BRONCHOALVEOLAR LAVAGE LYMPHOCYTE CYTOTOXICITY IN REJECTING AND INFECTED LUNG ALLOGRAFTS

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PREFACE

Transplantation of the lung has rapidly evolved in the last decade to become an accepted therapeutic modality for certain endstage lung diseases. Its wide clinical application is still hampered by the high incidence of graft rejection or infection. It is still difficult at times to make the distinction between graft rejection and infection especially chronic rejection and indolent opportunistic lung infection. The combination of transbronchial biopsy and bronchoalveolar lavage (BAL) is the diagnostic method widely used to differentiate between these two pathologic entities. The work presented in this thesis is part of an experimental study designed to characterize BAL cellular phenotypes and lymphocyte cytotoxic functions following different types of lung injuries, those that are frequently encountered following lung transplantation such as atelectasis, warm and cold ischemia/reperfusion injury, rejection and bacterial infection. The goal of the study is to explore the potentials of BAL as the non-invasive diagnostic method capable of differentiating rejection from other forms of lung injuries. Results of this comprehensive research project have been published in parts elsewhere: Warm Ischemia Induces Alteration in Lung Immune Cell Function (Journal of Thoracic and Cardiovascular Surgery 1991;101:1030-36), Altered Cellular Immune Function in the Atelectatic Lung (Annals of Thoracic Surgery 1991;51:76-80), Bronchoalveolar Lavage and Lung Transplantation (Annals of Thoracic Surgery 1991;51.335-40), Bronchoalveolar Lavage Lymphocyte Cytotoxicity: An Indicator of Adequacy of Lung Preservation (Canadian Journal of Surgery 1991,34.396).

The materials presented in this thesis focus on the alterations of BAL lymphocyte cytotoxic functions in lung allograft rejection or infection. This experimental work was supported by a grant from the Canadian Cystic Fibrosis Foundation. The author was a recipient of the Canadian Cystic Fibrosis Foundation Research Fellowship. This project was awarded the Stykeman Research Prize of the Department of Cardiovascular and Thoracic Surgery, McGill University (1990). Parts of the results were presented at the 70th Annual Meeting of American Association for Thoracic Surgeons (Toronto, Canada, 1990) and at the 1st Annual Meeting of the European Respirology Society (London, England 1990). The manuscript of this work has been accepted for publication in the journal Transplantation.

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ABSTRACT

Differentiation between rejection and infection of lung allografts remains difficult The effects of these two pathologic entities on the cytolytic activity of bronchoalveolar lavage (BAL) and peripheral blood lymphocytes (PBL) were investigated. Left lung allotransplantation was performed on sixteen mongrel dogs of which twelve were available for complete studies. All animals received cyclosporine A, azathioprine and prednisone for 2 weeks. Four grafts developed left lower lobe Gram negative pneumonia. The eight remaining recipients progressed gradually to severe rejection following acute reduction of immunosuppression. Cytolytic activity of blood and left lung BAL lymphocytes was quantitated by the natural killer (NK) and lectin dependent cell mediated cytotoxicity (LDCMC) assays. Two additional groups serving as controls were either given a ten day course of immunosuppressants or had right lower lobe pneumonia induced by transbronchial inoculation of gram negative bacteria. Immunosuppressed control animals showed significant depression of PBL and BAL lymphocyte LDCMC and NK activity. Similarly, BAL lymphocytes expressed very low LDCMC in normal allografts (2.8±.8%). Once rejection developed and progressed, LDCMC became significantly higher (15.6±2.2% and 52.7±2.8% in moderate and severe rejection respectively). There was no detectable NK activity in rejecting lung allografts. BAL lymphocytes from infected allografts, on the other hand, showed an elevation of both NK and LDCMC activity (9.1±1.1% and 14.6±1.0% respectively). Similarly, bacterial pneumonia in control animals manifested an increase in NK and LDCMC activity in lung and blood. Peripheral blood lymphocytes of lung allograft recipients, however, had increased NK and LDCMC activity in both rejection and infection LDCMC/NK activity ratio (LM/NK index) of lung lymphocytes was significantly higher in rejecting allografts (11.2±1.0 and 12.4±1.6 for moderate and severe rejection respectively) than in infected ones (1.2±.3, $p<0.0001$). It appeared, from this study, that rejection of the lung allograft results in alterations in BAL lymphocyte phenotypes and functions which differ from those associated with bacterial infection. Such differences may be useful in distinguishing episodes of acute allograft rejection from bacterial infection.

RESUME

La différenciation entre le rejet et l'infection des allogreffes pulmonaires demeure difficile. Les effets de ces deux entités pathologiques sur l'activité cytolytique du "bronchoalveolar lavage (BAL)" et des "peripheral blood lymphocytes (PBL)" furent investigués. L'allogreffe du poumon gauche fut effectuée sur seize chiens bâtards dont douze furent utilisables pour études complètes. Tous les animaux ont reçu de la cyclosporine A, de l'azathioprine et de la prednisone pour une période de deux semaines. Quatre greffés développèrent une pneumonie bactérielle du lobe inférieur gauche. Les huit autres progressèrent graduellement vers le rejet sévère à la suite d'une réduction aigue du système immunodépresseur. L'activité cytolytique du sang et des lymphocytes BAL du poumon gauche fut quantifiée par le "natural killer (NK)" et les essais du "lectin dependent cell mediated cytotoxicity (LDCMC)". On a donné à deux autres groupes servant de contrôle, soit des immunodépresseurs pour une période de 10 jours ou bien nous avons provoqué chez eux une pneumonie du lobe pulmonaire droit par l'inoculation transbronchique d'une bactérie. Les animaux immunodépressifs de contrôle ont démontré une dépression significative du PBL et des lymphocytes BAL LDCMC et de l'activité du NK. De façon similaire, les lymphocytes BAL ont démontré un LDCMC très bas dans les allogreffes normales (2.8 ± .8%). Une fois le rejet développé et progressé, le LDCMC est devenu significativement plus élevé (15.6 ± 2.2% et 52.7 ± 2.8% pour le rejet modéré et sévère respectivement). Il n'y avait pas d'activité du NK détectable dans le rejet des allogreffes pulmonaires. D'un autre côté, les lymphocytes BAL provenant des allogreffes infectées ont démontré une élévation du NK et de l'activité du LDCMC (9.1 ± 1.1% et 14.6 ± 1.0% respectivement). De façon similaire, la pneumonie bactérielle chez les animaux de contrôle a démontré une augmentation du NK et de l'activité du LDCMC dans les poumons et le sang. On a noté que les PBL, provenant des animaux ayant reçu l'allogreffe, avaient cependant subit une augmentation du NK et de l'activité du LDCMC lors du rejet ou de l'infection. Le rapport entre l'activité du LDCMC/NK (LM/NK index) des lymphocytes pulmonaires était significativement plus élevé dans les allogreffes rejetées (11.2 ± 1.0 et 12.4 ± 1.6 pour les rejets modérés et sévères respectivement) que dans celles infectées (1.2 ± .3, $p<0.0001$). Il apparaît, à partir de cette étude, que le rejet de l'allogreffe pulmonaire est

le résultat des altérations dans les phénotypes et des fonctions lymphocytes BAL qui diffèrent de celles associées avec l'infection bactérielle. De telles différences peuvent être utiles dans la distinction entre un épisode de rejet aigu d'allogreffe ou celui d'une infection bactérielle.

I. INTRODUCTION

1. An Overview of Lung Transplantation

Lung and heart-lung transplantation has rapidly evolved in the last decade to become an accepted therapeutic option for selected patients with endstage lung or combined heart-lung disease[1-3]. Up to 1,212 heart-lung and 1056 lung transplants performed before January 1 1992 from more than two hundred transplant centres worldwide were reported to the Registry of the International Society for Heart and Lung Transplantation [4]. The number of procedures has increased exponentially in the last few years. A great deal of knowledge on various aspects of lung transplantation has been accumulated through well-conceived experimental and clinical research. Various problems, seemed unsurmountable by lung transplant workers in the earlier days, have been and are being systemically addressed and solved. As clinical experience increases, the indications for pulmonary transplantation, donor and recipient selection criteria have changed to allow effective use of the scarce donor pool and to serve a larger and more heterogenous group of patients with endstage lung disease. It seems that problems related to organ preservation, to graft acute and chronic rejection as well as to opportunistic infection of the immunosuppressed hosts are the focus of attention of the lung transplant community in the years to come.

1.1 The Evolution of Lung Transplantation

The earliest experiments involving allo-transplantation of a pulmonary lobe, of an entire lung or heart-lung block were performed by Demikhov and associates in the late 1940's[5]. No immunosuppression was used and the longest surviving animal died at 10 days with a bronchopleural fistula. In the early 1950's, Metras from France and Neptune and co-workers from United States independently demonstrated the technical feasibility of single lung transplantation in a canine model[6,7]. They were able to decrease the incidence of thrombosis of the pulmonary venous anastomosis by transecting a cuff of left atrium which included the orifices of the pulmonary veins, thereby eliminating the need for individual vein anastomosis. The first successful long-term re-implantation of the entire lung in a dog was reported by Juvenelle and associates in 1951[8]. The animal survived for 5 months by the time of their report and retained normal lung function. Hardin, Kittle, and Schafer

published their preliminary observations on canine left lung allo-transplantation in 1954[9]. By performing right pneumonectomy immediately after left lung allotransplantation, these investigators demonstrated that the transplanted lungs could sustain pulmonary function and life on their own abeit for a short period of time. They also observed longer survival in recipients treated with cortisone or received lungs from their littermates. Numerous physiologic, pathologic as well as immunologic studies on auto and allotransplanted lungs were reported following these pioneering works[10]. The first human single lung allo-transplantation performed by Hardy and associates at the University of Mississippi in 1963[11]. The recipient suffered from advanced emphysema, chronic renal disease and squamous cell carcinoma completely obstructing the distal left main stem bronchus. The left lung was harvested from a patient who died from irreversible cardiogenic shock and pulmonary edema. Immunosuppression was achieved with azathioprine, corticosteroid and mediastinal irradiation. The patient survived 18 days with good pulmonary function and death was attributed to renal failure and malnutrition. There was no gross or histopathologic evidence of graft rejection on post-mortem examination of the transplanted lung. During the 20-year period subsequent to this limited clinical experience, there were 40 sporadic unsuccessful attempts of human lung transplantation performed by a large number of surgeons at various institutions none of whom had extensive experience with problems peculiar to transplantation of the lung[12,13]. It is interesting to note that one of the first few lung allotransplants was performed at the Royal Victoria Hospital, Montreal in 1963 on a patient suffering from advanced silicosis. The patient survived only 7 days after grafting. Postmortem examination revealed bronchopneumonia, bronchial infarction, lymphatic engorgement and mild rejection[14]. The clinical results were uniformly disappointing: the mean survival was only 8.5 days; 2 patients survived for 6 and 10 months. The only recipient to leave hospital, and the most notable success in this period, was a young man with endstage silicosis who received a right lung transplant by Derom and colleagues from Belgium[15]. The initial eight months were spent in hospital, with only a brief period of survival after discharge. Death was attributed to bronchial complication and infection. The main factors contributing to the uniformly poor outcome include inappropriate selection of recipients and donors, inadequate lung preservation, poor healing of the bronchial

anastomosis, ineffective immunosuppressive regimes, inability to differentiate between graft rejection and infection.

Despite the poor initial clinical results, research efforts on lung transplantation continued and concrete progress have been made to overcome various problems associated specifically with the transplantation of this organ. Three key problems were identified: rejection of the allograft, bronchial anastomotic complications, early mortality from respiratory failure. Azathioprine, adrenal corticosteroids, antilymphocyte immunoglobulins did not provide the lung allografts adequate protection against rejection. Moreover, their uses were associated with high incidence of graft loss and infections[16,17]. The discovery cyclosporin A has revolutionalized the field of organ transplantation. The utilization of this immunosuppressive agent in canine lung and primate hear-lung transplant recipients resulted in significant reduction in allograft rejection, easily reversible acute rejection episodes and long-term survivors with adequate pulmonary functions[18,19] Major complications relating to the bronchial anastomosis (disruption, mucosal necrosis, infection, stenosis) occurred and contributed directly to the deaths of nearly all patients who received unilateral pulmonary transplantation prior to 1982[16]. Factors thought to play a role in this complication include ischemia of the donor bronchus due to transection of the bronchial arterial supply, poor tissue preservation, detrimental effect of high dose corticosteroid used in the perioperative period and rejection. The addition of cyclosporin A to the immunosuppressive regime allows better control of rejection with the avoidance of corticosteroids in the perioperative period. Both of these factors improve significantly the healing of the bronchial anastomosis[19-22]. Wrapping of the bronchial anastomosis with an omental pedicle, intercostal muscle flap or peritracheal membranous adipose tissue flap has been shown to rapidly restore systemic circulation to the donor bronchus and decrease significantly the risk of bronchial anastomotic dehiscence in both experimental and clinical settings[23-27].

