1998

Immunopathogenesis of a Spectrum of Inflammatory Lung Diseases Induced by Experimental Reovirus Infection

Stephen Clifford Bellum
Medical University of South Carolina

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Stephen Clifford Bellum

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the College of Graduate Studies

Department of Microbiology and Immunology

1998

Approved by:

Steven D. London, D.D.S., Ph.D.
Chairman, Advisory Committee

Russell A. Harley, M.D.

Lucille London, Ph.D.

Maria Trojanowska, Ph.D.

William Tyor, M.D.

ACKNOWLEDGEMENTS

Thank you Steven and Lucille for providing me with this opportunity. Your guidance and the stimulating environment you have created have helped me to organize my curiosity and to think critically. These are skills that have given me great confidence in my abilities and enabled me to proceed to another level of training. I will always regard my graduate experience as a period of tremendous personal and academic growth.

Also, I know I am fortunate that the other members of my committee, Drs. Trojanowska, Tyor, and Harley were at all times generously helpful and interested in my project. The technical advice and scientific insight that you were able to dispense to me, by benefit of your expertise helped me to ask interesting and important questions. I look forward to future opportunities for discussions.

None of us are able to successfully reach our goals by working alone. My colleagues during the time of my graduate work; Damir Hamamdzic, Sanja Altman-Hamamdzic, Allison Stack, Amy Thompson, Elizabeth Majeski, Taetia Phillips-Dorsette, and Sherry Crowe all exposed me to a diversity of personalities and experiences that have and will continue to be a part of my life. In particular, I would like to especially

acknowledge Elizabeth and Sanja for their contributions to the initial characterization of the reovirus-induced inflammatory lung response in neonatally thymectomized CBA/J mice. Also, thank you Damir for the many thoughtful discussions that I have had with you throughout my graduate career.

I thank my family for granting me unyielding, loving support during my graduate career. My parents, Clifford and Shirlyn, and my sisters, Karen and Laura sacrificially invested themselves emotionally into each of my obstacles and successes. Furthermore, I acknowledge my maternal grandmother, Mazie Daniels, and my paternal grandfather Clifford Bellum Sr. who have supported me throughout this experience with love and prayer. Finally, my maternal grandfather, "Shorty", and my paternal grandmother, Mabel Bellum, who are deceased, also were and will remain important influences in the shaping of my life and of my interests.

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List of Abbreviations

ARDS	Acute Respiratory Distress Syndrome
BALT	Bronchus Associated Lymphoid Tissue
BOOP	Bronchiolitis Obliterans Organizing Pneumonia
CTL	Cytotoxic T Lymphocyte
DAD	Diffuse Alveolar Damage
ECM	Extracellular Matrix
FB	Follicular Bronchiolitis
GAG	Glycosaminoglycans
GI	Gastrointestinal Tract
GSR	Gordon and Sweet reticulin stain
IgA	Immunoglobulin A
i.n.	Intranasal
i.t.	Intratracheal
LN	Lymph Node
LALN	Lung Associated Lymph Node
MHC	Major Histocompadability Complex
NTx	NeoNatally Thymectomized
PA	Pulmonary Artery
PBS	Phosphate Buffered Saline
PFU	Plaque Forming Unit
PP	Peyer's Patches
UIP	Usual Interstitial Pneumonia
VVG	Verhoeff Van Gieson

Absract

Inflammatory lung diseases cause a significant amount of morbidity and mortality. However, the mechanisms that control the initiation and resolution of pulmonary inflammatory responses have not been well-elucidated. Histopathological data suggests that the lung responds with a similar pattern of inflammation to diverse range of injurious stimuli. In order to investigate the immunopathogenesis of lung inflammation, a series of *in vivo* models of lung inflammation were generated in CBA/J and CD-1 mice by respiratory reovirus 1/L infection. Using this approach, it was observed in CBA/J mice that a relatively low titer of reovirus 1/L induced a pattern of intraluminal fibrosis in the bronchoalveolar compartment of the lung that is characteristic of the disorder bronchiolitis obliterans organizing pneumonia (BOOP). When higher titers of reovirus 1/L were used to inoculate CBA/J mice, most animals developed acute respiratory distress syndrome (ARDS), which is characterized by widespread vascular endothelial and respiratory epithelial cell damage. Conversely, the pulmonary response of CD-1 mice to respiratory reovirus 1/L was not acute or fibrotic, but instead was characterized by a bronchiocentric, lymphocytic cellular infiltrate that recapitulates the disorder follicular bronchiolitis (FB). FB was also described as a simultaneous component of the inflammatory response in CBA/J mice in addition to BOOP and ARDS. Collectively the models of reovirus 1/L-induced FB, BOOP, and ARDS represent a spectrum of inflammatory lung disorders that are generated by one agent, thus allowing the exploration of cellular and molecular mechanisms that regulate the pulmonary inflammatory response.

CHAPTER ONE

General Introduction and Review of the Literature

General Introduction

The cellular and molecular interactions among pulmonary inflammatory and resident parenchymal cells following injury or infection of the lung are incompletely understood (Colby 1997). Histopathological observations of lung inflammation (including fibrosis) indicate that the lung responds with a similar pattern of inflammation to wide range of inflammatory stimuli as diverse as connective tissue disorders, lung transplantation, bone transplantation, exposure to toxic agents, and pathogenic infections (Epler 1992; Wright, *et al.* 1992; Wells and du Bois 1993; Myers and Colby 1993; Kanda, *et al.* 1997). The following investigations of the immunopathogenesis of lung inflammation are focused on three models of human lung disorders that primarily affect the distal airways, which include the small airways and air spaces of the lung. These surfaces collectively compose the largest interface that the body maintains with the outside environment (Pabst 1995). However, very little is known about the interactions of the respiratory epithelium with infiltrating inflammatory cells following injury or infection of the distal regions of the lung. Respiratory reovirus 1/L infection of selected mouse strains has been used to recapitulate Follicular Bronchiolitis (FB), Bronchiolitis Obliterans Organizing Pneumonia (BOOP), and Acute Respiratory Distress Syndrome (ARDS). FB, the first of these models to be described, is a lymphoid-proliferative disorder that primarily affects the small conducting airways of the lung, but does not usually result in alterations to the architecture of the lung (Yousem 1985; Kinane 1993). The second model is of BOOP, which is characterized by the presence of intraluminal fibrotic lesions in the small airways of the lung and in the alveolar spaces (Epler 1985). BOOP is also typically associated with a lympoid-proliferative component like that observed with FB (Epler 1995). Lastly, respiratory reovirus 1/L infection is also used to generate a model of ARDS, a severe inflammatory disease of the lung characterized by widespread, massive alveolar epithelial and vascular endothelial cell damage (Luce 1998).

FB, BOOP, and ARDS are disorders that primarily affect the distal regions of the lung, which include small airways (bronchioles) and air spaces (alveoli) as well as the intervening interstitial and vascular tissue. Bronchiolar lumens are characterized by a mucosal epithelial lining and then moving from the lumen towards the adventitia, a basement membrane, a thin layer lamina propria, an elastic tissue membrane, a layer of smooth muscle, and an adventitial connective tissue layer that is associated with the surrounding alveolar and perivascular interstitium (Colby 1998). The bronchioles lead into the alveolar ducts, which communicate directly with the alveolar air spaces (Wang 1988). In humans, as the bronchioles transition into alveolar ducts a number of air spaces may be found along the walls of the bronchioles (Wang 1988). These bronchioles are referred to as respiratory bronchioles; however, respiratory bronchioles are not typically found in rodents (Pabst 1992). A schematic representing the cellular and anatomic features of FB, BOOP, and ARDS is shown in Figure 1.1.

The lung alveolar epithelium covers a large surface area (Simon and Paine 1995). Two types of cells comprise the alveolar epithelium, Type I cells and Type II cells (Crapo 1982). Type I cells are characterized by an extremely attenuated cytoplasm that is spread over a relatively large area (Simon and Paine 1995). Approximately 93% of the alveolar epithelium surface area is composed of Type I cells (Simon and Paine 1995). A basement membrane underlies the alveolar epithelium and allows capillary endothelial cells to be juxtaposed to Type I cells (Crapo 1982). The other 7% of the alveolar surface is covered by Type II cells (Saffiotti 1996). Contrary to the thin, attenuated morphology of Type I cells, Type II cells are compact and cuboidal (Simon and Paine 1995). Each Type II cell covers only 3% of the basal lamina surface area as does each Type I cell, but Type II cells are progenitor cells for Type I cells and can differentiate into Type I cells in order to replace damaged epithelium (Simon and Paine 1995). However, Type I cells represent a terminally differentiated cell type and are not able to revert to a Type II state (Simon and Paine 1995). While Type I cells primary function is to mediate the exchange of gases between the

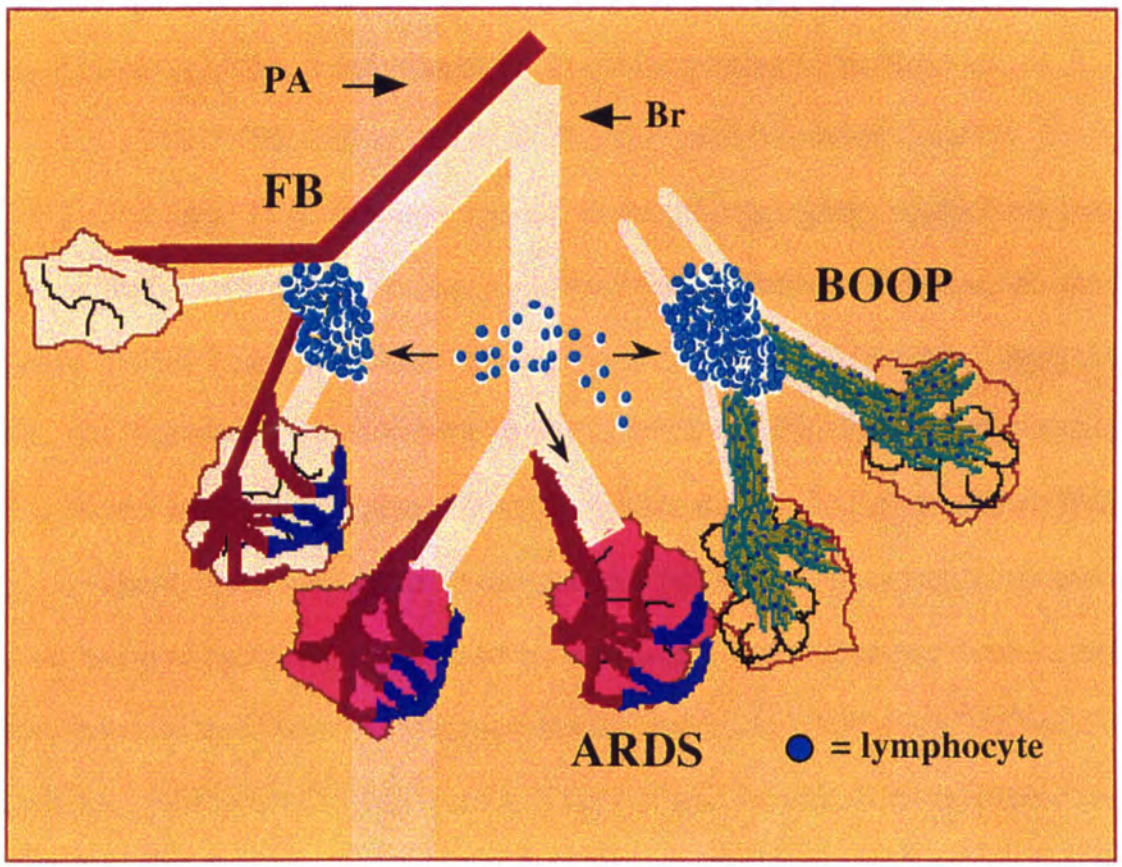

Figure 1.1: Schematic of FB, BOOP, and ARDS. **Br** = bronchiole, **PA** = pulmonary artery

alveolar lumen and the vasculature, Type II cells are more involved in the synthesis and recycling of factors that maintain normal lung physiology (Rooney 1994). The progression of lung inflammation to fibrosis appears to involve intimate cell-to-cell contact between the alveolar epithelium and fibroblasts in the lung interstitium (Rhodes, *et al.* 1989; Adamson, *et al.* 1988). The failure of type II cells to differentiate quickly enough to alveolar type I cells (type I) in order to replace damaged respiratory epithelium has been proposed as an important event leading to intra-alveolar fibrosis (Rhodes, *et al.* 1989; Adamson, *et al.* 1988). In addition, a rat model of silicosis-induced interstitial lung fibrosis showed that type II produce Transforming Growth Factor (TGFβ)-1, a potent stimulator of fibroblasts to secrete collagen and precedes extra cellular matrix (ECM) proteins (Williams, *et al.* 1993). There have also been investigations performed demonstrating that lung fibroblasts do not just passively respond to inflammatory and fibrogenic signals, but that they can also regulate their own proliferation via the secretion of Platelet-Derived Growth Factor (PDGF)A , Fibroblast Growth Factor (FGF)-2, and Insulin Growth Factor (IGF)-1 (Rapolee and Werb 1992; Story, *et al.* 1993; Shoji, *et al.* 1990).

