCR; (vi) HCMV was detected frequently in the respiratory tract of lung transplant recipients; however it was associated to a high viral load in bronchoalveolar lavage from only 3/16 patients with a lower respiratory tract infection.

As for the first point, the results of this study strongly support the conclusion that detection of respiratory viruses in the upper or lower respiratory tract of lung transplant recipients is mostly associated with the presence of upper or lower respiratory symptoms. In addition, the incidence of viral coinfections and sequential infections in lung transplant recipients does not seem to be substantially different from that reported in previous studies in the general population, representing a fair proportion of respiratory infections [Coiras et al., 2003; van den Hoogen et al., 2003, 2004; Rovida et al., 2005]. However, antirejection treatment appears to be a significant risk factor for occurrence of multiple infections.

The comparable frequency of detection of respiratory viruses in bronchoalveolar lavage and nasopharyngeal aspirate samples indicates that viral respiratory infections involve the lower as well as the upper respiratory tract in lung transplant recipients. In particular, in the current study, the significantly higher incidence of hMPV infections in bronchoalveolar lavage was surprising when compared to nasopharyngeal aspirate samples, while all the other respiratory viruses were detected at a comparable rate at the two sites. Although this respiratory virus [van den Hoogen et al., 2001] has been reported to be pathogenic for immunocompromised patients, the present findings suggest the need for careful monitoring of hMPV infections in lung transplant recipients. Molecular methods, such as RT-PCR, should be adjusted to detect all hMPV subtypes [Sarasini et al., in press], while hMPV-specific monoclonal antibodies have been recently developed in our laboratory as a complement to or an alternative to RT-PCR for diagnosis of hMPV infections [Percivalle et al.,

2005]. The reported lack of hMPV detection in bronchoalveolar lavage samples from lung transplant recipients is likely to reflect poor circulation of the virus in the local community [Garbino et al., 2004].

From a diagnostic standpoint, in this study the immunological approach for detection of respiratory viruses using monoclonal antibodies appeared to be much less sensitive than the molecular approach using RT-PCR, the former detecting about 1/3 of samples positive by RT-PCR. Monoclonal antibodies may provide test results within 2 hr after nasopharyngeal aspirate collection. On the other hand, direct fluorescent antibody staining cannot be applied reliably to bronchoalveolar lavage specimens, for which rapid viral isolation in shell vial cultures followed by monoclonal antibodies identification should be the test of choice. Use of appropriate and sensitive methods for rapid detection of respiratory viruses is of crucial importance in light of advances in antiviral therapy and potential threats from pandemic influenza. In our study, use of zanamivir in three patients with lower respiratory tract infection caused by influenza viruses and use of ribavirin in four patients with lower respiratory infection caused by hRSV contributed to resolve the acute episode.

As for HCMV detection in respiratory specimens, while its presence in nasopharyngeal aspirates is not significant clinically, presence in bronchoalveolar lavage seems to correlate both with clinical symptoms and histological detection of HCMV-specific inclusion bodies in trans-bronchial biopsies. However, HCMV infection in bronchoalveolar lavage must be considered in quantitative terms to predict reliably biopsy-proven HCMV lung infection [Bailey et al., 1995; Riise et al., 2000; Westall et al., 2004]. The presence of HCMV DNA at a level above a predetermined threshold value may indicate either a high risk of emergence or the presence of an overt lower respiratory tract disease, thus suggesting a timely initiation of antiviral treatment at a full dosage. Prophylaxis protocols using reduced ganciclovir dosage have not been found to prevent HCMV infection as detected in bronchoalveolar lavage samples [Westall et al., 2004].

It is well known that organ localization of HCMV infection may be dissociated from systemic infections, that is, from the presence of virus in blood, both in AIDS patients and lung transplant recipients [Gerna et al., 1994; Sanchez et al., 2001; Westall et al., 2004]. Hence, the need for monitoring HCMV in both blood and bronchoalveolar lavage samples in view of starting treatment even in the presence of high virus loads in bronchoalveolar lavage alone. Recently, Westall et al. [2004] have determined that DNA levels above 46,000 copies/ml in bronchoalveolar lavage samples are predictors of HCMV inclusions in the lung allograft of 100% lung transplant recipients. In addition, the same investigators have speculated that a HCMV load greater than 64,000 copies/ml is more likely to be associated with specific disease, such as HCMV pneumonitis [Sanchez et al., 2001]. In all three patients of our cohort with high HCMV load in bronchoalveolar lavage, local resolution of HCMV infection was achieved by reconstitution of specific T-cell immunity, which chronologically occurred in the absence of antiviral treatment in patients #2 and #3, and following a timely start of ganciclovir treatment at a full dosage in patient #1.

Finally, both respiratory virus and HCMV infections have been suggested as potential cofactors in the pathogenesis of bronchiolitis obliterans syndrome. However, as mentioned above the literature data in this respect are conflicting [Kroshus et al., 1997; Husain et al., 1999; Khalifah et al., 2004; Tamm et al., 2004; Westall et al., 2004]. Whether respiratory virus and HCMV infections of the respiratory tract may play a causative role in the pathogenesis of bronchiolitis obliterans should be investigated in multi-center large-scale prospective studies, by periodically determining the clinical, pathological and functional conditions of the respiratory tract of patients who received lung transplants.

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