Peri-operative respiratory failure was one of the important factor contributing to recipients' demise in the earlier attempts of human lung transplants. Poor lung preservation with prolonged inadequately protected ischemia cause tissue injury and the development of

increased capillary permeability pulmonary edema[12]. Lymphatic disruption and operative manipulations further compounded on ischemia/ reperfusion lung injury Major advances have been made in the field of lung preservation in the last 25 years. Early methods of lung and heart-lung preservation such as topical cooling after absorption atelectasis or normothermic autoperfusion of the heart-lung block have been practically abandoned[28,29]. Single flush perfusion of the lungs with cold modified Euro-Collins solution and donor treatment with prostaglandin E_1 or prostacyclin followed by static cold storage is now by far the most commonly used technique in clinical lung preservation today[30]. Prostaglandins have a wide variety of properties that are of theoretical advantages to lung preservation: they are powerful pulmonary vasodilators, they down regulate inflammatory reaction by attenuating leucocyte sequestration in damage tissues, inhibiting platelet aggregation, preventing lysosomal enzyme release and superoxide production by white cells, they also have cytoprotective effect[31]. This method of lung preservation has experimentally been shown to provide adequate protection for up to 24 hours of hypothermic ischemia. Clinically, however, the upper limit of cold ischemic time is probably 6 to 8 hours Donor-core cooling on cardiopulmonary bypass has been used with good success for heart-lung block transplantation[32]. Short and long term physiologic studies of the denervated lung implants provide valuable information about the pulmonary function and hemodynamic performance following transplantation. Auto-transplantation experiments have shown that the denervated lungs are capable of providing total pulmonary function and receiving the entire cardiac output at all times without abnormal elevation of the pulmonary artery pressure[33-38]. These observations have also been confirmed clinically[39-41].

1.2 The Current Clinical Experience

In 1982, Reitz and colleagues from Stanford University reported the first long term survivors of combined heart and lung transplantation[42]. Their patients had pulmonary hypertension of various etiologies. Two patients had excellent cardiopulmonary function 8 and 10 months after transplantation. The third patient with transposition of the great vessels and associated cardiac defects died 4 days post-operatively of renal, hepatic and pulmonary complications. Immunosuppressive regime consisted of cyclosporin A, prednisone,

azathioprine and peri-operative rabbit antihuman thymocyte globulin. In 1986, the Toronto Lung Transplant Group reported two cases of successful single lung transplantation for endstage pulmonary failure due to pulmonary fibrosis[26]. The two recipients were alive and well with excellent pulmonary function at 14 and 26 months after the procedure. Immunosuppression consisted of cyclosporin A and azathioprine with the addition of oral prednisone 3 weeks postoperatively. Three years later, in 1989, The Toronto Lung Transplant Group reported their preliminary clinical experience with 6 successful double lung transplants for patients with endstage obstructive lung disease. Those patients were alive and well 5 to 15 months after transplantations[43]. These initial clinical successes quickly renewed worldwide interests in pulmonary transplantation. There has been an exponential increase in the number of transplant centres and of lung and heart-lung transplants performed in the last few years (Figure 1). As clinical experience with lung transplantations accumulate, the indications for single, double and heart-lung transplants expand and change significantly[4]. As of 1992, the major indications for heart-lung transplants are primary pulmonary hypertension, Eisenmenger's syndrome secondary to congenital heart disease, cystic fibrosis and emphysema. For double lung transplants, the main indications are cystic fibrosis and emphysema. A smaller percentage of double lung transplants were performed for patients with pulmonary fibrosis, primary pulmonary hypertension, Eisenmenger's syndrome. The greatest change has occurred in the indications for single lung transplantation. It is still the procedure of choice for patients with endstage pulmonary fibrosis. Increasing number of patients with either pulmonary hypertension (primary or secondary) or emphysema received single lung grafts with good results on short term follow-ups. Double lung transplants has been the procedure of choice for these conditions. The concern of overexpansion of the emphysematous native lung compressing the graft or respiratory problems secondary to ventilation/perfusion imbalance have not been observed clinically following single lung transplantation for such conditions[40,41,44-49]. Even though surgical techniques have been well described and accepted[50-52], further refinements have decreased operative morbidity and mortality. Bilateral thoracosternotomy (clam shell) incision provides superb exposure of the thoracic cavities. Sequential bilateral lung transplantation allows avoidance, in certain cases, of cardiopulmonary bypass and its

associated bleeding complications[48]. The use of bilateral bronchial anastomosis instead of the tracheal one in double lung transplants has resulted in a decrease in the incidence of airway complication as reported by Noiclerc and associates[53]. According to the Registry of the International Society for Heart and Lung Transplantation, the overall one-year actuarial survival rate is 59% for heart lung, 69% for single lung and 62% for double lung. Intra-operative technical complications (including primary graft failure and hemorrhage) and cardiac complication account for about one third of all deaths. Infection is the most common cause of death in lung (55%) and heart-lung (34%) transplant recipients. Rejection is reported as the cause in 10% of the mortality in lung recipients[4].

1.3 The Problem of Allograft Rejection and Infection

Post-transplant acute and chronic rejection (AR and CR) as well as bacterial and opportunistic infection continue to inflict considerable morbidity and mortality to lung transplant recipients. Histopathologic classification of lung rejection is now standardized following a recent meeting of the Lung Rejection Study Group sponsored by the International Society for Heart Transplantation[54] (Table 1). Acute rejection has been observed as early as 7 days after transplantation, up to 60% of recipients have one or more AR episodes (clinical diagnosis or biopsy proven) in the first 3 months after grafting[55-57]. Although the likelihood of rejection appears to decrease over time, longitudinal follow-up has demonstrated that late episodes are not infrequent[56]. It is often clinically suspected on the basis of low grade temperature, dyspnea, hypoxia, crackles and wheezing on chest physical examination, new perihilar infiltrate on chest roentgenography, deterioration of lung flow spirometry (FEV_1, FVC). These signs and symptoms are sensitive but not specific for lung rejection as viral (CMV), protozoal (Pneumocystis carinii) lung infection may have similar manifestation. The majority of acute rejection episodes can be reversed with boluses of intravenous methylprednisolone (Solumedrol). Actually, initial clinical response to boluses of steroids is currently used as one diagnostic criteria for rejection. More powerful immunosuppressants such as the monoclonal antibody OKT3 or the polyclonal antithymocyte globulin have to be used for occasional steroid resistant rejection[58]. Chronic rejection of the graft continues to be a major cause of late post transplant morbidity and mortality. The

clinical manifestation of CR is bronchiolitis obliterans. It usually run an insidious course with gradual decline of lung function as noted on flow spirometry. If detected early, its progression can be arrested or reversed; however, if diagnosed late, it usually follows a relentlessly progressive and ultimately fatal course.

It is now well recognized that, in heart-lung transplant recipients, lung rejection can occur in the absence of and often precedes cardiac rejection; hence, endomyocardial biopsy is not a reliable method of detecting lung rejection. Conventional noninvasive methods such as chest roentgenographs, radionuclide scans, pulmonary function test used in conjunction with clinical assessment have been shown to be nonspecific for the diagnosis of allograft rejection. Changes of pulmonary function test parameters or chest roentgenographic appearance associated with rejection have similarly been observed with infection. Differentiation of graft rejection from infection remains difficult. Open lung biopsy provides ample tissue for histopathologic examination yet it is too invasive to be performed routinely. Transbronchial biopsy (TBB) as advocated by the Papworth group in UK has become the method of choice for diagnosing rejection in lung transplant recipients. When performed for clinical indications, TBB has an overall diagnostic yield for rejection or infection of approximately 85%[55]. For the detection of suspected rejection alone, sensitivity of 84% to 93% and up to 100% specificity have been reported[59-62]. Bronchoalveolar lavage (BAL) is frequently performed together with TBB to exclude infection as the cause of clinical abnormalities. Multiple biopsies are routinely done to reduce false negative rate[62]. This technique carries a low complication rate (pneumothorax 5.5%, self limited bleeding 12%) and 1 death (0.4%) as reported by Scott and associates[62]. Better survival rate has been achieved with frequent monitoring of the grafts using serial pulmonary function test, TBB, BAL allows early detection and specific treatment of asymptomatic graft rejection and/or infection[63]. Examination of BAL-derived lymphocyte specific immunoreactivity against donor antigens has 91% sensitive but much lower specificity as a diagnostic method for lung AR because positive results have been observed during bacterial, viral and protozoal infection[64]. However, this technique is better at detecting chronic rejection and bronchiolitis obliterans[65]. Measurement of bronchial mucosal blood flow using laser

flowmeter via a flexible bronchoscope as a method to detect lung AR has been reported with promising results[66,67].

Bacterial pneumonia is the most common infection in the early post transplant period[68-70]. Although the risk of bacterial pneumonia is highest in the early post-operative course, its prevalence is rather uniform throughout the post-transplant follow-up period. The problem is more serious in cystic fibrosis patients undergoing double lung transplantation as they frequently harbour multiply resistant Gram negative organisms notably Pseudomonas species[45]. The diagnosis is made on the basis of fever, pulmonary infiltrate on chest roentgenography and isolation of pathogens on sputum or bronchial washings. There have been a very high incidence of opportunistic infections associated with immunosuppression as the number of transplant recipients increases. Pneumocystis carinii infection was noted in high percentages of heart-lung transplant recipients[71,72]. The diagnosis was usually made by the presence of foamy exudate containing silver staining cysts on silver methenamine stain of the BAL samples. The incidence of infection was decreased by the institution of prophylactic trimethoprime-sulfamethoxazole. The most troublesome is cytomegalovirus (CMV) infection. Elevated incidence of CMV infection (especially gastroenteropathy and pneumonitis) and its associated high morbidity and mortality rate has been observed by various transplant centres. The diagnosis of CMV pneumonitis is made on cytologic and histologic examination of BAL, TBB samples and in certain cases open lung biopsy specimen. It is most frequent and highly fatal in seronegative recipients of seropositive donors. Cytomegalovirus infection in seropositive recipients is usually mild and carries a much lower mortality[45,46]. Cytomegalovirus pneumonitis poses a special problem because its clinical profile is so similar to acute rejection, but the therapy is quite different. Accurate differential diagnosis of these two pathologic entities is therefore essential. There have been evidence of increase risk of chronic rejection following episodes of infection[73]. It is possible that infection augments graft immunogenicity or reduction of immunosuppression in response to the presence of opportunistic infection or both result in higher chance of graft rejection[74]. Measures currently employed to reduce the rate of opportunistic infection include: avoid mismatching of CMV status, prophylactic anti-CMV

hyperimmunoglobulin, use of CMV negative blood products, prophylatic acyclovir (for both CMV and Herpes Simplex virus), and trimethoprime-sulfamethoxazole. The routine perioperative prophylactic use of OKT3 has been abandoned by few centres as it has been reported to associate with a high incidence of severe cytomegalovirus (CMV) infection[75]. Frequent and aggressive surveillance for asymptomatic opportunistic infection with BAL, TBB, viral/protozoal serology and early treatment would improve long term survival[55].

2. Bronchoalveolar Lavage in Lung Transplantation

2.1 An Overview

Lavage of the bronchoalveolar space to obtain alveolar macrophages for functional and morphologic studies was first reported in 1960[76]. Following the development of the flexible bronchoscope, bronchoalveolar lavage (BAL) was popularized as a diagnostic and research tool to study a variety of interstitial lung diseases[77]. Generally, BAL is done in conjunction with fiberoptic bronchoscopy and light sedation. Sterile buffered saline is the usual lavage medium, infused through the working channel of the bronchoscope with the tip wedged to a distal subsegmental bronchus. Aliquots of 50 ml are injected to an accumulative amount of 200 to 300 ml. The lavage fluid is retrieved by gentle suctioning. On average 50% to 60% recovery of the lavage fluid is expected. The application of BAL as a research and as a diagnostic tool in pulmonary transplantation has recently been reviewed by Nguyen and Shennib[78]. It offers a unique opportunity for the safe and repetitive harvesting of large quantities of graft infiltrating immunocompetent cells in the transplanted lung. Retrieved cell suspensions do not require complicated cell separation methods in order to obtain purified lymphocytes as done from tissue fragments of other organs. Phenotypic and immunologic studies of graft-infiltrating cells are essential to advance our understanding of the mechanisms of lung rejection as well as the development of a reliable method to detect and differentiate rejection from various infections in the lung allograft. Functional studies of graft lymphocytes retrieved by BAL and those propagated from transbronchial biopsy specimen demonstrated that BAL lymphocyte immunologic activities reflect those of the intragraft lymphocytes. This observation reaffirms the usefulness of BAL in the immunologic monitoring of lung allograft recipients[79].