Investigations into the mechanisms that control inflammation in the lung have traditionally focused on professional inflammatory cells (macrophages, lymphocytes, eosinophils, and neutrophils) which gather in the alveolar spaces following lung injury or pathogenic infection (Simon and Paine 1995). In general, organized lymphoid tissue is usually absent in normal adult human or murine lungs (Colby 1998). However, under conditions of chronic inflammation lymphoid follicles characteristic of FB may develop along bronchioles as a component of the mucosal immune response (Yousem 1991). Lung parenchymal cells, particularly epithelial cells and fibroblasts, have become increasingly recognized for their role in the regulation of the inflammatory response under pathologic conditions (Simon and Paine 1995). Type II cells have been shown to be capable of processing and presenting antigen to T lymphocytes (Schneeberger 1986). The role of type II cells as antigen presenting cells is further demonstrated by the fact that isolated type II

cells can stimulate allogeneic T lymphocytes to proliferate in a mixed lymphocyte reaction (Armstrong 1992).

Type II cells are also able to participate in the regulation of inflammatory reactions by secreting inflammatory cytokines such as, Tumor Necrosis Factor (TNF)α and Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) (Piguet, *et al.* 1993; Nash, *et al.* 1993; Tazi, *et al.* 1993). Messenger RNA transcripts for Macrophage Inflammatory Factor (MIP)-2, a potent chemoattractant of neutrophils and lymphocytes has also been detected in the cytoplasm of type II cells shortly after exposure of the lung epithelium to α-quartz in rats (Driscoll, *et al.* 1996). In general peptide mediators belonging to the cytokine and growth factor families are being increasingly recognized as important regulators of inflammation and fibrosis in the lung (Jordana, *et al.* 1993; Kumar and Lykke 1995). It is through an elaborate, organized cytokine network that all the cell types involved in a pulmonary inflammatory response are able to communicate (Jordana, *et al.* 93; Kumar and Lykke 95). Although many investigations into pulmonary cytokine production have demonstrated alveolar macrophages to be the primary source of cytokines in the lung, more recent studies suggest that fibroblasts, alveolar epithelial, endothelial, neutrophils, eosinophils, and lymphocytes are also important sources of cytokines (Kita, *et al.* 1991; Zhang, *et al.* 1995). Early inflammatory events occurring within hours after injury to the lung epithelium have been associated with the expression of Interleukin (IL-1)α and IL-1β, IL-8, Monocyte Chemotactic Protein (MCP), and TNFα (Jordana, *et al.* 1993; Zhang, *et al.* 1995). Upregulation of TNFα has been reported within hours after lung injury in experimental models of pulmonary fibrosis generated by exposure of the lung to the toxic chemotherapeutic agent bleomycin or to silica dust (Piguet, *et al.* 1990). TNFα has also been strongly associated with the transformation of a pulmonary inflammatory response into a fibrotic reaction (Piguet, *et al.* 1990; Jordana, *et al.* 1993; Zhang, *et al.* 1995). However, fibrosis cannot be initiated solely by the administration of recombinant TNFα suggesting that multiple factors are required *in vivo* for inflammatory and fibrotic processes

to proceed (Piguet, *et al.* 1990). Clinical studies of human lung inflammation as well as the bleomycin model of lung injury have also been used to show that TGFβ ECM deposition (Zhang, *et al.* 1995).

Initial experiments to define the pulmonary cytokine network focused on the release of cytokines into the cell free bronchoalveolar lavage (BAL) fluid of mice that were inoculated intratrachearly with influenza virus (Holt, *et al.* 1986; Hou, *et al.* 1992). These experiments demonstrated an early increase in a number of cytokines including IL-1α, IL-1β, TNFα, Interferon (IFN)γ and GM-CSF but did not find an increase in the T cell cytokines, IL-2, IL-3 and IL-4 (Holt, *et al.* 1986; Hou, *et al.* 1992). An evaluation of the cytokine mRNA expression by *in situ* hybridization analysis of cells obtained from both lung associated lymph nodes (LALN) and lungs following primary influenza virus infection demonstrated that the pulmonary T-lymphocyte response to influenza is detected first in the lymph nodes and then later in the lungs following the emigration of effector T cells from the LALN to the lungs (Becker, *et al.* 1991). $CD8^+$ T cells were shown to express mRNA for IFNγ and TNFβ, whereas mRNA for IL-4 and IL-10 was more prevalent in $CD4^+$ cells (Becker, *et al.* 1991). In addition, γ/δ T cells predominately expressed IL-2, IL-4, and IFN-γ (Becker, *et al.* 1991; Hou, *et al.* 1995). The frequency of cytokine-secreting cells from the BAL cells of influenza virus infected mice demonstrated numerous IL-2, IL-4 and IFNγ secreting cells with fewer TNF or IL-10 secreting cells (Ikonen, *et al.* 1994). Depletion of $CD4^+$ and $CD8^+$ cells significantly decreased the frequency of IL-2 and IL-4 secreting cells (Ikonen, *et al.* 1994). In contrast, cells obtained from the lung associated lymph nodes did not produce IL-4, IL-5 or TNF while IL-2, IL-10 and INFγ expression were prominent (Jackson, *et al.* 1987).

Respiratory Syncytial Virus (RSV) infection of the lung has also been used to study immune responses at respiratory surfaces. It was shown in vitro that RSV initially causes an abortive infection of alveolar macrophages and the production of TNFα, IL-6, and IL-8, all of which may modulate the inflammatory responses to the virus (Joel and Chanana

1985). Additionally, RSV induced TNFα and IL-1, but not IL-6 and IL-8 mRNA expression from bronchial epithelial cells, suggesting that cytokines produced by RSV-infected alveolar macrophages may be important in modulating the inflammatory response to RSV than directly interfering with viral infection or replication (Joel and Chanana 1985). With RSV immunization, it was shown that the pattern of cytokine expression observed after i.n. application was dependent on the form of RSV used (Jones and Ada 1987; Jung, *et al.* 1993). The most predominant cytokine found in the lungs after formalin inactivated (FI)-RSV inoculation was IL-4 (Jones and Ada 1987; Jung, *et al.* 1993). However, intranasal immunization with viable RSV predominately induced a TH1 like pattern (Jones and Ada 1987). The effect of cytokine depletion was evaluated upon restimulation with either FI-RSV or infection with RSV (Jung, *et al.* 1993). Simultaneous depletion of both IL-4 and IL-10 completely abrogated the pulmonary histopathology in FI-RSV-immunized mice, while depletion of IL-2 or IFNγ had no effect suggesting that FI-RSV inoculation primes for the TH2 cytokines (IL-4, IL-10) (Jung, et al 1993). In addition, treatment of mice with IL-4 at the time of immunization with FI-RSV reduced clinical illness after a live viral challenge that was associated with an augmented CTL response (Kaltreider, *et al.* 1987). Taken together, these results suggest that different subpopulations of lymphocytes can be activated by different forms of RSV and the subpopulation of lymphocytes that are activated can be selectively modulated by anti-cytokine treatment prior to re-stimulation (Kaltreider, *et al.* 1987; Jung, *et al.* 1993). These experiments collectively demonstrate the complexity of the inflammatory response of respiratory cell populations, which have been represented in Figure 1.3.

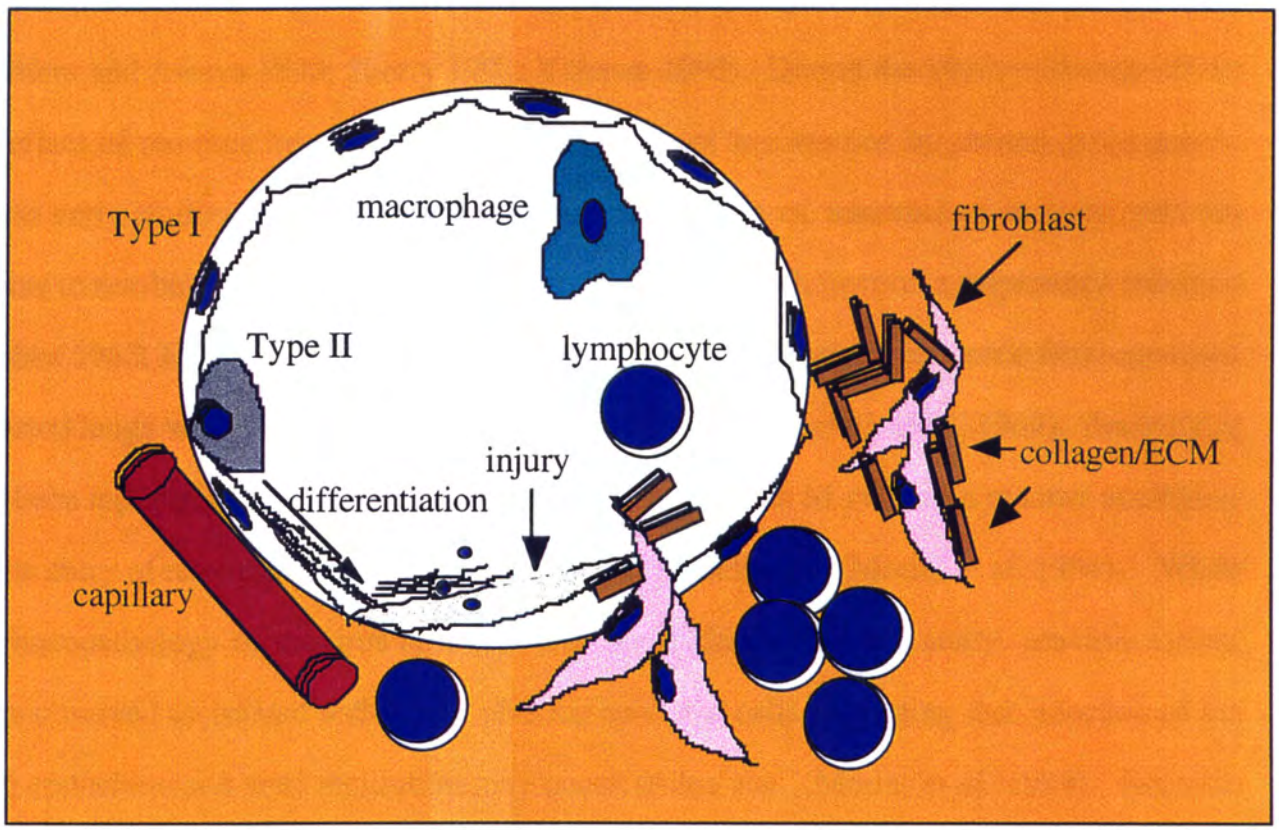

Figure 1.2: Schematic of general intra-alveolar inflammatory events.

The choice to use reovirus as an agent to stimulate an inflammatory response in the lungs is based on experiences gained from the utilization of reovirus in other model systems. A number of reovirus strains have been found to induce pathology in various organ systems including the CNS, heart, pancreas, and thyroid (Sharpe and Fields 1985; Onodera and Awaya 1990; Sherry 1993; Yukawa 1993). During the 1960s and early 1970s the effect of reovirus infection of the respiratory tract was studied by several investigators. These early studies demonstrated that the i.n. application of mammalian or avian reovirus strains to newborn or weanling mice resulted in the establishment of a respiratory infection (Hobbs 1965; Klein; *et al.* 1967; Stanley 1974). Delayed bacterial clearance from reovirus-infected lungs was also demonstrated in these studies (Klein, *et al.* 1967, 1969). Recently, it has been reported that reovirus enters the BALT of rats via M cells in a manner analogous to the entry of reovirus into the Peyer's patches (PP) of the gut (Morin, *et al.* 1994). While the histopathology of reovirus infection was not addressed in this study, reovirus virions were observed associated with type 1 alveolar epithelial cells suggesting that infection of the lung epithelium and viral replication may occur at this site (Morin, *et al.* 1994). Reovirus 1/L has been used extensively in our laboratory to study both the cellular and humoral components of the gut mucosal immune system (London, *et al.* 1987, 1989, 1990). In the gut, reovirus 1/L induced in PP a B cell response chiefly comprised of reovirus 1/L-specific IgA^+ memory cells (London, *et al.* 1987) as well as a switch in the immunoglobulin isotype potential of previously primed, but antigenically unrelated B cells to IgA (Cebra, *et al.* 1989). In addition, MHC restricted, $CD8^+$ virus specific precursor and effector cytotoxic T lymphocytes (CTL) were present in PP and the epithelium of the gut mucosa after enteric reovirus 1/L infection (London, *et al.* 1987, 1989). Therefore, reovirus 1/L is an effective mucosal immunogen eliciting a humoral as well as a cellular immune response.

The name reovirus is derived from respiratory enteric orphan virus (Sabin A, 1959). The terms respiratory and enteric refer to the anatomic locations where reovirus was first isolated and the word orphan indicates its nonassociation with a particular human disease

(Sabin A, 1959). Reovirus is a member of the *Reoviridae* family of viruses, which also include rotaviruses and orbiviruses (Nibert, *et al.* 1996). Structural characteristics of *Reoviridae* include a segmented double-stranded RNA genome as well as a non-enveloped double protein icosahedral capsid shell approximately 70 nm in size (Figure 1.2) (Sturm, et al 1980; Glaser and Morecki 1987; Nibert, *et al.* 1996). Replication occurs completely in the cytoplasm, is conservative, and does not require a DNA intermediate (Nibert, *et al.* 1996). Furthermore, *Reoviridae* possess all of the required enzymes for their own transcription (Tyler, *et al.* 1986). The segmented genome contains ten discrete genes that are classified according to their relative size (Nibert, *et al.* 1996). Included are 3 large-sized genes (L1, L2, L3), 3 medium-sized genes (M1, M2, M3), and 4 small-sized genes (S1, S2, S3, S4) (Nibert, et al 1996). S1, S3, and M3 genes code for nonstructural proteins (Nibert, *et al.* 1996). The remaining 7 genes and an additional gene on S1 code for core and outer capsid proteins (Nibert, *et al.* 1996). Variability at the S1-coded outer capsid protein, σ1 results in the classification of reovirus into three serotypes, 1/Lang, 2/Jones, and 3/Dearing, that can be identified by hemagglutination-inhibition analysis (Rosen 1960). The σ1 protein is also actively involved in the tropism, attachment, and uptake of reovirus (Verdin, *et al.* 1989; Weiner, *et al.*1980).

The adsorption of reovirus to its host cell can occur in about 1 hour at temperatures from 4^0 to 37^0 C (Silverstein and Dales 1968). The host cell receptor for reovirus has not been identified; however, there is some evidence to suggest that it might be the β-adrenergic receptor (Nibert, *et al.* 1996). Electron microscopy studies demonstrate that receptor-adsorbed reovirus particles are endocytosed and can found in host cell vacuoles

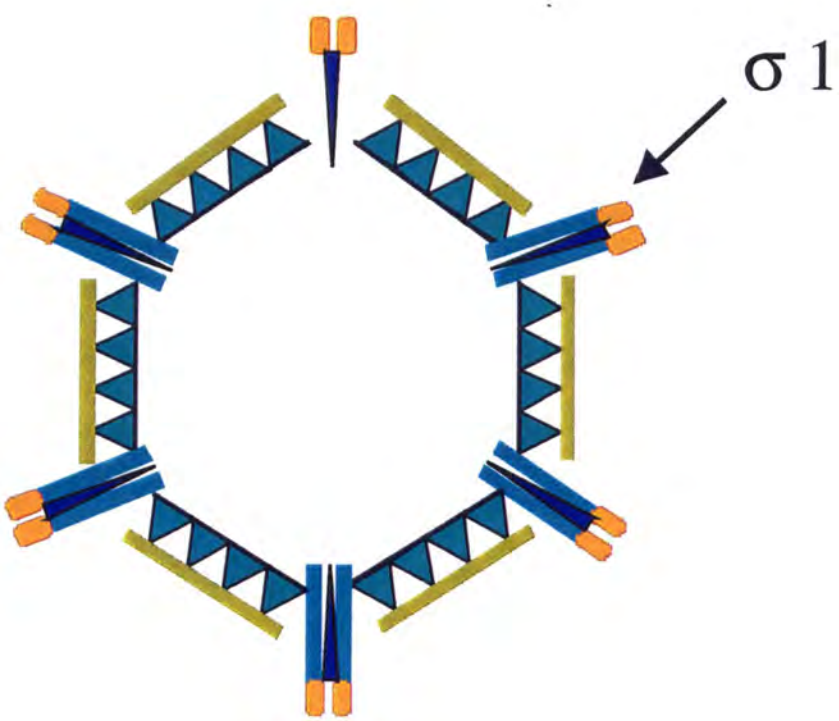

Figure 1.3: Schematic of reovirus

within 15 minutes after adsorption (Silverstein and Dales 1968). Then the reovirus-containing endosomes fuse with lysosomes within an hour (Silverstein and Dales 1968). Proteolysis of the outer capsid then takes about 2 to 3 hours (Silverstein and Dales 1968). Once the virus particles are uncoated, transcription and replication of new progeny genomes for all 10 genes and translation of new viral proteins can occur by 10 hours post infection (Zweerink and Joklik 1970). By that timepoint most of the protein production in the host cell is for viral proteins (Zweerink and Joklik 70). An alternative pathway may also exist for reovirus adsorption and penetration that allows reovirus to bypass receptor mediated endocytosis and lysosome proteolysis (Borsa, *et al.* 1982; Nibert, *et al.* 1996). This occurs when proteolytic lysis outside of the cell causes the partial uncoating of the viral particle, forming what is referred to as an intermediate subviral particle (ISVP) (Borsa, *et al.* 1982; Nibert, *et al.* 1996). The ISVPs have then shown to be able to directly enter the host cell and initiate transcription without interacting with lysosomes (Borsa, *et al.* 1982, Nibert, *et al.* 1996). Following the translation of viral proteins and the transcription and replication of new progeny genome segments, assembly of new virus particles occurs by a mechanism that is still not clear, but it only results in approximately 1 infectious reovirus particle out of a 100 assembled virions (Nibert, *et al.* 1996). Normally, the new reovirus particles are released from the host cell lytically; however, depending on the cell type reovirus may persistently infect the host cell (Ahmed and Fields 1982; Taber, *et al.* 1976).

CHAPTER 2

Materials and Methods

Animals and housing

The following mice were used for investigations relating to this thesis: Sixteen to eighteen gram female outbred CD-1 mice (Charles Rivers Laboratories, Wilmington, MA); 4 to 5 week old female Balb/C (H-2^d), C3H (H-2^k), and CBA/J (H-2^k) mice (Jackson Laboratories, Bar Harbor, ME); and 4 to 5 week old CBA/J that were neonatally thymectomized 4 days after birth (surgery performed by Jackson Laboratories, Bar Harbor, ME). All mice were maintained in micro-isolator cages under specific pathogen-free conditions in a BL-2 facility. Cages were housed in a HEPA-filtered animal isolator clean room (Nuaire Inc, Plymouth, MN).

Inoculation protocol

Animals were anesthetized with an intraperitoneal (i.p.) injection of 0.15 cc (CBA/J mice were inoculated with 0.1 cc because they appeared to be more sensitive to the anesthetic) of 20% Ketamine and 2% PromAce (Vetalar 100mg/ml and Acepromazine Maleate 10mg/ml respectively; Ayerst Laboratories, New York, NY). CD-1, Balb/C, and C3H strains of mice were then inoculated intranasally (i.n.) with 1 x 10^7 PFU of reovirus 1/L in 30 µl (15 µl in each nostril) of sterile injectable grade 0.9% NaCl (Baxter Healthcare Corp., Deerfield, IL). Control animals received only the saline solution or the inactivated reovirus 1/L by the identical inoculation procedure. CBA/J and neonatally thymectomized CBA/J mice were inoculated intranasally (i.n.) with reovirus 1/L (1 x 10^6 PFU or 1 x 10^7 PFU as indicated in the figure legends) in 30 µl of 0.9% NaCl (15 µl in each nostril) (Baxter Healthcare Corp., Deerfield, IL). Reovirus 1/L at 3.3 x 10^8 PFU/ml 0.9% NaCl in a P-60 petri dish was inactivated by exposure to an ultraviolet (UV) light source (30W/G30; Philips Electronics, Holland) placed 15 cm away for 15 minutes. Control animals received either 1 x 10^6 PFU of UV-inactivated virus or only the saline solution by the identical

inoculation procedure. Reovirus 1/L-inoculated mice of all strains were kept physically isolated from all other experimental and control mice.

Virus

Reovirus 1/L was originally obtained from Dr. W. Joklik (Duke University School of Medicine, Durham, NC). Third-passage gradient-purified stocks were obtained by recloning and amplifying parental stocks with a L-929 fibroblast cell line as previously described (Rubin, *et al.* 1985). Following the purification of new stocks, infectious viral titers were obtained by limiting dilution on L-929 monolayers.

Histology

Hematoxylin and Eosin staining was performed on 4 micron sections of lungs fixed overnight in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were prepared and stained by the Department of Pathology and Laboratory Medicine, Medical University of South Carolina.

Electron Microscopy

Cross sectional areas of lung lobes were examined for areas of pathologic change using a dissecting microscope. 1 mm^3 blocks were cut and processed by standard techniques using 2% aqueous osmic acid followed by dehydration through graded alcohols and propylene oxide. 0.5 μm sections were obtained from Epoxy embedded blocks and were placed on glass slides and stained with Toluidine blue The sections were examined by light microscopy and appropriate areas were sectioned at 800 Angstroms, placed on copper grids,

and stained with uranyl acetate-lead citrate contrast stains. The grids were examined with an Hitachi H-7000 transmission electron microscope.

Antibodies

The following monoclonal antibodies were used in this study: RA3-6B2, anti-B220; M1/70.15, anti CD11b (Mac-1); YTS 169.4, anti-CD8a; XMG1.2, anti IFNγ; BVD-24G2, anti IL-4 (Caltag, So. San Francisco, CA); H129.19, anti-CD4 (Gibco, Gaithersburg, MD); and reovirus specific rabbit serum (a gift from Dr. Donald Rubin; Vanderbilt University, Nashville, TN);

Immunohistochemistry

Animals were sacrificed with an i.p. injection of 0.2 cc Sodium Nembutal (50 mg/ml; Abbott Laboratories, No. Chicago, IL). The lungs were inflated *in situ* with OCT compound (TissueTek II, Miles Laboratories Inc., Naperville, IL) by tracheal intubation, removed, suspended in additional OCT compound, and snap-frozen in liquid nitrogen. The tissues were stored at -70°C. Five micron sequential sections were collected on poly-L-lysine (Sigma, St. Louis, MO) treated slides and immediately fixed for 10 minutes in 95% ethanol at room temperature. After two five-minute washes in PBS, the sections were blocked for 20 minutes with a solution of 2% normal serum blocking solution (serum from the species the secondary Ab was made) (Vector Laboratories, Burlingame, CA) and 0.5% nonfat dried milk in PBS. A 1:50 dilution (in blocking solution) of the primary antibody was added and incubated for 45 minutes, except for XMG1.2, anti IFNγ, and BVD-24G2, anti IL-4, which were incubated overnight at 4° C. After two five-minute PBS washes, the biotinylated secondary Ab (anti-IgG of the species in which the primary Ab was made)

(Vector Laboratories, Burlingame, CA) diluted 1:100 in blocking solution was added to the slides and incubated for 30 minutes. After two additional five-minute PBS washes, the tissues were treated for 30 minutes with a 1% solution of hydrogen peroxide (Sigma, St. Louis, MO) in methanol to quench endogenous peroxidase activity. Following two additional five-minute washes the slides were incubated for 30 minutes with the solution containing the avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA) which was prepared and used according to manufacturer's instructions. Diaminobenzidine (Sigmafast DAB tablets; Sigma, St. Louis, MO) was prepared according to the manufacturer's instructions and allowed to react with the peroxidase-labeled tissue for 20 minutes. After two, one minute rinses in distilled water, the tissue was hematoxylin counter stained (Gill's #1; Baxter, McGaw Park, IL), cleared and dehydrated by successive gradations through 70%, 95%, and 100% ethanol followed by a final passage in xylene. The slides were mounted with Accu Mount 60 (Stephens Scientific; Riverdale, NJ).