2.2 Morphologic and Flow Cytometric Analysis of BAL Cells

The composition of cells harvested from the normal bronchopulmonary compartment has been well characterized in various species[80-83]. Typically, human bronchoalveolar washings contain about 95% macrophages, 4%-5% lymphocytes and 0%-1% polymorphonuclear leucocytes (PML's). Normal canine BAL cells consist of 65%-85% macrophages, 10%-20% lymphocytes and 4%-10% PML's. Many studies were carried out in the different animal models to examine the effect of various types of lung injury (atelectasis, ischemia/reperfusion, allograft rejection) on BAL cell compositions[80-82,84-91]. The goal was to characterize changes in BAL cellular profile specific to each of these pulmonary injuries and hence, to attempt a differential diagnosis. Significant and overlapping increases in PML counts were observed following lung ischemia/reperfusion injury, acute allograft rejection or lung atelectasis. Allograft rejection is associated with a steady rise in the BAL absolute lymphocyte counts as the process advances. None of these BAL cellular changes is specific for a particular pathologic process. There certainly is strong evidence that conventional total and differential counts of BAL cells cannot differentiate any of these lung pathologies after lung transplantation. Similar observations were made in clinical lung transplantation[92]. Furthermore, the plausible presence of more than one pathologic entity in the post-operative period (i.e., atelectasis and rejection or pneumonia and rejection) leading to overlapping BAL cellular profiles makes morphologic examination of BAL cells alone a less valuable method to differentiate rejection from other types of lung injury. More sophisticated immunologic methods have recently been considered to potentiate the role of BAL in the diagnosis of rejection/infection in the lung allograft.

Examination of BAL mononuclear cell phenotypes using monoclonal antibodies against specific cell surface markers in rejection and infection may reveal differences in its cell subset that may be specific to each of these pathologic entities and, hence, may enable us to distinguish one from the other. Lung allograft rejection in a murine experimental model was associated with a significantly higher cytotoxic/suppressor to helper/inducer T lymphocyte ratio, and the inverse was observed for lung infection[93]. In a canine model, Shennib and co-workers showed different patterns of BAL mononuclear subsets following

rejection and bacterial infection[94]. The percentages of mature and cytotoxic/suppressor T lymphocytes were higher in the setting of graft rejection than in the presence of infection. The accumulation of T lymphocytes particularly cytotoxic/suppressor (CD8+) cells was observed in acute rejection and also in opportunistic infection (CMV and Pneumocystis carinii) of human lung allografts[95-97]. However, helper/inducer (CD4+) T cell subset was noted to increased in the presence of such infections but not rejection[98]. Although different phenotypic patterns of BAL lymphc were observed to associate with different pathologic lung conditions, no studies so far examined the predictive values of BAL phenotypic analysis as a diagnostic test for the differentiation of lung graft infection or rejection.

2.3 Functional Analysis of BAL Cells

Cytotoxic lymphocytes in the canine model of rejected lung allografts were quantitated using the lectin dependent cell mediated assay by Emeson and colleagues[99,100]. This assay is believed to detect the total T cell mediated cytotoxicity of a lymphocyte population since the lectin substitute for alloantigens in the binding phase of the lytic reaction[101,102]. Natural killer cells are not detected by this assay[103,104]. They demonstrated the presence of cytotoxic T lymphocytes in the lung grafts when only pathologic changes in the form of mononuclear cell cuffings of the small vessels were present before any clinical or radiologic manifestations of rejection. A strong correlation was noted between cytotoxicity of BAL T cells and the clinicopathological severity of rejection. Typical allospecific cytotoxic T lymphocytes were detected late in the course of rejection and their appearance was preceded by a population of cytotoxic cells characterized by their ability to lyse xenogeneic target in a lectin dependent cytotoxicity assay but an inability to kill allogeneic target cells from the donors. These cells may represent the nonspecific lymphokine activated killer (LAK) cells which are presumably generated in the presence of lymphokine mediators produced in the process of rejection[105,106]. It was also shown by these investigators that alveolar macrophages harvested during rejection inhibited graft cytotoxic T lymphocytes in vitro[107]. The presence of cytotoxic lymphocytes in the graft is not specific for rejection as elevation of BAL lymphocyte cytotoxic activity was noted in graft

infection as well[100]. Clinically, Zeevi and co-workers at the University of Pittsburgh had demonstrated that accumulation of donor specific lymphocytes (as shown by accelerated proliferation in a mixed lymphocyte culture or specific lysis of donor cells in cytotoxic assay) were present in graft chronic and acute rejection[98,108,109]. Once again, accumulation of donor specific lymphocytes was observed in the presence of bacterial, viral and Pneumocystis carinii infection without any evidence of graft rejection[64,73]. It seems that these types of infection initiates an inflammatory reaction with accumulation of various host immunocompetent cells, which in the presence of graft alloantigens, cell mediated immunity is facilitated leading to acute or chronic rejection. Functional study of graft infiltrating lymphocytes may serve as a screening method to detect immunologic abnormality of the graft which requires further more specific investigations.

2.4 Other Applications of BAL in Lung Transplantation

Not all the studies done on BAL are focused on the examination of its cellular components. BAL lavage fluid has also been analyzed for soluble markers of lung allograft rejection and/or infection. For instances, high levels of thromboxane B_2 (TXB_2) in BAL fluid from the lung grafts were strongly correlated with rejection[110]. Moreover, infection and/or rejection of the lung allograft resulted in distinctly different levels of protein, lipid and various components of pulmonary alveolar surfactant in BAL fluid[111]. Since rejection is known to associate with an increase in pulmonary vascular resistance and shifting of the blood flow to the native lungs, our laboratory is now exploring the role played by vasoactive substances such as angiotensin converting enzyme (ACE) and endothelin in the occurrence of this phenomenon. Bronchoalveolar lavage fluid samples are analyzed to see if a correlation between the levels of these vasoactive substances and various causes of lung allograft failure exists.

Finally, BAL has been a valuable and the most frequently used tool for the direct diagnosis of causative agents of lung graft infection. Viral and protozoal opportunistic infection can now be identified with relative ease. Gryzan and colleagues[70] from the University of Pittsburgh reported a very high incidence of post-operative Pneumocystis

Carinii (PC) infection (88%) in the heart-lung transplant recipients. They reliably and rapidly made the diagnosis of PC infection by examining cytocentrifuge preparations of BAL cells stained with silver methenamine for cysted forms. In addition to monitoring the raise of CMV titres, BAL fluid can be tested for cytomegalovirus by inoculation into human embryonic lung tissue culture and CMV "immediate early antigens" may be detected by immunofluorescence using monoclonal antibody at intervals after inoculation. This technique allows relatively rapid and accurate detection of cytomegalovirus in BAL fluid[112]. Whether the identification of infectious agents in BAL necessarily indicates the presence of lung infection by these organisms remains controversial.

3. Cellular Events of Rejection and Infection

3.1 Rejection

On the basis of the timing, the morphology and the underlying mechanisms, rejection reactions are classified as hyperacute, acute and chronic[113]. Hyperacute rejection is a humoral-mediated phenomenon attributable by the presence of preformed circulating antibodies directed against donor specific antigens. The antibody-antigen reactions occur at the level of vascular endothelium leading to diffuse microvascular thrombosis and rapid graft destruction. Chronic rejection results in gradual attrition of graft function secondary to chronic immunologic inflammation directed at the vascular endothelium which is mediated by both cellular and humoral immunity. Acute rejection is predominantly a T-lymphocyte-mediated phenomenon. However, humoral immunity also plays a role in this process. Initiation of the rejection process occurs when alloantigens on parenchymal cells or passenger leucocytes of transplanted tissues are processed and presented to the host lymphoid cells by antigen presenting cells (APC - thought to be of monocyte/macrophage lineage). Interactions between APC carrying alloantigens and appropriate host CD4+ T helper/inducer (MHC class II dependent) and CD8+ T cytotoxic/suppressor (MHC class I dependent) lymphocytes lead to a series of clonal proliferation and differentiation which produce antigen-specific lymphocytes capable of inflicting damage to the graft. Much initial sensitization to allografts probably takes place within the peripheral lymphoid tissue of the host by antigens shed from the graft or on donor lymphocytes transplanted with the

organ[114]. Moreover, there is good evidence that sensitization can occur within the graft itself by virtue of circulating host lymphocytes that migrate to the graft[115]. Activated CD4+ lymphocytes under the influence of APC-derived interleukin 1 elaborate interleukin 2 which induces clonal expansion of the allospecific CD4+ cells in an autocrine fashion and also stimulates antigen-specific clones of CD8+ T effector cells to proliferate and mature into antigen-specific cytotoxic T lymphocytes (CTL). Moreover, interleukin 2 permits the proliferation of all T cells so that nonspecific as well as antigen-specific proliferation of T lymphocytes occurs. Gamma interferon elaborated by activated CD4+ cells amplifies the entire process by inducing and intensifying class I and class II MHC antigen expression on the graft, stimulates B cells to increase antibody production, and augments alloaggressiveness of previously uncommitted monocytes and macrophages[116]. Other interleukins produced by activated CD4+ cells also stimulate B cell maturation. Activated macrophages also elaborate other cytokines such as tumor necrosis factor alpha and beta which contribute directly and indirectly to graft destruction. Recent experimental evidence substantiates the role of delayed type hypersensitivity in allograft rejection[117,118]. This mechanism relies on sensitized T helper cells which, upon recognition of alloantigens, release lymphokines (macrophage chemotactic factor, macrophage migration inhibitory factor, interleukin 1, tumour necrosis factor, etc.), leading to accumulation and activation of nonspecific inflammatory cells. The net result of the rejection process is the accumulation within the allograft (either by in situ proliferation or by recruitment from the circulation) a variety of host cells including T cell subpopulations, B cells, natural killer (NK) cells, macrophages and neutrophils. One can identify specific antidonor cytotoxic T cell activity, nonspecific macrophage mediated killing, antibody dependent cell mediated cytotoxicity which all contribute to graft destruction. Natural killer (NK) cells are cytotoxic non-T, non-B mononuclear leucocytes which are capable of destroying certain tumour cells in vitro without prior sensitization. They are thought to play a role as the first line of defense against tumours and virus infection[119]. Natural killer cells have been shown to be the principle effectors of bone marrow rejection in the mouse[120]. The exact role of NK cells in the process of solid organ allograft rejection is unclear. They have been shown morphologically and functionally to be the first lymphoid cells to infiltrate the kidney allograft or the sponge

matrix allograft model[121,122]. However, deletion experiments in graft recipients using specific anti-NK antibodies show that they are not critical in the acute rejection process[123]. In antibody-antigen interactions where the constant Fc portion of the antibody is exposed, NK cells adhere to the Fc molecules and presumably contribute to the rejection process.

3.2 Infection

Viral infection evokes both humoral and cellular immune responses[124]. Interferons mediate the earliest antiviral defenses either by directly inhibiting intracellular viral replication or by boosting cell-mediated responses. Natural killer cells together with activated macrophages can destroy infected cells or viral particles. Recognition of viral antigens on the surface of infected cells by antigen-specific CD4+ T lymphocytes activates the cell-mediated immune system with the generation of specific CD8+ cytotoxic T lymphocytes. These effector cells recognize specific viral antigens in association with MHC class I and proceed to destroy host cells bearing viral neoantigens. CD4+ lymphocyte-mediated delayed type hypersensitivity is also instrumental in host cellular response to viruses. The cellular events following viral infection is similar to those of the allograft rejection phenomenon. Specific neutralizing antibodies facilitate clearance of viral particles or virus-infected cells via the antibody-dependent-cell-mediated cytotoxicity pathway. Bacterial infection results in acute inflammatory reactions characterized by accumulation of leucocytes (mainly monocytes/macrophages and neutrophils) and generation of various humoral mediators such as vasoactive substances, activated complement factors, prostaglandins, cytokines particularly interleukin 1 and tumour necrosis factor at the site of infection. Opsonizing antibodies against bacterial soluble and particulate antigens produced by appropriate B cell clones later in the course of infection. Infectious agents are eradicated by phagocytosis. Lymphocytes play a more well-defined role in chronic than in acute inflammation. NK cells can kill certain bacteria, fungus, protozoa in vitro either by secreting soluble factors or by cell-mediated lysis of targets. The role of NK cells in infections caused by those organisms in vivo, however, is unknown[125].

4. Hypothesis

Bronchoalveolar lavage is a quick and non-invasive method of retrieval of immunocompetent cells from the lung allografts. The working hypothesis is that BAL, by allowing better identification of cellular events in the setting of lung graft rejection or infection, can be useful in differentiating the etiology of graft dysfunction. Furthermore, it may be useful as a screening procedure for the detection of subtle subclinical rejection or infection. Cytotoxic T lymphocytes but not NK cells play the dominant effector role in the rejection process. Natural killer cell, on the other hand, is one of the host's defense mechanisms against infection. Detection of increased NK activity in the BAL space may serve as a marker of local lung infection. In this study, one examined the cytotoxic activity of BAL lymphocytes using the lectin-dependent-cell-mediated cytotoxicity (LDCMC) and natural killer assays in the models of progressive acute graft rejection and bacterial lung infection. Cytotoxicity of peripheral blood lymphocytes was also concomitantly quantitated for comparison.