Titration of infectious virions

Freshly harvested lungs without the associated lymph nodes were removed and freeze-thawed three times in 2 mls of 0.5% gelatin (Difco Laboratories, Detroit, MI) dissolved in PBS (gel-saline) followed by homogenization for 45 seconds with a microprobe tip of a Virsonic 475 sonicator (Virtis, Gardner, NY). Serial dilutions of the lung homogenate in gel-saline were used in a standard plaque assay on L-929 cell monolayers in 6-well tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ) as previously described (Rubin, et al. 1985).

RNA Preparation

Total cellular RNA was isolated from freshly harvested lungs by guanidinium denaturation according to manufacturer's instructions utilizing RNazol (Tel-Test Laboratory, Inc., Friendswood, TX) for RNA work performed in chapters 3 and 4 and Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH) for RNA work performed in Chapter 5.

RNA Blot Analysis

RNA was subjected to electrophoresis through a 1% agarose, 7% formaldehyde gel followed by transfer to nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by capillary blotting with 10 X SSC buffer. Nitrocellulose membranes were prehybridized in phosphate buffer (500 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, pH 8.0, 7% SDS, 1% bovine serum albumin) at 65°C for 60 minutes. Incubation was continued for 12-15 hrs. in the same solution containing the [^{32}P]-labeled probe created by nick translation (Promega, Madison, WI) of a full length reovirus serotype 3/Strain Dearing M3 gene inserted into a pUC vector (gift from Dr. Joklik, Duke University, Durham, NC). The hybridized blots were washed twice at 65°C in buffer containing 40 mM Na2HPO4, pH 7.5, 1% SDS and 1 mM EDTA, pH 8.0 for 60 min. The blots were dried and exposed to FUJI RX film at -70°C with a Kodak Lanex Regular intensifying screen for 18 hours.

CHAPTER 3

Respiratory Reovirus 1/L Induced Follicular Bronchiolitis: A Model for the Study of the Lung Inflammation in the context of the Mucosal Immune System

Introduction

The mucosal immune system provides the first line of defense against pathogens which invade at the wet epithelial surfaces of the body (Croitoru and Bienenstock 1994). These sites include the gastrointestinal (GI), respiratory, and urogenital tracts as well as other wet mucosal surfaces of the body (Phillips-Quagliata and Lamm 1994). The predominance of immunoglobulin A (IgA) antibodies at mucosal surfaces has allowed the definition of an interrelated immune response which operates among these mucosal sites (Tomasi 1992). Thus, immunization at one mucosal site frequently results in the generation of antigen-specific IgA antibodies at other mucosal sites as opposed to systemic sites (Phillips-Quagliata and Lamm 1994). The linking of mucosal sites by lymphocytes which preferentially recirculate to mucosal areas defines the common mucosal immune system. Since specific IgA antibodies at mucosal surfaces have been correlated with protection against a number of mucosally related pathogens (Murphy 1994; Underdown and Schiff 1986), knowledge concerning the details of immunity generated at mucosal surfaces is critical for the control of many human and animal pathogens.

The GI tract has served as a paradigm for the study of mucosal immunity since this site is thought to be important in the generation of many mucosal immune responses and a number of distinct lymphoid components can be obtained from this tissue (Kiyono, *et al.* 1992). Lymphoid populations of the GI tract include lymphocytes contained within the PP that line the wall of the intestine, lymphocytes contained within the epithelium, and lymphocytes contained within the lamina propria connective tissue underlying the gut epithelium (Kiyono, *et al.* 1992). Specialized epithelial cells (microfold, M cells) present within the dome epithelium overlying PP facilitate the cytosolic passage of antigenic material from the intestinal lumen into the PP allowing the sampling of intestinal contents (Owen 1994). An immune response generated in the PP results in the emigration of primed lymphocytes into the lymph and circulatory systems (Croitoru and Bienenstock 1994;

Kiyono, *et al.* 1992; Phillips-Quagliata and Lamm 1994). Ultimately, the mucosally-primed immune effector cells home to the mucosal epithelium of the GI tract and may also potentially seed the lung and other distal mucosal sites (Kiyono, *et al.* 1992; Wallace, *et al.* 1991).

Organized mucosally-associated lymphoid tissue found in the lung is referred to as BALT (bronchus associated lymphoid tissue) and is most commonly associated with branch-points of conducting airways (Bienenstock and Befus 1984). Although organized lymphoid tissue is not a constitutive feature in human or murine lungs the lymphoid accumulations along the walls of bronchioles that are characteristic of FB may possess specialized epithelial cells analagous to M cells in the gut (Bienenstock and Befus 1984; Pabst 199; Neutra 1996). Lymphatics leading from BALT eventually drain into the lung associated lymph nodes (LN) where a local immune response may be generated (Morin, *et al.* 1994).

While a number of studies have examined the nature of the immune response to particular mucosal pathogens, many of these organisms are able to colonize and stimulate specific immunity at only one mucosal site. Thus, it has been difficult to study cross priming between mucosal sites using the same pathogen. Reovirus serotype 1/strain Lang (reovirus 1/L) has been used extensively in our laboratory to study both the cellular and humoral components of the gut mucosal immune system (London, *et al.* 1987, 1989, 1990). In the gut, reovirus 1/L induced in PP a B cell response chiefly comprised of reovirus 1/L-specific IgA^+ memory cells (London, *et al.* 1987) as well as a switch in the immunoglobulin isotype potential of previously primed, but antigenically unrelated B cells to IgA (Cebra , *et al.* 1989). In addition, MHC restricted, $CD8^+$ virus specific precursor and effector cytotoxic T lymphocytes (CTL) were present in PP and the epithelium of the gut mucosa after enteric reovirus 1/L infection (London, *et al.* 1987, 1989). Therefore, reovirus 1/L is an effective gut-mucosal immunogen eliciting a humoral as well as a cellular immune response. Since reoviruses are isolates of both the lung as well as the gut (Sabin

1959), we chose to investigate whether reovirus could serve as an effective respiratory pathogen which stimulates the appearance of a lymphocytic infiltrate in the lung. In this report we describe a reovirus 1/L-induced murine model of respiratory mucosal immunity which will allow investigations of the mucosal immune response generated at respiratory surfaces as well as the interaction between the lung and other mucosal immune sites. In addition, the follicular organization of the lymphoid infiltrate in the lungs of CD-1 mice following reovirus infection is similar to the subacute human condition termed follicular bronchiolitis (FB).

Results

Clinical and pathological observations of reovirus 1/L-infected CD-1 mice

To assess the affect of respiratory reovirus infection in an experimental animal model system, CD-1, C3H, and Balb/C mice were observed over time after intranasal (i.n.) inoculation with 1 X 10^7 plaque forming units (PFU) of reovirus 1/L. This inoculation dose was selected since we had previously demonstrated this dose to be an effective stimulus for reovius-specific immune responses in the GI tract of mice (London, *et al.* 87). The viral inoculum was administered in a volume of 30 µl. We determined this was a sufficient volume to ensure the widespread distribution of the inoculate by i.n. administering 30 µl of a 1:1000 dilution of india ink to CD-1 mice. We then immediately sacrificed the animal and removed the lungs for gross observation, which demonstrated that the india ink had been distributed to all regions of the lung (Figure 3.1). Following reovirus 1/L-inoculation experimental and control animals were visually monitored for clinical manifestations of disease over time. No mortality of reovirus 1/L-inoculated mice was observed over an 80 day period of observation. However, reovirus 1/L infected mice showed obvious clinical signs of infection. By day 7, experimental animals were significantly less physically active compared to control saline inoculated mice and these mice developed a matted, unhealthy coat. These clinical changes were accompanied by a decrease in body weight which peaked 7 days after inoculation with an average 10% loss of total body weight versus controls (Figure 3.2A). The mice appeared to resolve the infection since they regained normal physical activity levels, their coats returned to a normal appearance, and their body weights returned to normal by the end of the third week (Figure 3.2A).

Gross observation of reovirus 1/L-infected CD-1 mouse lungs revealed a marked alteration in their appearance during the first two weeks of the infection. As compared to

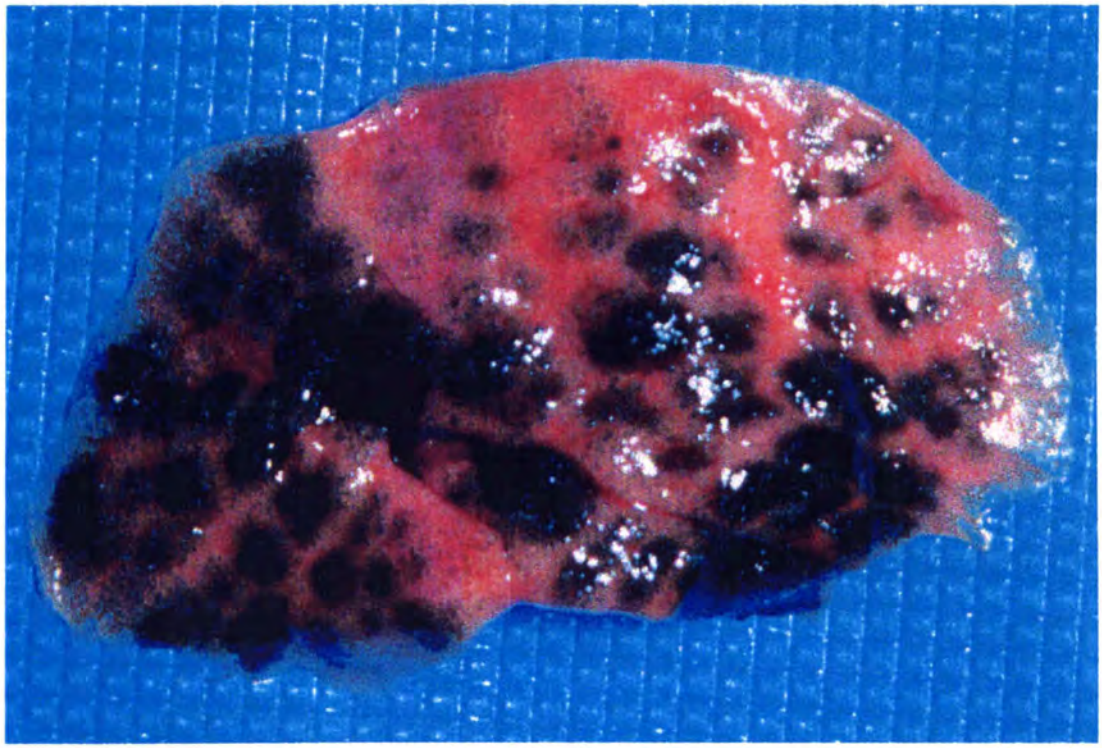

Figure 3.1: An India ink colored test inoculum is widely distributed in the lung. A 30 µl volume of India ink, diluted 1:1000 in water was administered either i.t. or i.n. to CD-1 mice. The animals were then sacrificed immediately and the lungs were removed for gross observation.

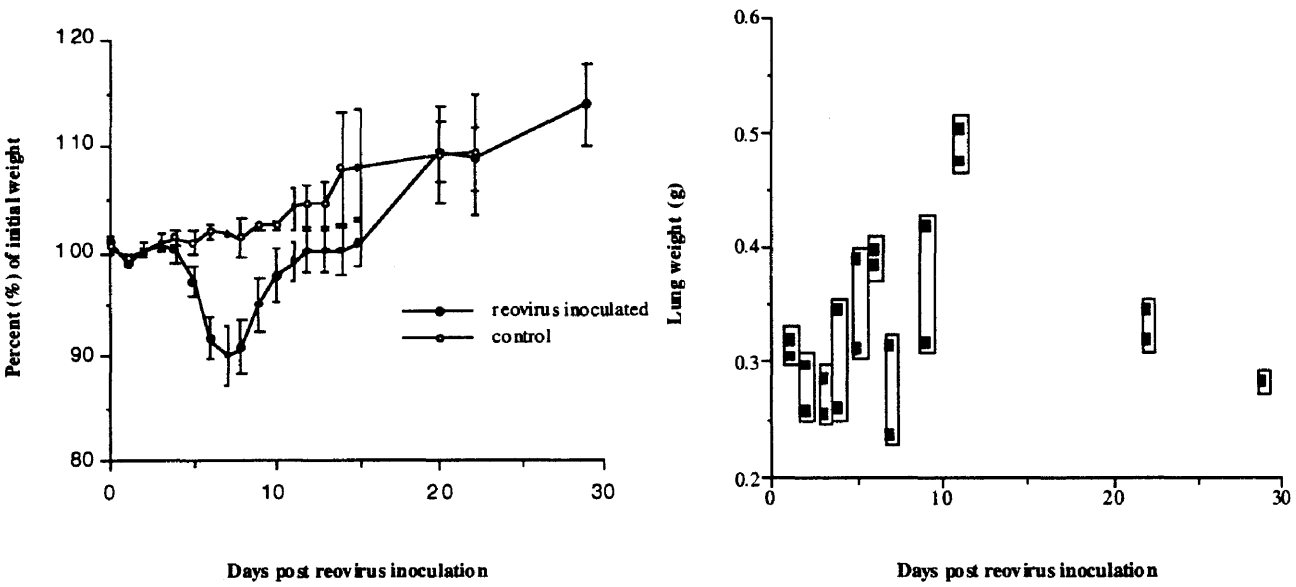

Figure 3.2. Body and lung weights of reovirus 1/L-infected mice. (A) The body weights of reovirus 1/L-inoculated (——●——) and saline inoculated (——o——) CD-1 mice are represented as the average percentage of the initial body weight at each timepoint. The initial body weight of an animal was determined by averaging the weight of the animal during the first three days of the experiment. Data represents the average of at least 6 reovirus inoculated mice and 3 control mice per timepoint with the exception of the control mice on day 22 where N = 2. (B) The lung weights of two reovirus inoculated mice are represented at each timepoint.

control, saline inoculated lungs, reovirus 1/L-infected lungs were edematous with pale subpleural zones alternating with areas of hemorrhage (Figure 3.3). Hyperplasia of the lung-associated LN occurred during the first week of the infection. An example of hyperplastic lung-associated LN 9 days following reovirus infection is shown in Figure 3.3. In contrast, the lung-associated LN are not observable in control animals (Figure 3.3). This hyperplasia persisted until at least day 21 (data not shown). Lung weights were determined to quantitate the gross pathological changes observed in reovirus 1/L-infected mice. These measurements demonstrated an increase in lung weights which peaked on day 11 with an approximate 2.5 fold increase in weight as compared to control lungs (Figure 3.2B).

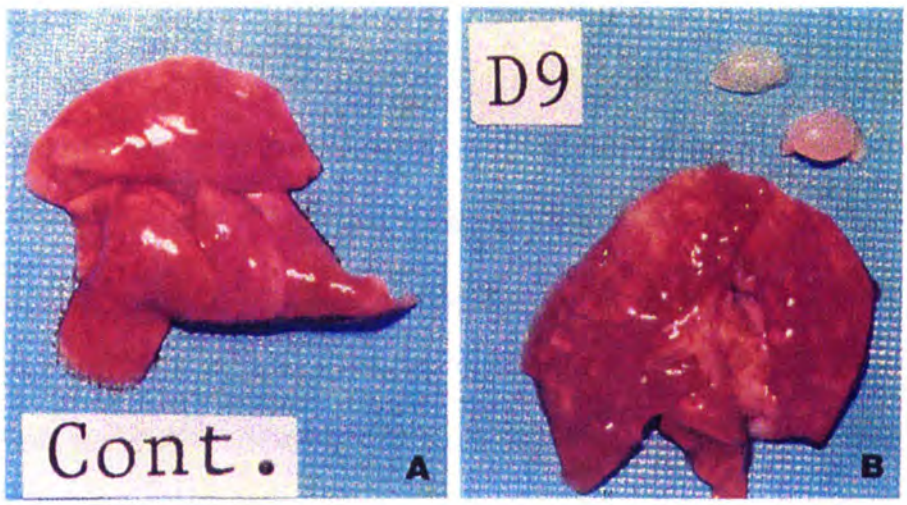

Figure 3.3: Gross pathology of reovirus 1/L-infected lungs. (A) Saline inoculated control lung. (B) Reovirus 1/L-infected lungs 9 days after inoculation. Hyperplastic lung-associated lymph nodes, unobservable in saline inoculated control lungs (A) are shown in reovirus infected lungs (B, arrow).

Replication and clearance of reovirus 1/L from the lungs of infected CD-1 mice

To assess reovirus 1/L replication in the lung, northern analysis of total cellular RNA from the lungs of CD-1 and C3H mice infected with reovirus 1/L over time or from saline inoculated controls was performed (Figures 3.4, 3.5). The reovirus M3 gene was used as a probe since the M3 RNA is an abundantly represented reovirus species in infected cells (Schiff and Fields, 1990). In CD-1 mice this analysis demonstrated that viral replication occurred on days 1 through 4 followed by a rapid decrease through day 7 of the infection (Figure 3.4). Viral replication was not detected on day 14 or in control lungs (Figure 3.4). Reovirus 1/L-infected L929 cells served as a positive control for the detection of the M3 RNA (Figure 3.4). Analysis of reovirus replication in the lungs of C3H mice demonstrated the replication kinetics of reovirus were similar to that observed in CD-1 (Figure 3.5). In addition, reovirus M3 mRNA was not detected from the lungs of CD-1 or C3H mice during the first 12 hours of the infection demonstrating that the original viral inoculum was not detected by this assay (data not shown).

The clearance of virus from the lungs of CD-1 mice infected with reovirus 1/L was assessed by a standard plaque assay of lungs homogenized by sonication (Figure 3.6). At day 1 of the infection the average titer of infectious virions was 2.4 x 10^8 PFU/lung representing an approximately 1 log increase from the initial viral innoculum of 1 x 10^7 PFU. This titer remained relatively constant through day 5. However, at day 7 a 1.5 log decrease of infectious virions was observed (3.4 x 10^6 PFU/lung) as compared to the titer of infectious virus present on day 1. By day 14, infectious virions were not detected within the limits of the assay. In addition, infectious virions were not detected from homogenized control lung tissues (data not shown).

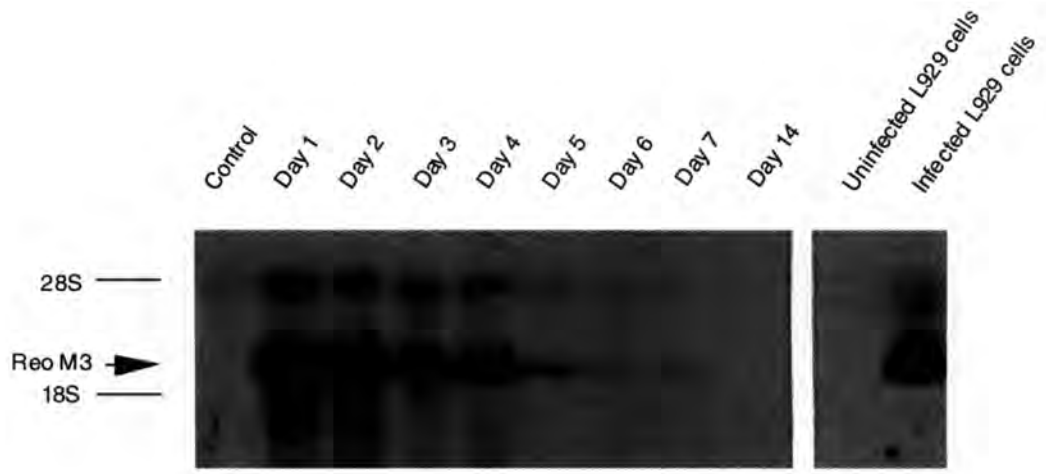

Figure 3.4. Detection of reovirus replication in the lungs of CD-1 mice. Northern analysis was performed using a [^{32}P]-labeled probe derived from the full length reovirus M3 gene on total cellular RNA from lungs of reovirus 1/L-infected or saline inoculated control mice. Lane 1, uninfected control ; Lane 2, day 1 post inoculation; Lane 3, day 2 post inoculation; Lane 4, day 3 post inoculation; Lane 5, day 4 post inoculation; Lane 6, day 5 post inoculation; Lane 7, day 6 post inoculation; Lane 8, day 7 post inoculation; Lane 9, day 14 post inoculation.

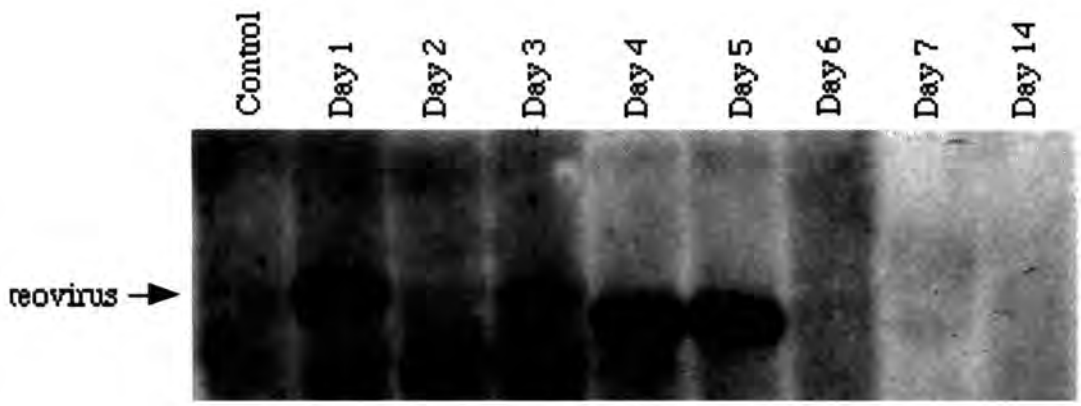

Figure 3.5. Detection of reovirus replication in the lung of C3H mice. Northern analysis was performed using a [^{32}P]-labeled probe derived from the full length reovirus M3 gene on total cellular RNA from lungs of reovirus 1/L-infected or saline inoculated control mice. Lane 1, uninfected control ; Lane 2, day 1 post inoculation; Lane 3, day 2 post inoculation; Lane 4, day 3 post inoculation; Lane 5, day 4 post inoculation; Lane 6, day 5 post inoculation; Lane 7, day 6 post inoculation; Lane 8, day 7 post inoculation; Lane 9, day 14 post inoculation.

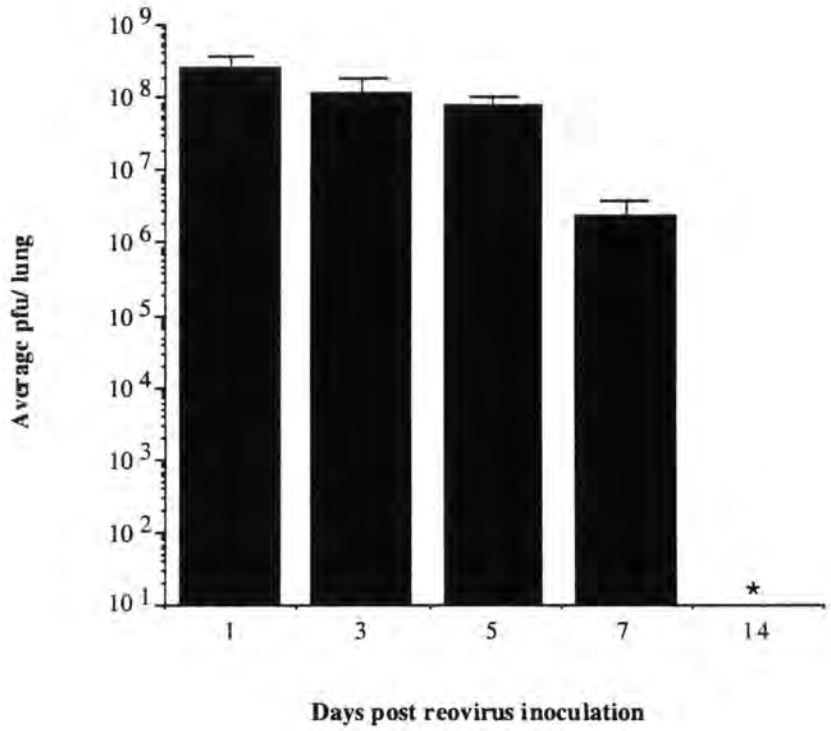

Figure 3.6. Infectious virus titers of the lungs of reovirus 1/L-infected CD-1 mice. The average +/- S.E. number of plaque forming units (PFU) per whole lung was determined from the lungs of three mice per timepoint. *No plaques were detected at the limit of detection (50 PFU/lung) from the lungs of saline inoculated controls or from the lungs of reovirus 1/L-inoculated mice 14 days after infection.