II. MATERIALS AND METHODS

1. Left Lung Allotransplantation

Lung transplantation was performed between 16 pairs of size-matched mongrel dogs (25 to 28 kgs). Recipient animals received prophylactic peri-operative broad spectrum antibiotics Cefazolin 1 gm IV and Tobramycin 40 mg IV TID for 24 hours. Post-operatively they all received Cephalexin 500 mg p.o. QID and Tobramycin 40 mg i.m. TID for 10 days. General anaesthesia was induced with pentobarbital 30 mg/kg body weight and maintained with 1% to 1.5% halothane. Oral endotracheal intubation was done using 8.5 mm ET tube. The volume cycle ventilator was set at: tidal volume of 15 -20 ml/kg body weight, rate of 14 to 16 breaths/ minutes, FiO_2 of 0.5, positive end expiratory pressure of 5 cm H_2O. Through the left posterolateral thoracotomy, hilar structures were identified and isolated. The three pulmonary veins were isolated intrapericardially. The left mainstem bronchus was exposed from the carina to the left upper lobe bronchus, vascularised peribronchial tissue was mobilized and used to buttress the bronchial anastomosis. The pulmonary artery was skeletonized from the bifurcation to its lobar branches. In the recipients, following systemic

heparinization (100 units/kg body weight), the pulmonary artery was occluded with a small Satinsky clamp and transected, three pulmonary veins were divided between silk ligatures and a large Satinsky clamp was placed where the left pulmonary veins join the left atrium taking a small cuff of atrium with it. The atrial cuff was then opened by transecting tissue bridges between the vein orifices. The bronchus was divided about 1 to 1.5 cm from the carina. In the donor, intrapericardial pneumonectomy was perform following systemic heparization, leaving the left pulmonary vein attached to a generous cuff of the donor left atrium. The donor lung was drained off all residual blood and immediately reimplanted to the recipients. No preservation was required as the warm ischemic time was around 25 minutes to 35 minutes. The left atrial cuff was attached first using continuous 4-0 prolene making sure good endocardial apposition to avoid thrombosis. The atrial clamp was removed upon completion of the anastomosis to allow retrograde flow to the pulmonary circulation. The pulmonary artery was anastomosed with continuous 5-0 prolene suture. The bronchial anastomosis was performed last with continuous 3-0 prolene suture, taking one bronchial cartilage ring on each side and telescoping the donor bronchus inside the recipient one. The suture line was then buttressed with the peribronchial vascular connective tissue. The animals were extubated few hours after surgery. Intramuscular demerol (1mg/kg) was used for postoperative analgesia. They were put on protective isolation in the animal care facility. Chest roentgenograms were performed on post-operative days 1 and 4 then weekly until sacrifice or more frequently if pulmonary infiltrations occurred or persisted.

Pre-operative immunosuppression was induced by intravenous methylprednisolone (250 mg) and cyclosporin (50 mg). Intravenous methylprednisolone (250 mg/day) was continued for three days and then replaced with oral prednisone. Post-operative immunosuppression consisted of cyclosporin A (15 to 17 mg/kg/day) given orally in oil suspension twice a day, azathioprine (2 mg/kg/day) and prednisone (2 mg/kg/day) given orally in the morning. Daily blood samples were obtained for complete cell counts and cyclosporin level in the immediate post-operative period and then twice weekly. Whole blood cyclosporin levels were maintained in the range of 400 to 600 ng/l, determined by

radioimmunoassay. After first lavages, cyclosporin, azathioprine and prednisone dosages were acutely reduced to 50% to allow progressive rejection to occur. The cyclosporin level was kept in the subtherapeutic range of 50 to 80 ng/l.

Serial bronchoalveolar lavages of the left lower lobes were performed pre-transplant and on the 10th to 14th post-transplant day, then every week after effective reduction of immunosuppressive therapy, whenever a new infiltrate appeared on chest roentgenograms or clinical deterioration of the recipients. Bronchoalveolar lavages were always followed by open lung biopsies for assessment of lung pathology including rejection and bacterial infection. Lavage fluid samples were quantitatively cultured for bacterial organisms. Animals which developed bacterial infection of their allografts in the early post-operative period during therapeutic immunosuppression were separated from others and studied as another group (ie. infected allografts).

2. Control Immunosuppression and Bacterial Pneumonia

Gram negative pneumonia was induced by transbronchial instillation of 10^8 colony forming units of Pseudomonas Aeruginosa mixed with melted agar to distal subsegmental bronchi of the right lower lobe of 7 dogs. This method was shown to consistently induce pneumonia in dogs three to four days after bacterial inoculation[94]. The development of pneumonia was noted with fever, coughs, leucocytosis on complete blood counts and pulmonary infiltrates in the right lower lobes on chest roentgenography. Pneumonia was confirmed by histologic examination of an open lung biopsy specimen and by positive culture of the same bacterial species from bronchoalveolar lavage fluid. Another group of dogs (n=8) was given a ten-day course of systemic immunosuppression similar to the lung transplant recipients in order to study the effect of this treatment on BAL cell types and lymphocyte cytotoxicity. Bronchoalveolar lavage of the right lower lobes were carried out in the pneumonia group when clinical and radiologic signs of pneumonia were present (3 to 4 days after instillation of bacteria) and after 10 days of systemic immunosuppression. Bronchoalveolar lavage fluid samples were also sent for bacterial cultures.

The experimental groups of this study are: 1/ Gram negative infection of the graft, 2/ lung grafts without rejection and 3/ lung grafts with evidence of rejection of different severity as evaluated by histologic grading. The three control groups are: 1/ pre-transplant non-immunosuppressed (normal) 2/ immunosuppressed, non-transplant and 3/ Gram negative pneumonia, non-transplanted.

All animals were given humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No 80-23, revised 1978).

3. Cell Preparations

3.1 Bronchoalveolar Lavage Lymphocytes

Bronchoalveolar lavage was performed at the time intervals described above. The animals were gently anaesthetized with intravenous pentobarbital 10mg/Kg body weight. They all maintained spontaneous breathings and weak cough reflex which were very helpful in obtaining good return rate of high quality BAL fluid. Lavages and handling of BAL cells were performed using strict aseptic techniques. Under bronchoscopic guidance, several 50 ml aliquotes of sterile phosphate buffered saline were injected into the selected lung segments through a sterile polyethylene tube wedged into the segmental bronchus and gently suctioned via a threeway stopcock at the other end of the tube after waiting few minutes to allow lavage fluid to reach distal alveoli. On the average 400 ml of saline were injected with 60% to 70% retrieval. Serial lavages of a given lung were performed on different pulmonary segments to avoid BAL cellular changes secondary to repeated lavage which have been described previously in dogs[126]. Lavage fluid was kept at room temperature during the whole procedure and brought to the laboratory for immediate processing. Gentle aspiration of BAL fluid was done with a narrow-tipped pipette to disperse mucus strands and then fluid was filtered through a loose cotton gauze to remove remaining clumps of mucus. Cells were then collected by centrifuging BAL fluid at 1500 rpm for 10 minutes. Bronchoalveolar lavage cells were washed three times with RPMI-1640 medium enriched with 10% fetal calf

serum, penicillin (100 I.U./ml), streptomycin (100 ug/ml) and glutamine (2 mM) (RPMI complete). Total BAL cell counts were performed manually with an improved Neubauer hemocytometer using 0.01% gentian violet in 3% acetic acid in water (v/v) (Turk's solution) as the counting fluid. The viability of BAL cells, usually more than 90%, was determined by the Trypan blue dye exclusion test. Slide preparations were made by a cytocentrifuge using 0.1 ml of the BAL cell suspension (concentration 5 X 10^6 cells/ml) and spinning at 500 rpm for 5 minutes. Differential BAL cell counts were made from total counts of 400 cells using a leukostat stain kit (Fisher Scientific, Orangeburg, N.Y., U.S.A.) and a non-specific esterase stain (Sigma Diagnostic, St. Louis, Missouri, U.S.A.) for better definition of mononuclear phagocytes.

Bronchoalveolar lavage cells were passed through a nylon wool column which removed alveolar macrophages and polymorphonuclear leucocytes (PML), thus enriching the lymphocyte population. One gram of nylon wool (FT-242 Fenwal Laboratories, Morton Grove Ill.) were washed in 0.2 N HCl and multiple large volume of distilled water, dried at 60°, packed to the barrel of a 10-ml disposable plastic syringe to 8-ml mark and autoclaved. Before the cells were applied, the nylon column was saturated and rinsed with RPMI complete and incubated at 37°C. After this preincubation, BAL cells suspended in 2 ml of RPMI complete were applied to the column and allowed to flow into the column. It was then incubated for 45 minutes in humidified atmosphere containing 5% CO_2. After this incubation, the non-adherent cells were recovered by adding warm RPMI complete medium to the column and adjusted the outflow with a threeway stopcock until 50 ml of the effluent was collected. Almost all (96% to 98%) of the eluted cells were stained negative for nonspecific esterase and possessed lymphocyte morphology. Natural killer cells were not removed by this purification step[127]. Lymphocytes were then adjusted to appropriate concentration (5 X 10^6 cells/ml) for cytotoxic assays. Nylon wool adherent phagocytes were separated from the effector cell population to eliminate the nonspecific lysis of target cells by these cells and also to remove the inhibitory effect of alveolar macrophages on cytotoxic T lymphocytes in vitro as described by Norin and associates[100].

3.2 Peripheral Blood Lymphocytes

Heparinized venous blood samples were obtained simultaneously with BAL's. Peripheral blood mononuclear cells were isolated by the Ficoll-Paque (specific gravity 1.077) discontinuous gradient density technique. Ten mililiters of whole blood were layered on a Ficoll-Paque cushion in a 50 ml test tube and centrifuged at 400 g for 30 minutes at room temperature. The mononuclear interface was carefully removed, washed three times with RPMI complete medium. Peripheral blood lymphocytes were isolated by nylon wool filtration. Cells were adjusted to 5 X 10^6 cells/ml for cytotoxic assays.

4. Cytotoxic Assays

4.1 Natural Killer Cell Assay

Canine thyroid adenocarcinoma cells served as targets for NK cells[128]. One hundred microliters of effector cells (either blood or lung lymphocytes) were added to 10^4 ^{51}Cr labelled target cells in round bottom 96-well tissue culture plates at effector:target ratios of 50:1, 25:1, 12.5:1. Since a fixed number of target cells were used, the decreasing number of effector cells were added to each well in a serial dilution manner to achieve the desired effector to target ratios. Total number of effector cells required was 6 X 10^6 cells for both cytotoxic assays. Assays were done in triplicate. In some experiments in which limited effector cells were available (some normal and sham immunosuppressed animals) it was necessary to use half the number of target cells to get the required effector to target ratios. After incubation at 37° C, in 5% CO_2 humidified air for 6 hours, the culture plates were centrifuged at 0° C for 5 minutes at 1500 rpm. From each well 0.1 ml of supernatant were harvested and radioactivity was measured in a gamma scintillation counter. Percentage cytotoxicity (% specific target lysis) was calculated by a standard formula, using the mean values of triplicate cultures.

$$\text{Percentage cytotoxicity} = \frac{\text{CPM experimental - CPM spontaneous release}}{\text{CPM total release - CPM spontaneous release}} \times 100$$

CPM : Count per minute.

Spontaneous release was determined in wells containing target cells and medium alone. Total release was determined by incubating target cells with 0.1 ml of 0.5% Triton X100 detergent. Spontaneous release was always less than 5% of total release.

4.2 Lectin-Dependent-Cell-Mediated Cytotoxicity (LDCMC) Assay

This assa is regarded as a quantitative measurement of the total cell mediated cytotoxicity of a T cell population[101,102]. Raji cells (derived from human Burkitt's lymphoma) were used as targets. It was similar to the NK cell assay, with the addition of the lectin Concavanalin A to the assay medium at a concentration of 8 microgram/ml.

4.3 Handling of Target Cells

Target cells were maintained in culture in RPMI-complete medium and split 1 to 5 every 4 days. Three days before the assays, a portion of the cells was brought to a concentration of 2 X 10^5/ml to ensure that the target cells were actively dividing on the day of cytotoxic assays. Labelling of target cells was performed as follows: 2 X 10^6 cells were suspended in 0.3 ml of RPMI-complete medium and 0.2 ml of Tris-phosphate buffer solution (pH = 7.4). One hundred microCi of sodium chromate ^{51}Cr (I.C.N. Canada, specific activity 250 Ci/mg Cr) were added to cell suspensions. They were incubated at 37° C, in 5% CO_2-humidified air for one hour with frequent shaking every 15 minutes. Cells were washed twice with the culture medium and then adjusted to 10^5/ml. For assays, 0.1 ml (10^4 cells) were added to each well.

5. Statistical Analysis

Results were presented as mean $\pm$ SEM. Different means from the same group of animals were compared using repeated measurement analysis of variance (ANOVA). Statistical difference between pairs of means was then determined by the Bonferroni test. Means from different groups of animals were compared using Student's t test. $P < .05$ was considered statistically significant.

III. RESULTS

1. Animals

All animals tolerated the sham immunosuppression well without any infectious or metabolic complications. The animals with pneumonia developed a cough 48 to 72 hours after transbronchial bacterial inoculation and showed a mild to moderate febrile reaction and peripheral blood leucocytosis. Chest roentgenography showed mild to moderate right lower lobe infiltrates. There was no mortality in this group. Bronchoscopic examination of right lower lobes revealed inflamed bronchial mucosa and various amounts of purulent secretion. Bacterial cultures of BAL fluid revealed Pseudomonas Aeruginosa species. Biopsies of the affected lung segments confirmed the presence of bacterial pneumonia. Neutrophils containing intracellular bacteria were frequently seen on the cytospin preparations

There were four peri-operative deaths in the transplant group. One animal had acute pulmonary vein thrombosis at the anastomotic site, three had rapidly progressing rejection and infection. Of the remaining twelve lung allografts, four were found to be infected with gram negative bacilli at the time of the first post-transplant BAL and biopsy despite a benign clinical appearance. Bacteria cultured from BAL samples were of the Pseudomonas and/or E. Coli species. Animals with infected lung allografts were sacrificed after the first lavage. Post-mortem histologic examination of the entire grafts did not reveal any signs of rejection. Intracellular bacteria in BAL neutrophils and macrophages were observed in 3 animals.

There were no evidence of rejection in the remaining eight transplants on the immunosuppressive regime. Histopathologic examination of the lung biopsy at the time of the first BAL did not show any signs of rejection. However, rapid, progressive acute rejection of the lung gratts 4 to 6 days following reduction of immunosuppression. Clinically, the animals developed low grade fever, became anorexic, appeared dyspneic, had occasinal coughs. Chest roentgenograms varied widely from normal to variable degrees of pulmonary opacification. Lung biopsies done at the time of BAL's showed progressive degrees of rejection with the presence of perivascular and peribronchiolar mononuclear cell infiltrates consistent with grade 2 acute rejection[54]. Three to 4 days later, productive coughs were

quite evident and chest roentgenograms showed opacification of the graft. Histopathologic examination showed severe rejection (grade 3 to 4) with dense perivascular, peribronchial and alveolar septal round cell infiltration and cellular desquamation in the alveolar space with necrosis.

2. Bronchoalveolar Lavage Cell Enumerations

Bronchoalveolar lavage of either normal controls or immunosuppressed animals yielded similar total and differential counts (Table 2). Lavages of infected lungs in either control animals or allograft recipients showed similar increase in total BAL cell counts (9.4 ± 0.2 and $8.4\pm1.6 \times 10^5$ cells/ml), both of which were considerably higher than normal controls ($1.9\pm0.2 \times 10^5$ cells/ml) and consisted mainly of PML ($65.6\pm6.2\%$ in infected allografts and $67.5\pm5.0\%$ in pneumonia controls). There was significant accumulation (two to three times the levels of normal or immunosuppressed controls) of inflammatory cells in the graft bronchoalveolar space even in the abscence of rejection or infection. Lavages of the lungs having mild rejection (M/R) or severe rejection (S/R) yielded $11.4\pm1.9 \times 10^5$ cells/ml and $15.6\pm3.0 \times 10^5$ cells/ml respectively which were significantly higher than the non-rejection (N/R) level of $4.8\pm1.4 \times 10^5$ cells/ml ($p<0.001$). The number of bronchoalveolar lymphocytes harvested from lung allografts increased progressively as rejection advanced ($9.6\pm2.0 \times 10^5$/ml in N/R, $23.5\pm4.8 \times 10^5$/ml in M/R and $42.5\pm8.1 \times 10^5$/ml in S/R). High percentages of PML were also seen in mildly and severely rejecting lung allografts ($39.2\pm2.8\%$ and $51.6\pm4.9\%$ respectively). Even though higher cell counts were generally obtained with lavages of the rejecting lungs, there is no clear distinction between BAL cellular patterns of allograft rejection or infection.

3. Bronchoalveolar Lavage and Peripheral Blood Lymphocyte Cytotoxicity:

Lymphocyte cytotoxicity was expressed as percentage of lysis of appropriate target cells at 50:1 effector:target cell ratio. Normal lungs possessed a small but detectable level of BAL NK activity ($5.1\pm1.0\%$) which appeared to be depressed by the administration of systemic immunosuppression ($2.4\pm0.3\%$) (Figure 2). In the presence of bacterial infection, NK activity of lymphocytes harvested from the affected lobes increased threefold to

14.7±1.7% ($p<0.05$). In the lung allografts, NK activity was not readily detectable (ranging from 1.2±0.2% in non rejecting grafts to 2.6±0.5% in severely rejected grafts). This was comparable to the value noted in the control immunosuppressed animals. Bacterial graft infection, on the other hand, resulted in a mild increase of BAL NK activity (9.1±0.6% $p<0.01$).

The extremely low LDCMC activity of BAL lymphocytes observed in normal control (3.9±0.6%) increase to 12.1±0.7% in the presence of bacterial infection ($p<0.01$). Immunosupressed, non-rejecting lungs had a very low level of LDCMC activity (2.8±1.1%). It significantly increased with moderate acute lung allograft rejection to 15.6± 2.2% ($p<0.01$) and reached 52.4±2.1% ($p<0.001$) with severe rejection. Interestingly, BAL lymphocyte LDCMC in infected, non-rejecting allografts was also mildly elevated to the same degree as that observed with mild graft rejection (14.3±1.0% vs 15.6±2.2%, p=NS) (Figure 3).

A distinctive pattern of cytotoxicity emerged with respect to bacterial graft infection and rejection when considering BAL NK and LDCMC activity together. With infection, both NK and LDCMC activity were increased, while with rejection, only LDCMC was increased. An elevated ratio of LDCMC over NK cytotoxicity (the LM/NK index) in BAL was associated with acute graft rejection (LM/NK = 11.2±1.0 in mild rejection and 12.4±1.6 in severe rejection) while a low LM/NK index was present in graft infection (1.2±0.3) ($p<0.0001$) (Figure 4).

Peripheral blood NK activity was significantly decreased in animals receiving systemic immunosuppression compared to that of the normal controls (8.2±1.4% vs 15.1±2.8%, $p<0.05$). Bacterial pneumonia, on the other hand, was associated with a two-fold increase in the PBL NK activity (29.6±1.9 vs 15.1±2.8%, $p<0.01$) (Figure 5). Similar changes were noted with PBL LDCMC activity (Figure 6). Analysis of PBL NK and LDCMC activity in the presence of graft infection and rejection yielded a less defined picture. Peripheral blood NK activity was quite similarly elevated whether graft rejection or infection existed

(16.2±2.7% in mild rejection, 22.5±2.2% in severe rejection and 14.4±1.5% in graft infection groups) (Figure 5). The same observation was made with PBL LDCMC (10.1±3.1% in mild rejection, 16.3±2.4% in severe rejection and 9.0±2.5% in infected graft groups) (Figure 6).

IV. DISCUSSION

Rejection and infection in lung allograft recipients can each result in graft dysfunction and decreased survival. Transbronchial biopsy and bronchoalveolar lavage may complement each other in establishing the correct etiology of graft dysfunction[1,55,63,129,130]. The specificity and sensitivity of each in confirming a diagnosis remains to be defined. Among other factors, variation in the techniques, frequency of samplings, number of retrieved cells, adequacy of biopsy size and methods of culturing or labelling of infectious agents can determine the diagnostic accuracy of TBB and BAL[61,62,131].

More recently, experience from the Papworth group suggests that unless approximately 15 TBB samples were taken, it was possible that important pathology such as rejection or bronchiolitis obliterans could be overlooked[62]. This reflects the patchy heterogenous nature of lung allograft rejection. The reliance on such an aggressive approach can be justifiable in the presence of clinical signs and symptoms indicative of lung dysfunction. However, for an otherwise clinically stable lung transplant recipient, an alternative equally reliable but less invasive approach would be preferable. We and others have been impressed with the frequent occurrence of episodes of rejection which were seen on routine TBB in otherwise asymptomatic patients. Such pathologic findings could occur without an observed deterioration of pulmonary flow rates or an abnormality on chest roentgenograms. Bronchoalveolar lavage has the advantage of allowing retrieval of free lung cells in adequate numbers to be tested for various purposes. It has also been shown that BAL cells represent those involved in the primary pathology just as if it were extracted from the lung tissue[79]. Bronchoalveolar lavage also has the additional advantage of its cells being in suspension, thus avoiding the time consuming and complicated purification techniques frequently employed to isolate lymphocytes from other solid allograft tissues.

In this study, we observed an increase in the total number of cells harvested by BAL when lung allograft rejection occurred. While BAL cells from moderately rejecting lungs showed modest elevation of the percentage of PML's and lymphocytes when compared to nonrejecting adequately immunosuppressed animals, marked increase in PML and lymphocyte populations was observed in and severely rejecting lung allografts (Table 2). This finding reflects the nonspecific inflammatory response expected to manifest once rejection was severe as such changes were similarly observed in infected allografts. Moreover, significant accumulation of lymphocytes in the lung allografts indicates the ongoing alloreactivity and is a special feature of BAL cellular profiles of graft rejection. Short term systemic immunosuppression (10 days as in this study) has no effects on the bronchoalveolar cell populations. Bacterial pneumonia results in a significant influx of inflammatory cells into the bronchoalveolar space, consisting mainly of macrophages and PML's. Although there are subtle differences in BAL differential leucocyte counts following graft rejection or infection, they are not specific and distinctive enough to be of diagnostic value. Therefore, enumeration of BAL cells, by itself, yields limited information as to the identification of the underlying pathology in lung allografts.

Experimentally, Shennib and associates[94] studied the difference in cellular phenotypes of BAL and peripheral blood mononuclear cells in a controlled canine model of allograft rejection and gram negative bacterial pneumonia. Using murine monoclonal antibodies raised specifically against canine mononuclear cell subpopulations, they found that the percentage of mature T-lymphocytes was higher in BAL cells from rejecting lungs as compared to infected ones. The percentage of cytotoxic/ suppressor cells as well as their precursors was higher in BAL and PBL cells of rejecting animals than in those of pneumonic ones. They noted that the ratio of macrophage/ T cell percentages was lower in BAL cells in rejection compared to infection. Except for the information achieved as to the cellular events in lung allograft infection and rejection, the diagnostic value of these findings have not been tested prospectively. Clinically, Zeevi and coworkers from the University of Pittsburgh performed phenotypic analysis of BAL cells in lung allograft recipients during episodes of rejection and infection. They found that during acute lung

rejection there was an increase in numbers of macrophages, CD8+ cells (cytotoxic/ suppressor T-lymphocytes) and neutrophils; during infection, especially with pneumocystis carinii (PC) and cytomegalovirus (CMV), both CD4+ (inducer/ helper T-cells) and CD8+ subsets were markedly increased[71,96,98]. They also noted that an increased CD4/CD8 ratio and a positive primed lymphocyte test in BAL cells may be risk factors for the development of chronic lung rejection in long-term survivors[73]. They suggested that these parameters may be useful in monitoring the effectiveness of immunosuppressive therapy. More recently various investigators have observed that there was a gradual increase in the number of CD8+ lymphocyte recovered from non-rejecting, non-infected lung allografts (by clinical criteria) 6 and 12 months after transplantation. There were significant differences of BAL percentage and absolute lymphocyte counts between acute lung rejection and normal or infected lung on retrospective analysis of BAL cells and clinical status of the lung allografts[91,95,97]. Although reports on phenotypic analysis of BAL cells with monoclonal antibodies in the investigation of lung allograft infection and rejection are still scanty and preliminary, this technique appears to have tremendous potential in the management of lung transplant recipients. It is less time consuming than functional assays currently investigated and results can be promptly received within a few hours. So far, various studies have suggested that different patterns of recovered BAL cells seem to be associated with different pathological lung conditions. However, the question remains how reliable and accurate can BAL cellular changes predict the underlying pathology within the lung allograft if evaluated as a diagnostic test in a prospective manner?