Histopathological alterations in reovirus 1/L-infected CD-1 mouse lungs

Histological observations of reovirus 1/L-infected or control CD-1 lungs were made on H&E stained paraffin embedded lung sections. By day 6 numerous foci of edema accompanied by mononuclear cell infiltration (Figure 3.7B) were observed as compared to control saline inoculated lungs (Figure 3.7A). Few neutrophils, eosinophils, or mast cells were observed. The cellular infiltrate was primarily located in the peri-bronchiolar alveolar spaces and became more tightly condensed by day 11 (Figure 3.7C). At three weeks much of the edema and original cellular infiltrate had disappeared (Figure 3.7D). However, peribroncholar lymphoid follicles indicative of the condition "follicular bronchiolitis" had formed (Figure 3.7D, arrows). Normal bronchioles, alveolar ducts, and alveoli can be observed in control saline inoculated lung tissue (Figure 3.7A).

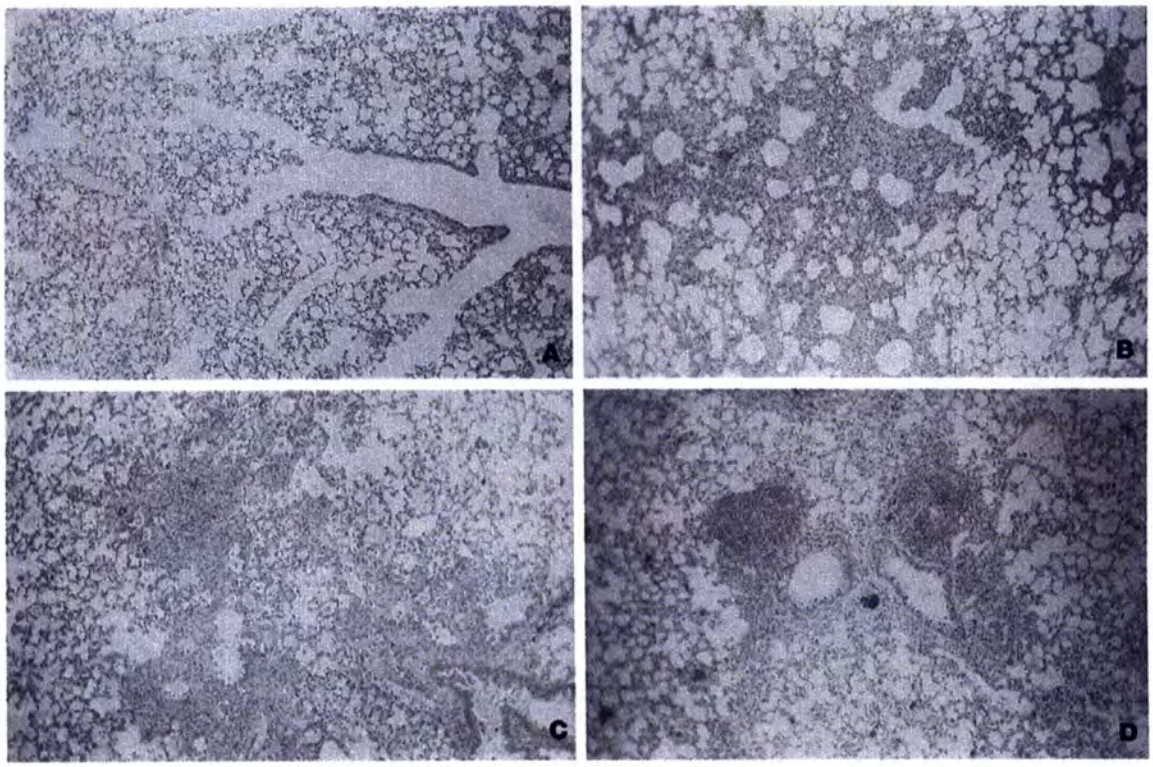

Figure 3.7. Histological characterization of the lung of reovirus 1/L-infected CD-1 mice. H&E staining was performed on formalin-fixed, paraffin-embedded lung sections. (A) Saline-inoculated controls; (B) 6 days post reovirus inoculation ; (C) 11 days post reovirus inoculation; and (D) 21 days post reovirus inoculation. All photographs were taken at 100X magnification.

Observation at a higher magnification was used to reveal the mononuclear characteristics of the interstitial and intra-alveolar infiltrate in the lungs of CD-1 mice. Two distinct yet overlapping processes were observed following reovirus 1/L infection. The first and predominant process involved the generation of cellular damage in the interstitial and alveolar spaces during the inflammatory response initiated by reovirus infection. At day 6 proteinaceous residue of pulmonary edema was present in the airspaces (Figure 3.8A). In addition, nuclear fragmentation was observed in active inflammatory lesions (Figure 3.8A, arrow). A Gordon and Sweet reticulin stain (GSR) demonstrated most of the alveolar supporting reticulin was intact with only occasional evidence of fragmentation of the reticulin framework (Figure 3.9B, arrow). It was also evident by GSR staining that the alveoli were filled with and expanded by a cellular infiltrate (Figure 3.9B). In addition to the largely lymphocytic population that had been observed throughout the infection, at day 11 large numbers of macrophages were also found in the airspaces and interstitium (Figure 3.9C, small arrows). Surfactant secreting alveolar type II cells were also observed to be enlarged at day 11 (Figure 3.9C, large arrows). At three weeks, prominent peri-bronchiolar interstitial lymphoid follicles were present (Figure 3.9D) and foamy macrophages could be observed in the air spaces (Figure 3.9D, arrows).

The second process was evident later in the infection and is characterized by an association of plasma cells with the vasculature of the lung. At day 11 lymphocytes were present adjacent to the pulmonary arteries in the lymphatic and connective tissue spaces (Figure 3.9A, arrows). By day 21, the lymphatic and connective tissue space adjacent to the pulmonary arteries contained cells predominantly of a plasmacytic nature (Figure 3.9B, arrows).

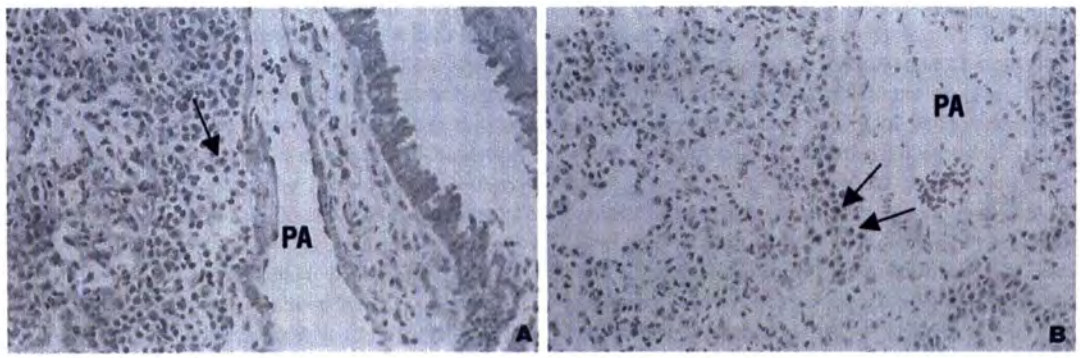

Figure 3.8. Histological characterization of the reovirus 1/L-induced inflamatory infiltrate in CD-1 mice. (A) H&E staining at 11 days post reovirus inoculation showing lymphoid populations in the connective and lymphatic tissue (arrows) adjacent to a pulmonary artery (PA); (B) H&E staining at 28 days post reovirus inoculation showing plasma cells (arrows) adjacent to a pulmonary artery (PA). Both photographs were taken at 400X magnification.

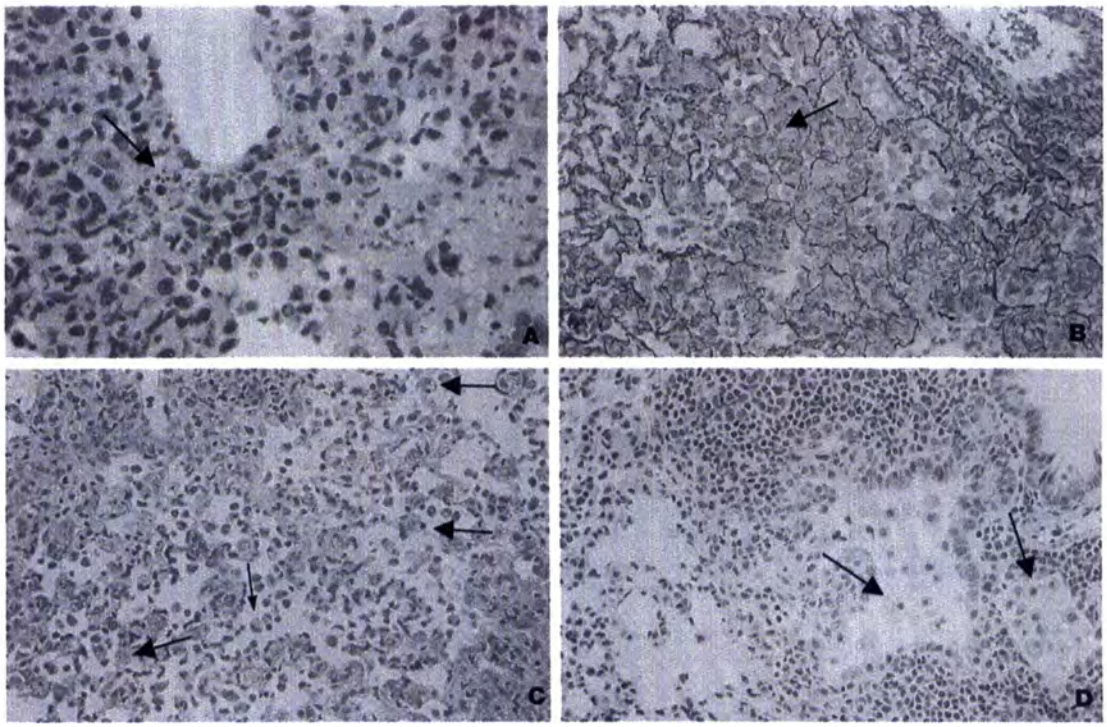

Figure 3.9. Histological characteristics of the lymphoid plasmocytic reaction to reovirus-1/L infection in CD-1 mouse lungs lung. (A) H&E staining showing nuclear fragmentation (arrow) at 6 days post reovirus inoculation; (B) GSR staining showing minor reticulin damage (arrow) at 5 days post reovirus inoculation; (C) H&E staining showing increased number of macrophages in the airspaces (small arrow) and swollen alveolar type II cells (large arrows) at 11 days post reovirus inoculation; (D) H&E staining showing foamy macrophages (arrows) at 28 days post reovirus inoculation. All photographs were taken at 400X

Immunohistochemical localization of lymphocyte populations in reovirus 1/L-infected lungs

Immunohistochemistry was used to examine the phenotype of the cells infiltrating the lungs after reovirus 1/L infection. Saline inoculated mice were used as controls. At day 6 of the infection $CD4^+$ (Figure 3.10B) and $CD8^+$ (Figure 3.10D) lymphocytes were diffusely scattered throughout the lesion and had infiltrated into the interstitial and alveolar spaces. $CD4^+$ (Figure 3.10A) and $CD8^+$ (Figure 3.10C) lymphocytes were not observed in saline inoculated control lungs. In addition to a T cell response, a B cell infiltrate was observed in reovirus 1/L-infected lungs (Figure 3.10F). Although $B220^+$ lymphocytes could be found scattered throughout all compartments of the lung, the majority of $B220^+$ lymphocytes surrounded the pulmonary arteries accompanying the conducting airways (Figure 3.10F). $B220^+$ lymphocytes were not observed in saline inoculated lungs (Figure 3.10E). In comparison to the resident alveolar macrophage population found in the air spaces of control lungs (Figure 3.10G), a large increase in the number of Mac 1^+ cells was observed in reovirus 1/L-infected lungs (Figure 3.10H). These Mac-1^+ cells were more numerous, phenotypically larger and more irregularly shaped (Figure 3.10H) than the Mac-1^+ cells of the control lung (Figure 3.10G).