In addition, to the increased number of lymphocytes in the rejecting allografts, BAL cells appeared to express a higher LDCMC than when the lungs were adequately immuno-suppressed. In fact, non-rejecting allografts had a very low lymphocyte cytotoxicity, similar to control animals given cyclosporin A, cortisol and azathioprine which reflected the effects of immunosuppression on the BAL lymphocyte cytolytic activity (Figure 2). LDCMC from BAL lymphocytes increased significantly with progression of rejection indicating the accumulation of cytotoxic lymphocytes in the rejecting grafts. This is consistent with the previous findings of the role of cytotoxic T lymphocytes in solid organ rejection.

Immunologic functions of bronchoalveolar lymphocyte and macrophages from a normal lung have extensively been characterized[132-134]. Emeson, Norin and colleagues from the Montefiore Medical Center in New York adapted the LDCMC assay to study the cytolytic immune function in BAL and peripheral blood lymphocytes in a canine lung allograft rejection model[99,100,105 107]. This assay quantitates total T cell cytotoxicity using readily available allogeneic or xenogeneic target cells and the plant lectin Concavanalin A or phytohemagglutinin[101,102]. They showed 1/ that a strong correlation was noted between the intensity of bronchoalveolar T-cell cytotoxicity and the clinical/pathological severity of rejection, 2/ that changes in LDCMC activity in the peripheral blood, on the other hand, failed to correlate with lung allograft rejection; 3/ that the separation or addition of alveolar macrophages into the assay resulted in augmentation or suppression of LDCMC activity, illustrating the suppressive effect of alveolar macrophages on the cytotoxicity of graft infiltrating lymphocytes and finally 4/ that cyclosporin was noted to induce a tolerance to the lung allograft in the canine model. This allogenic tolerance state appeared to be both a selective and a specific phenomenon. It is selective in that lymphocytes from a tolerant recipient were noted to proliferate but not to generate specific cytotoxic T-cells to donor antigen in mixed lymphocyte cultures. It is specific in that the immune nonresponsiveness was limited to donor antigen, but not to a third party antigen neither related to donors nor recipients. It was noted, however, that LDCMC assay not only detected cytotoxic T-lymphocyte activity, but also lymphokine activated killer (LAK) cells. Natural killer cell cytotoxic activity, however, is not detected by this assay[99,100]. Of additional importance in this study is the observation that changes in BAL cell cytotoxicity were noted at an early phase of lung rejection when only pathologic changes in the form of mononuclear cell infiltration around small vessels were present; changes which are usually clinically concealed and precedes the later phase of more advanced rejection and graft damage with alveolar space involvement and detectable roentgenographic manifestation. Interestingly, donor specific cytotoxic T-lymphocytes are detected in the graft 24 to 48 hours after the detection of LDCMC during a rejection episode. Therefore, surveillance of both LDCMC and donor specific cytotoxic lymphocytes in BAL cells is essential for early and accurate detection of an impending acute allograft rejection. However, results of the LDCMC assay must be

interpreted in conjunction with the overall clinical picture since there has been instances of transient increases in intragraft cytotoxic activity without evidence of rejection. Those turned out to be due to non-bacterial pneumonia in which virus induced local cytotoxic T-lymphocytes were detected by LDCMC[100]. Clinical limitation of these assays is the need to retrieve a large quantity of effector cells (1 to 5 x 10^7), and the commitment of time and personnel trained to do such assays. This may, for now, be the limiting factor of its clinical application.

In clinical lung transplantation, Zeevi and her associates subjected BAL cells harvested from the lung allograft recipients to a battery of functional studies. They performed 1/ spontaneous proliferation assay which quantitates the degree of allogenic interaction between donor and recipient immunocompetent cells, (also referred to as allogenic bronchoalveolar macrophage and lymphocyte reaction)[108,135]; 2/ primed lymphocyte testing (PLT) which quantitates the allo-reactivity of graft infiltrating lymphocytes to cells bearing donor antigens[96]; 3/ interleukin-2 response assay which indicates the presence of activated T- lymphocytes in BAL cells[108] and; 4/ cell mediated lympholysis assay which detects donor specific cytotoxic T-lymphocytes in the BAL cells[109]. They concluded that:

- Lung allografts were infiltrated with reactive donor specific lymphocytes which could be detected early in the course of rejection and before roentgenographic changes became apparent.

- Bronchoalveolar macrophage and lymphocyte reaction (BMLR) was observed to be high in both rejection (frequently within 2 months after engraftment) and Pneumocystic carinii (PC) infection (often 3 - 5 months post transplantation). The first reaction may represent an alloreactive response of recipient lymphocytes to donor antigens. This reaction may play an important role in allograft rejection. The increase of BMLR during or following PC infection might represent a physiologic response in which autologous macrophages and lymphocytes interacted to fight invading organisms.

- Similarly, PLT was also noted to be increased in both rejection and pneumocystic infection which suggests that an increased accumulation of donor specific alloreactive T-cells existed with this type of infection. The significance of this increase in donor specific T cells is not clear. It is possible that PC infection facilitated the development of a chronic allograft rejection.

- BAL was a valuable tool in the early diagnosis and screening for pulmonary infections, particularly Pneumocystis carinii, in heart-lung and lung transplant recipients[48].

Two interesting observations were also made. First, there was a suggestion that donor lymphocytes and macrophages were rapidly replaced with those of the recipient within 4 weeks after transplantation[136]. These local resident donor immunocompetent cells no doubt played an important role in the rejection process which frequently occurred in the first 4 to 6 weeks after transplantation. Prop and associates[137] demonstrated an active participation of passively transferred bronchus associated lymphoid tissue in the rejection response. This might explain why lung grafts were more vigorously rejected than other transplanted organs such as hearts, kidneys. Second, there was an increase of PLT and CD4/CD8 ratio in BAL cells at or immediately after Cytomegalovirus, Ebstein Barr virus, Pneumocystis carinii and occasionally after bacterial pneumoniae[73]. These infection-induced changes have been observed to preceed the development of chronic lung rejection which might be a sine qua non to obliterative bronchiolitis. If infection contributes subclinically to the occurrence of chronic rejection, then one may use BAL to reveal the presence of either and hence the need for defining further the type of infection or if negative, the need for augmenting immunosuppression. Primed lymphocyte testing (PLT) has 83% sensitivity and 91% specificity in detecting broncholiolitis obliterans. Phenotypic analysis and PLT of BAL lymphocytes have been currently employed by the Pittsburgh group to monitor chronic lung rejection and the development of obliterative bronchiolitis in human heart-lung transplant recipients[65].

Kirby and associates studied the frequency of donor specific cytotoxic lymphocytes

(effector cells and their precursors) in alveolar and peripheral blood mononuclear cells in canine lung allografts using the limiting dilution assay technique[138,139]. Frequency of donor specific cytotoxic lymphocytes peaked 9 to 14 days after transplantation and reached a magnitude of 8 to 15 times of pre-operative value in nonimmunosuppressed lung allograft with acute rejection. Interestingly, in adequately immunosuppressed lung transplant recipients, there was a very high sequestration of donor specific lymphocytes in the grafts, reaching 15 to 25 times the pre-operative level. Those grafts showed evidence of the subacute, alveolar type of rejection on histologic examination. Their conclusion was that cyclosporin A may have modified the pathogenesis of pulmonary rejection, even though it had not prevented the accumulation of donor specific lymphocytes within the grafts. Such studies emphasize the important potential variation in the expected outcome of any BAL test based on the type of immunosuppressants used and the dose at which they are administered.

Natural killer cells are mononuclear leucocytes which, without prior sensitization, are capable of lysis of several types of tumour-derived or virus-infected cultured target cells in vivo. They are morphologically large granular lymphocytes, functionally and phenotypically (surface cell markers as identified by monoclonal antibodies) distinct from T and B lymphocytes[140-142]. Natural killer cell cytolytic activity is detected in spleen, bone marrow, liver, lung, intestine and most significantly in the peripheral blood. They are implicated in several activities in vivo such as destruction of tumour cells, resistance to viral infection and regulation of haematopoiesis[141,119]. Natural killer activity is augmented by interleukins (particularly 1 and 2), all classes of interferons and tumour necrosis factor[125]. This function is down regulated to various degrees by cortisone, prostaglandin E, cyclosporine A, and macrophages[143-148]. Functional, morphologic and partial phenotypic analysis of canine natural killer cells have been studied by Loughran and his colleagues[128] This lymphocyte subpopulation has been implicated to be the effector mediating allogeneic bone marrow transplantation rejection in mice and canine experiment models[120,149]. The role of NK cells, on the other hand, in the rejection process of solid organ allografts is not clear. They have been functionally and phenotypically detected preceeding the appearance of donor specific

cytotoxic T lymphocytes in different models of allograft rejection[120,121]. Whether NK cells mediate specific or nonspecific graft destruction is not known. In the canine lung transplant rejection model employed in this study, BAL natural killer activity was not functionally detectable in the grafts in the presence of moderate or severe rejection. The rejection process seemed to be associated with a selective stimulation of LDCMC but not NK cytotoxicity. However, it was interesting to observe that bacterial infection, of either the non-rejecting allografts or controls, was associated with a significant increase in both lung lymphocyte LDCMC and NK activity. Nonspecific augmentation of lymphocyte cytotoxic function in the presence of bacterial infection has not been systematically documented before. Norin and co-workers, during their initial studies on LDCMC in BAL cells in lung allograft rejection, had incidentally observed an increased BAL cytotoxicity in the absence of rejection in a dog which was subsequently found to have lung infection[100]. The cause of such increase is not clear but it may reflect the nonspecific activation and/or recruitment to the infected grafts of lymphocyte subpopulations by inflammatory mediators such as interleukins, gamma interferon, etc...known to be locally generated at the site of infection by the host[150]. Increased BAL NK activity was also noted in nonspecific lung inflammation induced by ischemia/reperfusion injury [90]. It was previously shown that lymphokine activated killer (LAK) cells were detected early in the rejected lung allograft before the apprearance of allospecific cytotoxic lymphocytes[105,106] Why these cells were not functionally detected by the spontaneous cytolytic (NK) assay in the setting of allograft rejection is not known. Lymhocyte activated killer cells constitute a heterogenous group of effector subpopulations, predominantly activated NK cells and cytotoxic T lymphocytes[151,152]. One can postulate that nonspecific recruitment and/or activation of lymphocyte subpopulations including NK cells occurs in the presence of inflammatory reaction (such as in the presence of bacterial pneumonia) and therefore their cytolytic activity is detected by both fuctional assays employed here; other lymphocytes but not NK cells constitute the LAK population in early graft rejection and hence detected by the LDCMC but not by the NK assay.

A limitation of the lectin dependent assay employed in this study is that it quantitates

all (donor specific and non-specific) cytotoxic lymphocyte activity. In itself, therefore, LDCMC alteration could not distinguish mild graft rejection from infection. It seems apparent from our results that BAL-harvested lymphocyte subpopulations respond differently to bacterial infection and rejection of the lung allografts. By taking both BAL lymphocyte LDCMC and NK activity into consideration, as exemplified by the LM/NK ratio, different patterns of lymphocyte cytotoxicity for graft infection and rejection emerged: a high ratio corresponding to rejection (due to high LDCMC and low NK activity observed in this kind of lung pathology) and low ratio for lung infection (increase in both LDCMC and NK activity with graft bacterial infection) (Figure 4). Low LM/NK ratio is also observed in non-rejecting/non-infected lung allografts where both LDCMC and NK activities are low. This is easily distinguishable from low LM/NK associated with graft rejection where BAL lymphocyte cytotoxicity is high. The practical advantage of using LDCMC and NK assays over donor specific cytotoxicity testing as reported recently by Dal Col and Zeevi[100] to determine intragraft pathology is that the target cells for the assays employed in this study (Raji and Canine Thyroid Adenocarcinoma cell lines) are always readily available. This is in contrast to the maintenance of a large collection of donor lymphocytes. Furthermore, these assays could be performed over 8 hours which is more expedient than other immunologic tests reported earlier[64,134].

Analysis of peripheral blood lymphocyte CTL and NK cytotoxicity did not show any particular pattern of alterations specific for either graft rejection or infection. Peripheral blood LDCMC activity was previously noted to bear a very poor correlation with lung graft rejection[100]. Cytoimmunologic monotoring of the PBL has been used to detect organ rejection in heart-lung, heart transplantation. The sensitivity and specificity were reported to be in the range of 73% to 83% with high incidence of false positivity which was associated with a viral or bacterial infection and not a graft rejection[96,153,154]. Functional study of PBL cytotoxic activity, in this study, is unreliable in differentiating between moderate or severe lung graft rejection and infection.

In conclusion, BAL of the lung allograft results in the retrieval of cells with different

phenotypic and functional patterns in rejection or bacterial infection. We suggest that bronchoalveolar lavage can be used to monitor immunologic phenomena in the lung allografts. One would expect an adequately controlled allograft to have a BAL cell population predominantly consisting of macrophages, and its lymphocytes to show a very low NK and LDCMC activity. In the presence of an elevated cytotoxicity, subtle rejection or infection should be suspected. When the LM/NK index is high, it is most likely that rejection rather than bacterial infection has occurred.

FIGURES AND TABLES

FIGURE 1: The numbers of heart-lung (H-L) and lung transplantations performed in the years 1986 through 1991. As more single (S-L) and double lung (D-L) transplantations are performed, the number of heart-lung procedure is decreasing (Adapted from the Registry of the International Society for Heart and Lung Transplantation: Ninth Official Report 1992. J Heart Lung Transplant1992;11:599-606).

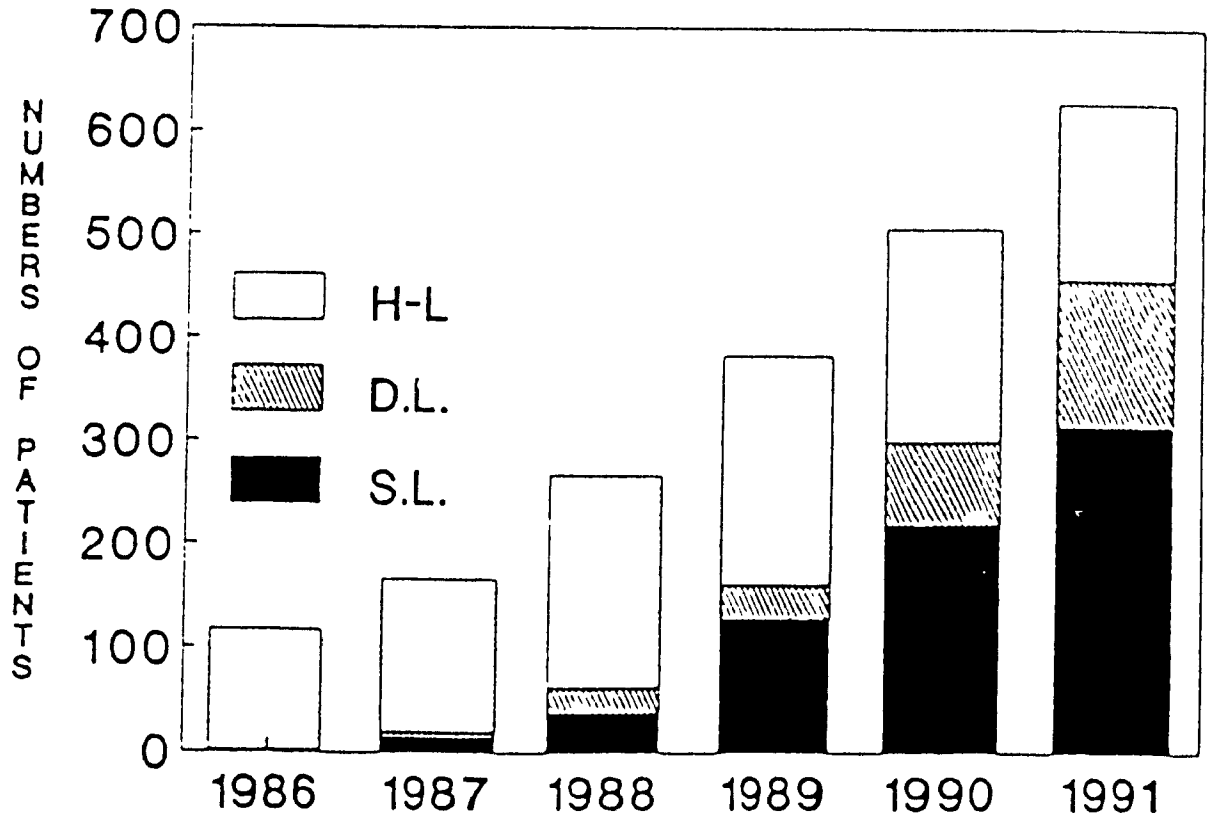

FIGURE 2. Natural killer activity of BAL lymphocytes of various control and experimental groups (at 50:1 of effector:target cell ratio). Bronchoalveolar lavage lymphocytes of normal lung expressed a low level of NK activity which was significantly reduced by systemic immunosuppression. It was also barely detectable in allografted lungs with or without rejection. Natural killer cytotoxicity was strikingly high in the infected lungs of both groups, more so in the infected controls (group abbreviations are similar to those in Table 2).

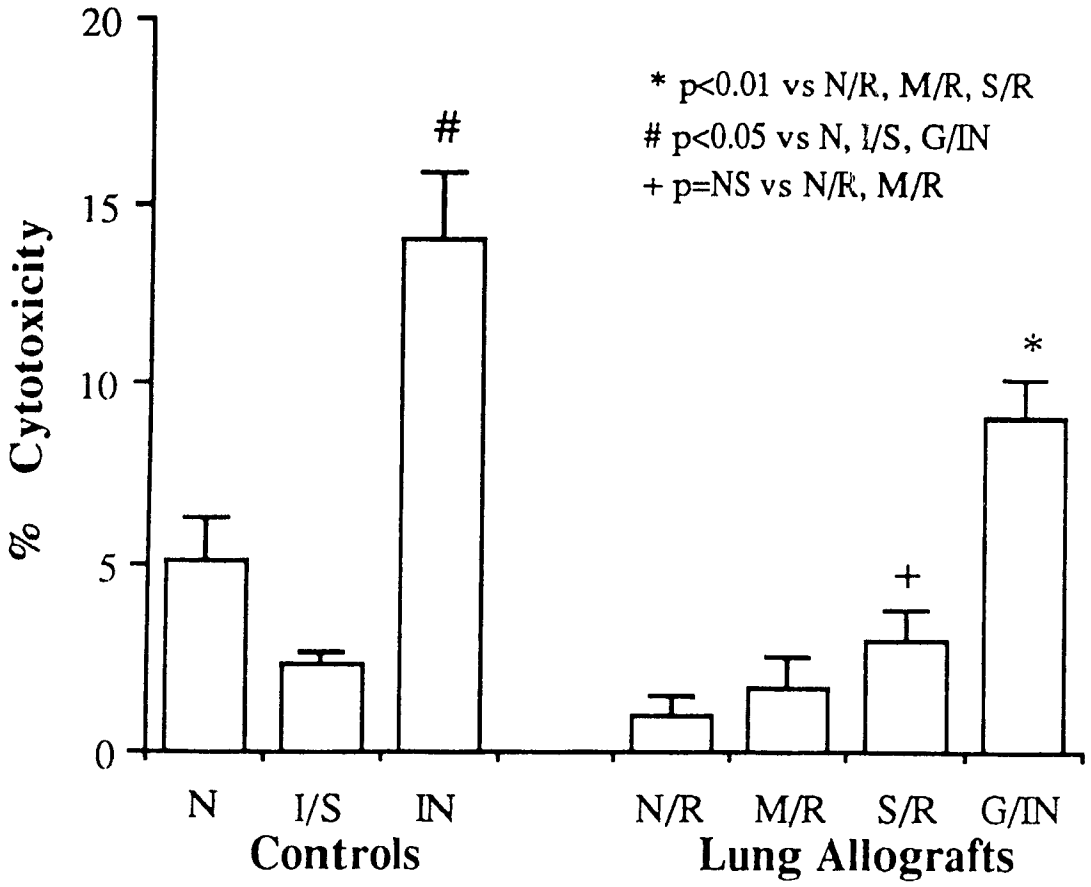

FIGURE 3: Lectin dependent cell mediated cytotoxic activity of BAL lymphocytes of various experimental and control groups (at 50:1 effector to target cells ratio). Lungs of normal or immunosuppressed controls possessed a very low level of LDCMC. It was also abscent in lung allografts without rejection nor infection (N/R). It was significantly elevated in the severely rejecting lung allografts. Infection and moderate rejection were associated with a similar increase in LDCMC activity (group abbreviations are similar to those in Table 2).

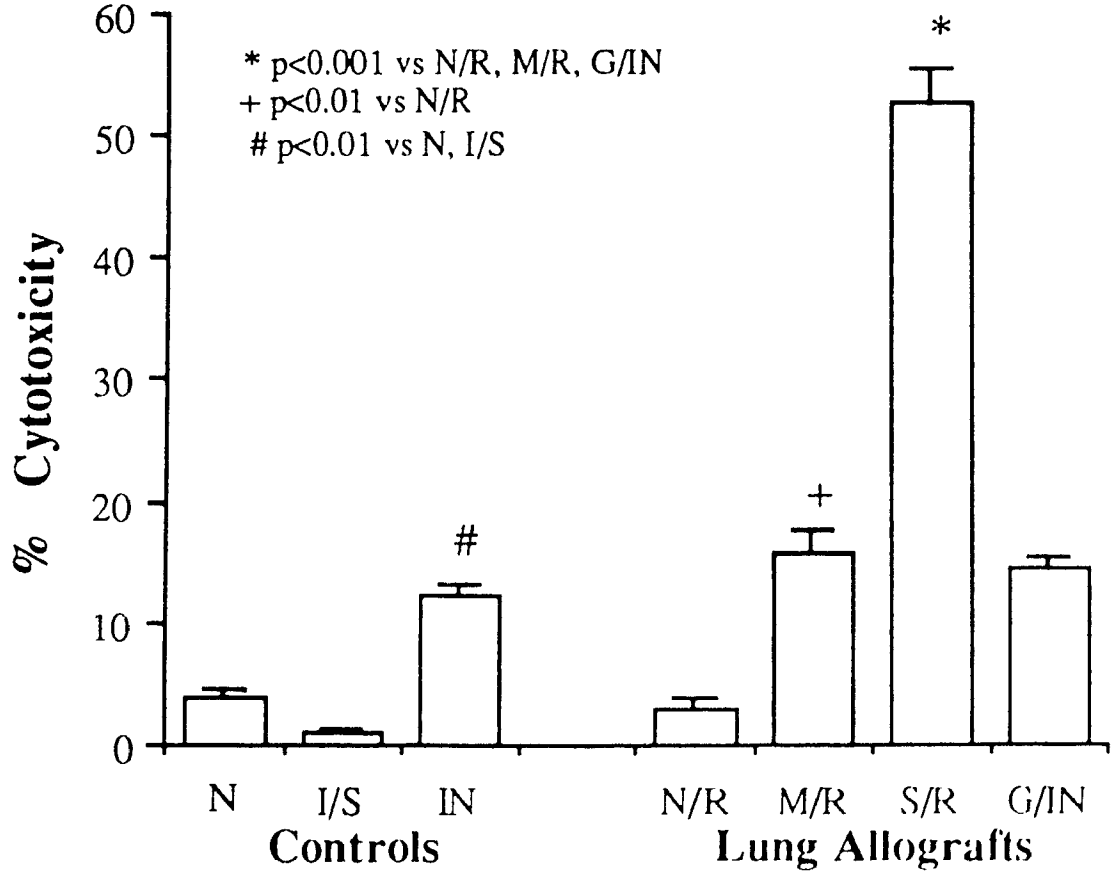

FIGURE 4: LM/NK cytotoxicity index of BAL lymphocytes harvested from lung allografts with or without acute rejection or bacterial pneumonia. This index allows easy identification of rejecting and infected lung allografts. In the absence of rejection and infection BAL LDCMC or NK activity were both low, giving a low index. In the presence of bacterial infection of the lung grafts, both LDCMC and NK activities were increased to the same degree, the index is also low. On the other hand, with acute rejection of the grafts LDCMC but not NK activity was significantly elevated, giving a very high LM/NK index, distinctly different from the index of infected grafts (N/R: no rejection, M/R: moderate rejection, S/R: severe rejection, IN: infected allograft).