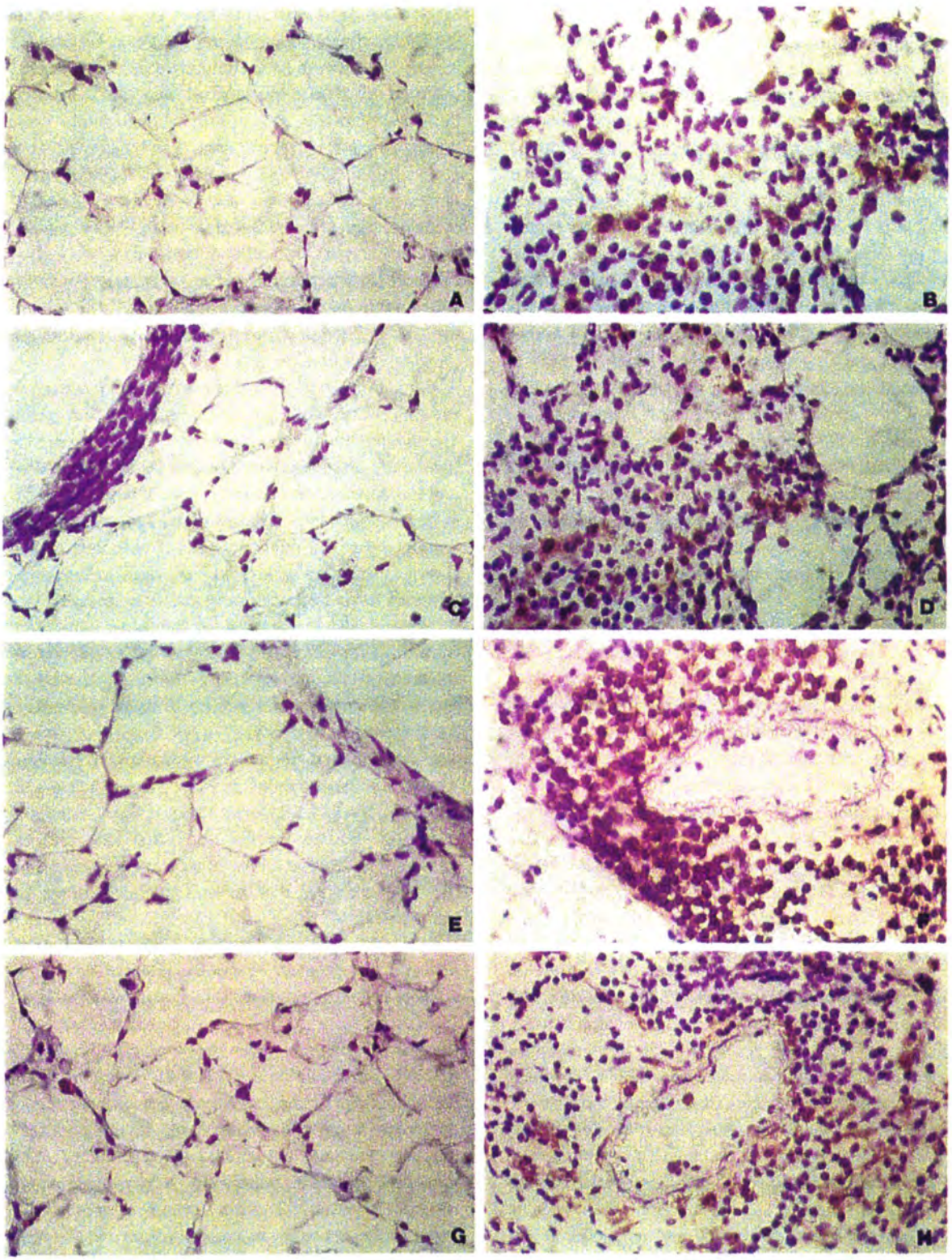

Figure 3.10: Immunohistochemical analysis of the reovirus induced cellular infiltrate in CD-1 mice. Frozen sections prepared from control lungs 6 days post saline inoculation were stained with: (A) anti-CD4 ; (C) anti-CD8 ; (E) anti-B220 ; and (G) anti-Mac-1 . Identically treated lungs 6 days post reovirus 1/L-infection were stained with: (B) anti-CD4 ; (D) anti-CD8 ; (F) anti-B220 ; and (H) anti-Mac-1. The sections were counterstained with Hematoxlyn after the immunostaining procedure. All photographs were taken at 400X magnification.

Discussion

Reovirus infection of the GI tract has provided a useful model for addressing fundamental questions of gut mucosal immunity (London, *et al.* 1987, 1989, 1990). Although the immune response at the mucosal surfaces of the lung has been studied extensively by utilizing various microbial models of infection (Allan, *et al.* 1990; Graham, *et al.* 1991, Hou, *et al.* 1992, Murphy 1994), the majority of these models have been limited by the microorganism's restricted immunogenicity to a single mucosal site. Therefore, it has been difficult to study the interaction of the respiratory and gut-mucosal immune systems in response to a single pathogen. In this report we demonstrate that respiratory reovirus infection elicits an acute lymphocytic inflammatory infiltration of the lung and hyperplasia of the lung-associated LN. Thus, respiratory reovirus infection provides a useful model for the study of respiratory-mucosal immunity and the relationship of respiratory and gut-mucosal immunity to the "common mucosal immune system".

Since the characterization of the *Reoviridae* family of viruses as respiratory and enteric isolates (Sabin 1959), a number of reovirus strains were found to induce pathology of a number of organs systems including the CNS, heart, pancreas, and thyroid (Sharpe and Fields 1985; Onodera and Awaya 1990; Sherry 1993; Yukawa 1993). During the 1960s and early 1970s the effect of reovirus infection of the respiratory tract was studied by several investigators. These early studies demonstrated that the i.n. application of mammalian or avian reovirus strains to newborn or weanling mice resulted in the establishment of a respiratory infection (Hobbs 1965, Klein, *et al.* 1967 Stanley 1974). Delayed bacterial clearance from reovirus-infected lungs was also demonstrated in these studies (Klein, *et al.* 1967, 1969). Recently, it has been reported that reovirus enters the BALT of rats via M cells in a manner analogous to the entry of reovirus into the PP of the gut (Morin, *et al.* 1994). While the histopathology of reovirus infection was not addressed

in this study, reovirus virions were observed associated with type 1 alveolar epithelial cells suggesting that infection of the lung epithelium and viral replication may occur at this site.

We have found that i.n. inoculation of CD-1 mice with reovirus 1/L results in a severe acute viral bronchopneumonia that is histologically similar to the murine model of respiratory syncytial virus infection (Connors, *et al.* 1992, Murphy 1994). The most obvious characteristic of the reovirus I/L induced respiratory disease is an intense mononuclear inflammatory infiltrate related to small bronchioles and pulmonary arteries. This disease pattern can be divided into two overlapping processes. The first and predominant process is characterized by an inflammatory response in the airspaces and interstitium of the lung. The peri-bronchiolar location of the inflammatory infiltrate, which can be visualized as early as day 3 of the infection, coupled with the observation of nuclear fragmentation at this site several days later suggests that viral infection and replication occurs in this region of the lung. As the infection progresses, the initially diffuse cellular infiltrate becomes more focused around small bronchioles. Although the airspaces become packed with lymphocytes, the reticulin framework of the lung remains largely intact. While viral replication diminishes during the later part of the first week of the infection, and infectious virions are eliminated during the second week, the acute phase of the inflammatory response persists and continues to organize during this time. Within the lesion a second process occurs resulting in the accumulation of plasma cells in the lymphatic and connective tissue adjacent to pulmonary arteries. As this second cellular event progresses, the original infiltrate concurrently becomes more condensed and organized, primarily around bronchioles, and includes an increasing number of macrophages. By the third week, foamy macrophages, indicative of macrophage mediated clearance of cellular debris and edema, are present in airspaces and terminal bronchioles.

We also demonstrate the probability that the potent lung inflammatory response induced by reovirus 1/L is not simply a nonspecific reaction to viral infection or damage to the alveolar epithelium. First, the absence of a potent inflammatory lung response by CD-1

mice after i.n. inoculation with reovirus 3/D demonstrates that the respiratory reovirus model of lung inflammation is reovirus serotype specific. In addition, by moving the model of respiratory reovirus 1/L infection to outbred mouse strains Balb/C and C3H we show that the genetic background of the host animal may influence the intensity of the inflammatory response to reovirus 1/L. Intranasal inoculation of Balb/C mice with reovirus 1/L induces a pattern of pulmonary inflammation that closely parallels the peri-bronchiolar accumulation of mononuclear cells observed in CD-1 mice after i.n. inoculation with reovirus 1/L. However, the pulmonary response of C3H mice to i.n. inoculation with reovirus 1/L is considerably less severe and does not result in the prominent FB-like organization of infiltrating mononuclear cells. Although the variability observed in the reovirus 1/L-induced pulmonary responses among CD-1, Balb/C, and C3H mice may seem to indicate that infectious reovirus 1/L would be cleared from the lung with kinetics that would be directly related to the intensity of the inflammatory response, assessment of viral replication by northern analysis indicates that the reovirus replication kinetics are similar between the strains.

In situ immunohistochemical analysis of reovirus-infected CD-1 lungs was performed to characterize the composition of the cellular infiltrate of the lung. This analysis revealed the presence of $CD4^+$ and $CD8^+$ T-lymphocytes, B-lymphocytes, and macrophages in the airspaces and interstitial tissues of reovirus 1/L-infected lungs. Thus, any of these cellular populations may be involved in either the clearance of the viral infection and/or be associated with the persistence of the inflammatory infiltrate. Clearance of infectious viruses from the lung is frequently associated with a CTL response mediated by MHC class I restricted $CD8^+$ T lymphocytes (Allan, *et al.* 1990; Hou, *et al.* 1992; Taylor, *et al.* 1985). It is possible that the $CD8^+$ lymphocytes infiltrating reovirus-infected lungs are performing an analogous function. Our observation that infectious reovirus virions are cleared with similar kinetics to that observed in experimental influenza and Sendai virus infections (Eichelberger, *et al.* 1991; Hou and Doherty 1995) may support this hypothesis.

In addition, we have demonstrated lysis of reovirus-infected target cells by both pulmonary and lung-associated LN lymphocytes obtained from reovirus-infected but not normal Balb/c mice (Thompson, *et al.* 1996). The function of $CD4^+$ helper T cells in the mediation of viral clearance from the lung is unclear, making their role in lung immunity difficult to assess (Allan, *et al.* 1990; Connors, *et al.* 1992). *In vivo* depletion of $CD4^+$ cells in the influenza model only delays the clearance of influenza virus by four days (Allan, *et al.* 1990), although the $CD4^+$ population also appears to be a primary source of TH_2 associated cytokines (Sarawar and Doherty 1994). Conversely, *in vivo* depletion of $CD4^+$ but not $CD8^+$ cells abrogates the histopathology observed in respiratory syncytial virus infected mice (Connors, *et al.* 1992). The role of the $CD4^+$ T lymphocytes in reovirus infected lungs remains to be determined.

Although the humoral component of the respiratory reovirus 1/l response in this model has not been investigated, the presence of $B220^+$ cells adjacent to pulmonary arteries during the first week of infection and the appearance of plasma cells at the third week in the same location suggests that a reovirus-specific component of the pulmonary B cell response is occurring. If reovirus-specific B cells exist in the lung, they may either be locally generated or be the progeny of viral-specific B lymphocytes which have emigrated from the lung-associated LN. Most investigations of the humoral immune response in the lung indicate that IgG is the predominate isotype in lower respiratory tract secretions (Reynolds, 1991). One group, however, reports that IgA predominates over other isotypes in an experimental influenza model (Fazekas, *et al.* 1994). It will be of interest to determine the isotype profile of reovirus-specific antibodies in the lung and to correlate the isotype profile of reovirus-specific antibodies of the lung and gut after various mucosal routes of immunization.