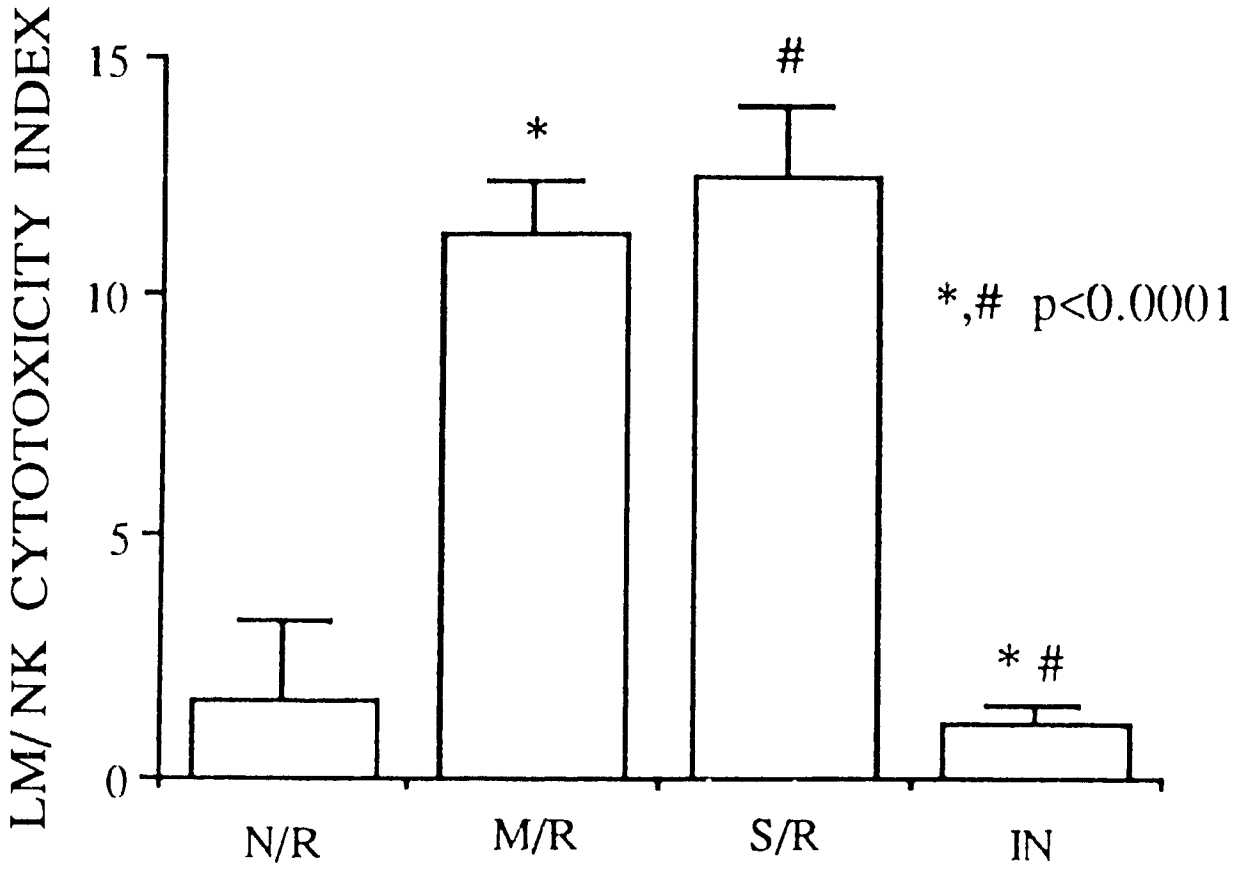

FIGURE 3. NK cytotoxicity of blood lymphocytes from various control and allotransplanted groups. Systemic immunosuppression was associated with a significant reduction of PBL NK activity. On the other hand, bacterial pneumonia resulted in two fold increase in the blood NK cytotoxicity. With regards to lung transplant recipients, there was no difference of blood NK activity between lung recipient subgroups except when rejection was severe (group abbreviations are similar to those in Table 2).

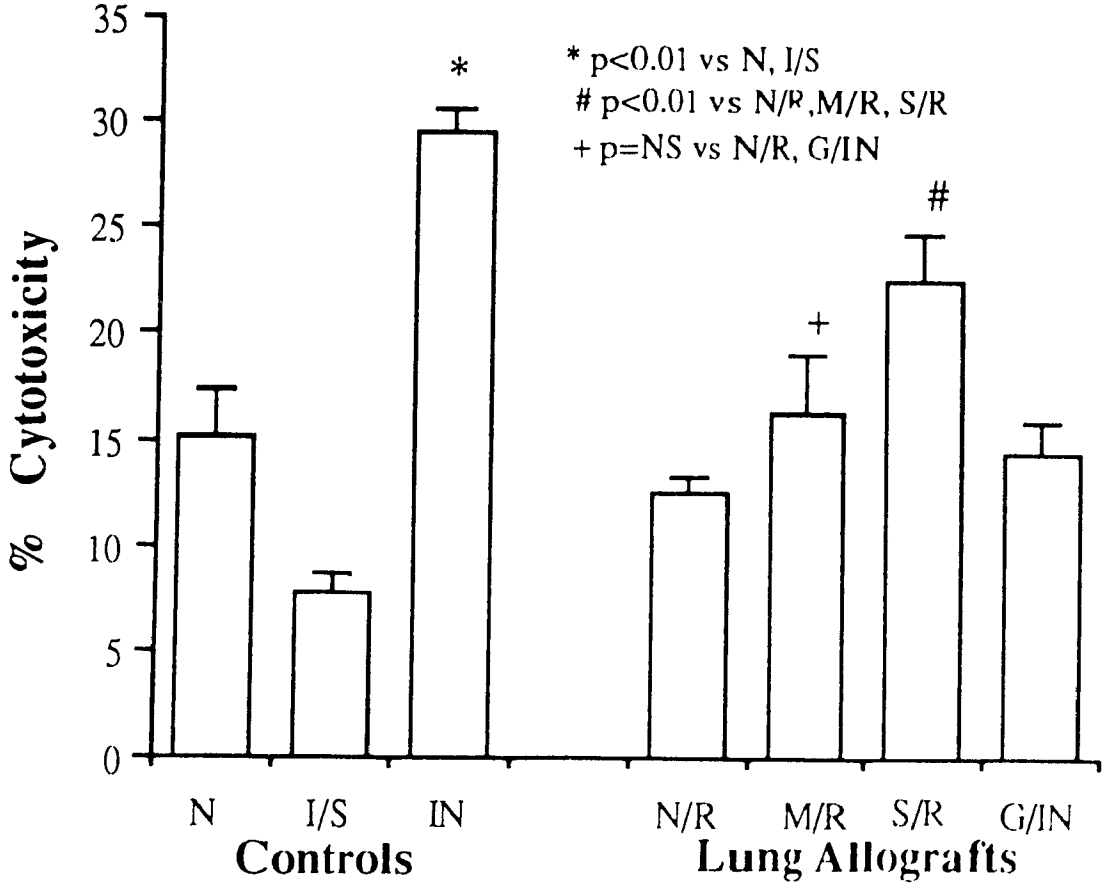

FIGURE 6: LDCMC activity of blood lymphocytes from controls and lung allograft recipients. Normal controls expressed low blood LDCMC activity which was abolished by systemic immunosuppression. Bacterial pneumonia was associated with a more than two-fold increase in PBL LDCMC activity. Note the low peripheral blood lectin dependent cytotoxicity in the absence of allograft rejection. Progressive and significant elevation of cytotoxic activity occurs as rejection advanced (group abbreviations are similar to those in Table 2).

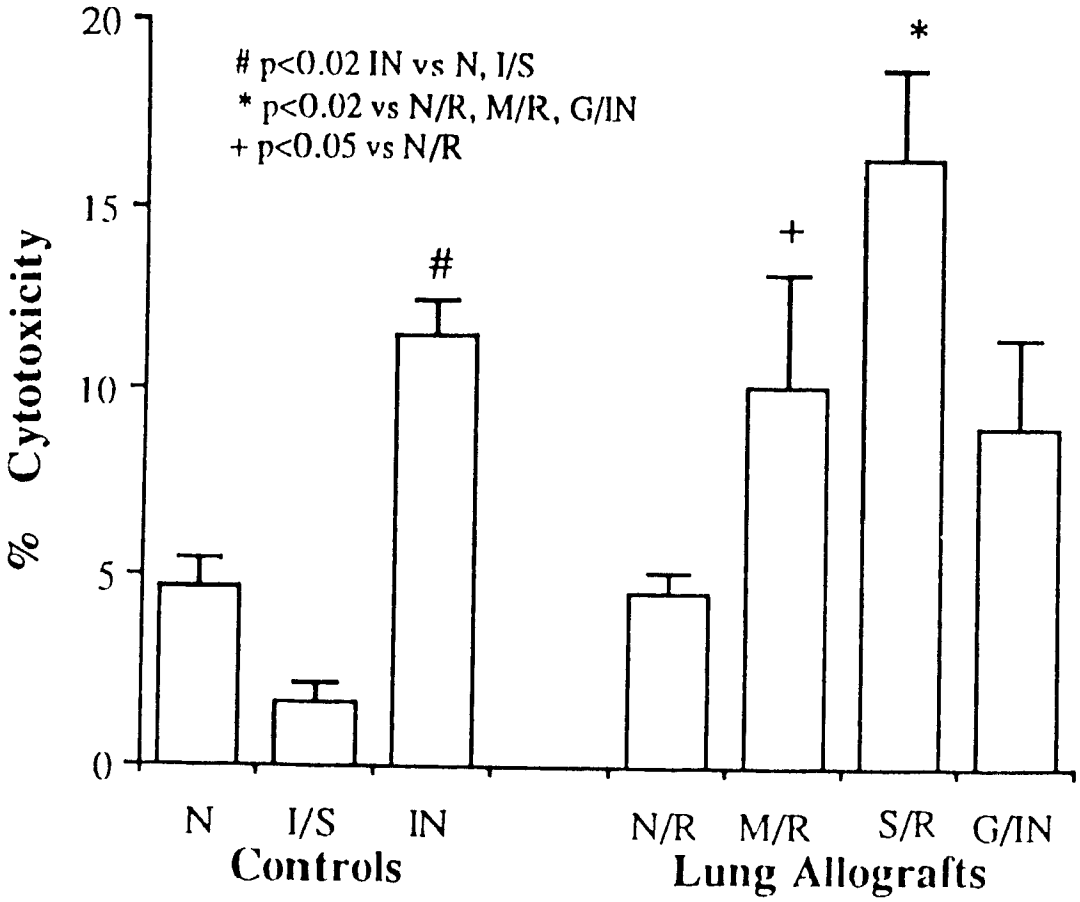

TABLE 1

Working Formulation for Classification and Grading of Pulmonary Rejection

A. Acute rejection
   0. Grade 0 - No significant abnormality
   1. Grade 1 - Minimal acute rejection
      a. with evidence of bronchiolar inflammation
      b. without evidence of bronchiolar inflammation
      c. with large airway inflammation
      d. no bronchioles are present
   2. Grade 2 - Mild acute rejection
      a. with evidence of bronchiolar inflammation
      b. without evidence of bronchiolar inflammation
      c. with large airway inflammation
      d. no bronchioles to evaluate
   3. Grade 3 - moderate acute rejection
      a. with evidence of bronchiolar inflammation
      b. without evidence of bronchiolar inflammation
      c. with large airway inflammation
      d. no bronchioles to evaluate
   4. Grade 4 - severe acute rejection
      a. with evidence of bronchiolar inflammation
      b. without evidence of bronchiolar inflammation
      c. with large airway inflammation
      d. no bronchioles to evaluate
B. Active airway damage without scarring
   1. Lymphocytic bronchitis
   2. Lymphocytic bronchiolitis
C. Chronic airway rejection
   1. Bronchiolitis obliterans - subtotal
      a. active
      b. inactive
   2. Bronchiolitis obliterans - total
      a. active
      b. inactive
D. Chronic vascular rejection
E. Vasculitis

From: A Working Formulation for the Standardization of Nomenclature in the Diagnosis of Heart and Lung Rejection: Lung Rejection Study Group. J Heart Lung Transplant 1990;9:593-601.

TABLE II: TOTAL AND DIFFERENTIAL COUNTS OF BAL CELLS FROM LET LUNG ALLOGRAFTS, NORMAL CONTROLS, INFECTED AND IMMUNOSUPPRESSED ANIMALS

Group	N	IN	I/S	N/R	M/R	S/R	G/IN
N=	8	7	8	8	8	8	4
TOTAL 10^5/cc	1.9 ± .2	9.4 ± .2	1.4 ± .6	4.8 ± 1.4	11.4 ± 1.9	15.6 ± 3.0	8.4 ± 1.6
Mac 10^4/cc	14.4 ± 1.4	22.8 ± 4.8	11.0 ± 2.1	27.4 ± 3.4	46.2 ± 4.7	25.8 ± 7.4	16.7 ± 3.4
(%)	75.8 ± 4.8	24.3 ± 4.6	78.5 ± 3.0	57.2 ± 2.3	40.0 ± 3.8	18.2 ± 4.2	21.5 ± 2.1
Lymph 10^4/cc	3.3 ± 0.4	8.0 ± 2.1	2.2 ± 0.4	9.6 ± 2.0	23.5 ± 4.8	42.5 ± 8.1	9.9 ± 1.6
(%)	17.7 ± 3.3	8.5 ± 2.8	16.0 ± 4.0	20.0 ± 1.3	20.8 ± 1.3	30.2 ± 3.0	12.8 ± 2.1
PML 10^4/cc	1.2 ± 0.3	63.4 ± 7.5	0.7 ± 0.2	11.0 ± 2.5	44.6 ± 4.6	66.6 ± 11.2	55.1 ± 9.2
(%)	6.5 ± 1.0	67.5 ± 5.0	5.5 ± 1.9	23.0 ± 3.8	39.0 ± 2.8	51.6 ± 4.9	65.6 ± 6.2

N = Normal
IN = Infection
I/S = Immunosuppression
N/R = No rejection
M/R = Moderate rejection
S/R = Severe rejection
G/IN = Infected graft

Mean±SEM

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