Respiratory reovirus infection generates a potent mucosal inflammatory response that makes reovirus a suitable probe for respiratory mucosal investigations. The results reported in this chapter suggest that reovirus can be used to develop a practical model of

respiratory mucosal immunity that will contribute to an understanding of the pulmonary immune response and more broadly, its relationship to the common mucosal immune system. Most importantly this model will provide an opportunity to compare immune responses to the same immunogen at remote mucosal surfaces, namely between the lung and GI tract. In addition to these applications, respiratory reovirus infection provides a potentially useful model for the study of a spectrum of inflammatory lung diseases. The follicular organization of the lymphoid infiltrate in the lungs of CD-1 mice following reovirus infection is similar to the human condition termed follicular bronchiolitis (FB). FB is characterized by enlarged peri-bronchiolar lymphoid follicles that restrict airflow by the compression of the bronchioles (Wells and du Bois 1993). In humans FB may be associated with environmental factors such as viral infections or the inhalation of tobacco smoke (Wells and du Bois 1993). Additionally, FB may be a component of systemic disorders with a predilection for connective tissue diseases which are characterized by increased fibroblast activity and matrix deposition (Myers and Colby 93, Wells and du Bois 93). Additionally, as addressed in the subsequent chapter, I observed that CBA/J mice in response to respiratory reovirus 1/L infection develop the intraluminal fibrotic syndrome Bronchiolitis Obliterans Organizing Pneumonia (BOOP), or the acute inflammatory disorder, Acute Respiratory Distress Syndrome (ARDS) (Bellum, *et al.* 1997). BOOP, and ARDS, like FB, are considered to belong to a spectrum of lymphocytic inflammatory diseases of the small airways of the lung (Myers and Colby 1993; Wright, *et al.* 1992). However, BOOP is characterized by the formation of intraluminal fibroblastic plugs in terminating bronchioles, alveolar ducts, and airspaces in addition to the usual presence of an organizing lymphocytic (Myers and Colby 1993). The development of ARDS also has underlying similarities to the generation of FB and BOOP, but is primarily characterized by extensive vascular damage that results in the flooding of the air spaces with edema (Myers and Colby 1993; Wright, *et al.* 1992). While BOOP, ARDS and FB are different syndromes which to date have not been linked in a causal relationship, our results suggest

that comparative differences in the respiratory response to reovirus among strains of mice could be used to investigate relationship of FB and BOOP as a continuum of inflammatory lung disease.

CHAPTER 4

Respiratory Reovirus 1/L-Induced Bronchiolitis Obliterans Organizing Pneumonia: A Model for the Study of Bronchoalveolar Intraluminal Fibrosis

Introduction

Acute, inflammatory responses that occur in the distal air spaces of the lung (bronchioles, alveolar ducts, and alveoli) may develop into one of a limited number of nonspecific pulmonary disorders characterized by fibrosis (Cordier, *et al.* 1994; Myers, *et al.* 1994; Katzenstein and Askin 1990; Kuhn, *et al.* 1989). The pathologic features of a fibrotic response in the lung may include interstitial collagen deposition as observed with usual interstitial pneumonia (UIP), interstitial fibroblast proliferation as observed with diffuse alveolar damage (DAD), or intraluminal fibroblast proliferation as observed for a relatively recently described disorder termed bronchiolitis obliterans organizing pneumonia (BOOP) (Myers, *et al.* 1994; Epler, *et al.* 1985). BOOP lesions have a patchy distribution in the lung, are frequently associated with a peri-bronchiolar organizing pneumonia and also with the presence of lipid - laden foam cells in the alveolar spaces (Colby 1992). The structural integrity of alveolar ducts and walls within regions of BOOP lesion development are normally preserved; however, the alveolar septa may be thickened with an infiltrate of inflammatory mononuclear cells (Colby 1992). Since the initial description of BOOP by Epler and co-workers in 1985, an increasing number of reports of BOOP have appeared in the literature and BOOP is now accepted as a distinct clinical syndrome. While it has been documented that BOOP lesion development can be a secondary disorder to a number of pulmonary infections (which are frequently believed to be viral), most BOOP cases are not associated with any identifiable pathogen and are classified as idiopathic BOOP (Kuwano, *et al.* 1990; Epler 1992). Furthermore, a smaller group of BOOP cases have been associated with toxic fume exposure, connective tissue disorders, and as a clinical complication associated with a subset of lung and bone marrow transplantation (Myers, *et al.* 1994; Martinez and Lynch 1994; Mathew, *et al.* 1994; Thirman, *et al.* 1992). BOOP is normally treated with corticosteroids; however, some patients experience a spontaneous remission of BOOP and approximately one third of patients that have been administered

corticosteroids still show evidence of BOOP lesions at the end of treatment (Izumi 1994). Additional recent literature also suggests that not all BOOP cases respond well to steroids and that BOOP can be fatal in a small subset of cases (Nizami, *et al.* 1995; Epler 1995) Although BOOP is a well established syndrome, a small animal model developed for investigations of BOOP lesion development has not been described in the literature. Therefore, little is known concerning the cellular and molecular mechanisms that mediate the formation and resolution of BOOP lesions. Although existing models of experimental pulmonary fibrosis have been useful for histopathological and functional investigations of other types of fibrotic events in the lung, they fail to accurately reflect the intraluminal and fibroblastic nature of the bronchoalveolar obliteration observed in BOOP lesions (Piguet, *et al.* 1990; Zhang, *et al.* 1996). Therefore, while these models are of interest for the study of interstitial lung fibrosis or collagen scarring they are not directly relevant for the study of BOOP. This chapter describes the first virus-induced model of BOOP in which CBA/J mice are intranasally (i.n.) inoculated with reovirus 1/L and subsequently develop BOOP lesions, which morphologically resemble BOOP in humans. These BOOP lesions, like those observed in humans, are characterized by the patchy distribution of intraluminal plugs of granulation tissue that appear to be chiefly composed of fibroblast like cells and by limited collagen deposition. The development of BOOP lesions in this animal model is preceded by a similarly patchy distribution of peri-bronchiolar mononuclear cell inflammatory lesions which progress into characteristic well developed BOOP lesions during the second and third week post infection.

Results

Respiratory reovirus 1/L infection of CBA/J mice induces intraluminal fibrotic lesions

We have previously described the pulmonary inflammatory response of CD-1 and Balb/c mice after the intranasal inoculation of 1 x 10^7 PFU of reovirus 1/L (Bellum, *et al.* 1996, Thompson, *et al.* 1996). Both strains responded with a well delineated, peri-bronchiolar lymphocytic inflammatory response with 100% of the experimental animals surviving the infection. In contrast, the intranasal inoculation of CBA/J mice with 1 x 10^7 PFU of reovirus reovirus 1/L resulted in a severe clinical illness characterized by extensive damage to the lung vasculature and resulted in a mortality rate approaching 90% in the second week after reovirus 1/L inoculation (Table I, chapter 5). This acute pattern of inflammation recapitulated the human small airway disease termed acute respiratory distress syndrome (ARDS). When the lungs of surviving mice were examined histologically, we observed the presence of lesions which were similar to those observed in humans and characterized as bronchiolitis obliterans organizing pneumonia (BOOP). In Figure 4.1, a well formed BOOP lesion 21 days after reovirus 1/L infection of CBA/J mice is shown. In this figure a characteristic fibrous cellular plug is present in an alveolar duct, which indicates the lesion has a proximal acinar location in the lung. As shown in this figure, these well-formed lesions are frequently present in areas of the lung which are otherwise histologically unaffected and normal.

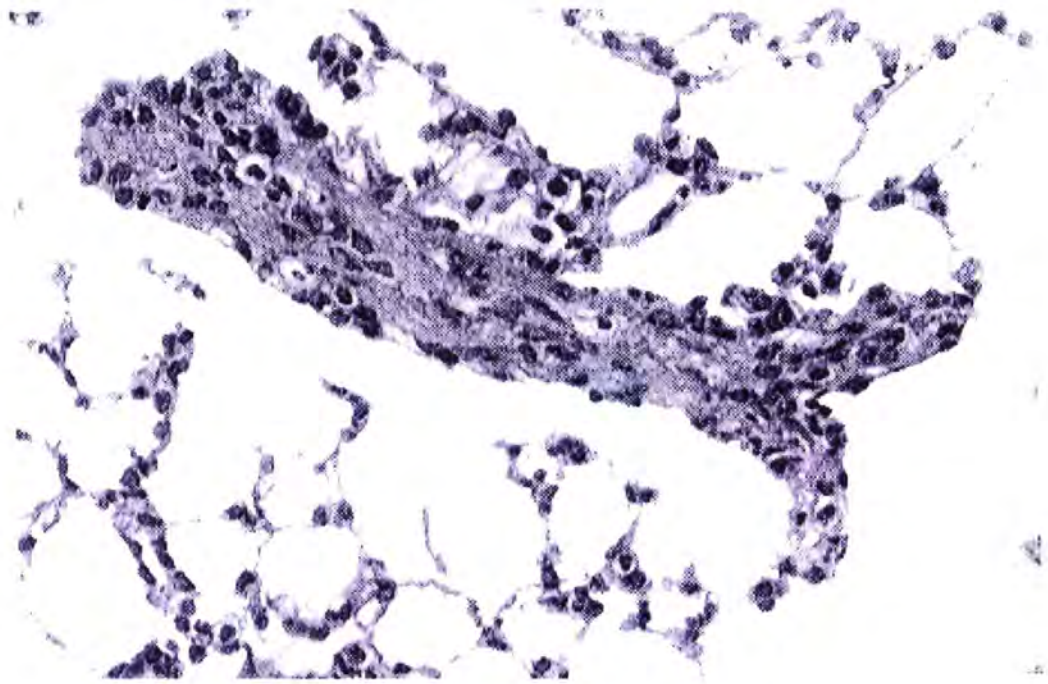

Figure 4.1. Histologic characterization of a well formed BOOP lesion . H & E staining of a characteristic fibrous cellular plug found in an alveolar duct at 21 days post i.n. inoculation with 1 x 10^7 PFU of reovirus 1/L. Objective magnification, X 20.

Histopathological Analysis of the Temporal Development of BOOP Lesions in CBA/J Mice

While we have demonstrated the presence of BOOP lesions in CBA/J mice 21 days after respiratory reovirus 1/L infection, a particular advantage of this model system is the ability to study the development of such lesions over time after the initiation of a known viral infection. These studies clearly demonstrate that the formation of BOOP lesions are preceded by a patchy inflammatory mononuclear cell infiltrate that progressively becomes more organized during the first three weeks of the infection. A kinetic analysis of the formation of BOOP lesions in CBA/J mice is shown in Figure 4.2. In Figure 4.2A, the response to reovirus 1/L at day 7 is typically characterized by an inflammatory cellular infiltrate that demonstrates a typical focus of an active viral pneumonia. At day 7, the cellular infiltrate is characterized by mononuclear cells and macrophages that are in the air spaces and the interstitium (Figure 4.2A). An analysis of lung sections at low power demonstrates that these diffuse inflammatory infiltrates have a patchy distribution throughout the lung and that these are often in a proximal acinar location (data not shown). At day 14, an increase in the number of macrophages and the formation of condensed focal lymphocytic accumulations are observed (Figure 4.2C, arrow). At day 21 the peribronchiolar mononuclear infiltrate is characterized by a more prominent lymphocytic proliferation (Figure 4.2E, arrows). The infiltrate at day 21 is still present in the interstitium and airspaces, including the presence of intra-alveolar macrophages.

In addition to the mononuclear cell inflammatory response, the development of fibrotic lesions can also be observed to occur during the three week time course of the infection. At day 7, the proximal acinar development of young fibrous polyps of collagenous tissue (FP) can be observed (Figure 4.2B). While these types of lesions are not commonly found at day 7, when present, they demonstrate that proliferative fibroblastic lesions can develop in association with an area containing an inflammatory infiltrate both within the alveolar spaces and within the interstitium (Figure 4.2B). At day 14, young fibroblastic polyps continue to

organize in the air spaces and foamy macrophages (arrow) are observed in the air spaces surrounding the lesion (Figure 4.2D). The relationship of this lesion to a small pulmonary artery (PA) indicates that the location of the lesion in the lung is proximal acinar (Figure 4.2D). The fibroblastic polyps appear to develop simultaneously in terminal bronchioles, alveolar ducts, and alveolar spaces. In contrast to humans, respiratory bronchioles (RB) are typically absent in mice, and when present, are poorly developed and defined by the presence of only a few ciliated bronchiolar epithelial cells.(Phalen 1984) The location of fibroblastic polyps observed in this model include regions of the acinus analogous to RB in humans. The further development of these lesions can be observed at day 21 where a well - formed fibroblastic polyp is observed in an alveolar duct (Figure 4.1 and 4.2F).

In contrast to the above described pattern of the development of mononuclear cell inflammatory infiltrates and BOOP lesions, control mice inoculated with saline do not develop an observable inflammatory (Figure 4.2G). Infectious reovirus virions are required to initiate the inflammatory response and the development of fibroblastic lesions. This is demonstrated by the inoculation of CBA/J mice with a non-replicating preparation of reovirus 1/L (produced by exposure of infectious virions to a UV light source) which contains less than 5 PFU/ml of infectious virions as determined by titration on L-929 fibroblast monolayers (data not shown). CBA/J mice inoculated with non-replicating virions do not develop an observable inflammatory response (Fig. 4.2H).

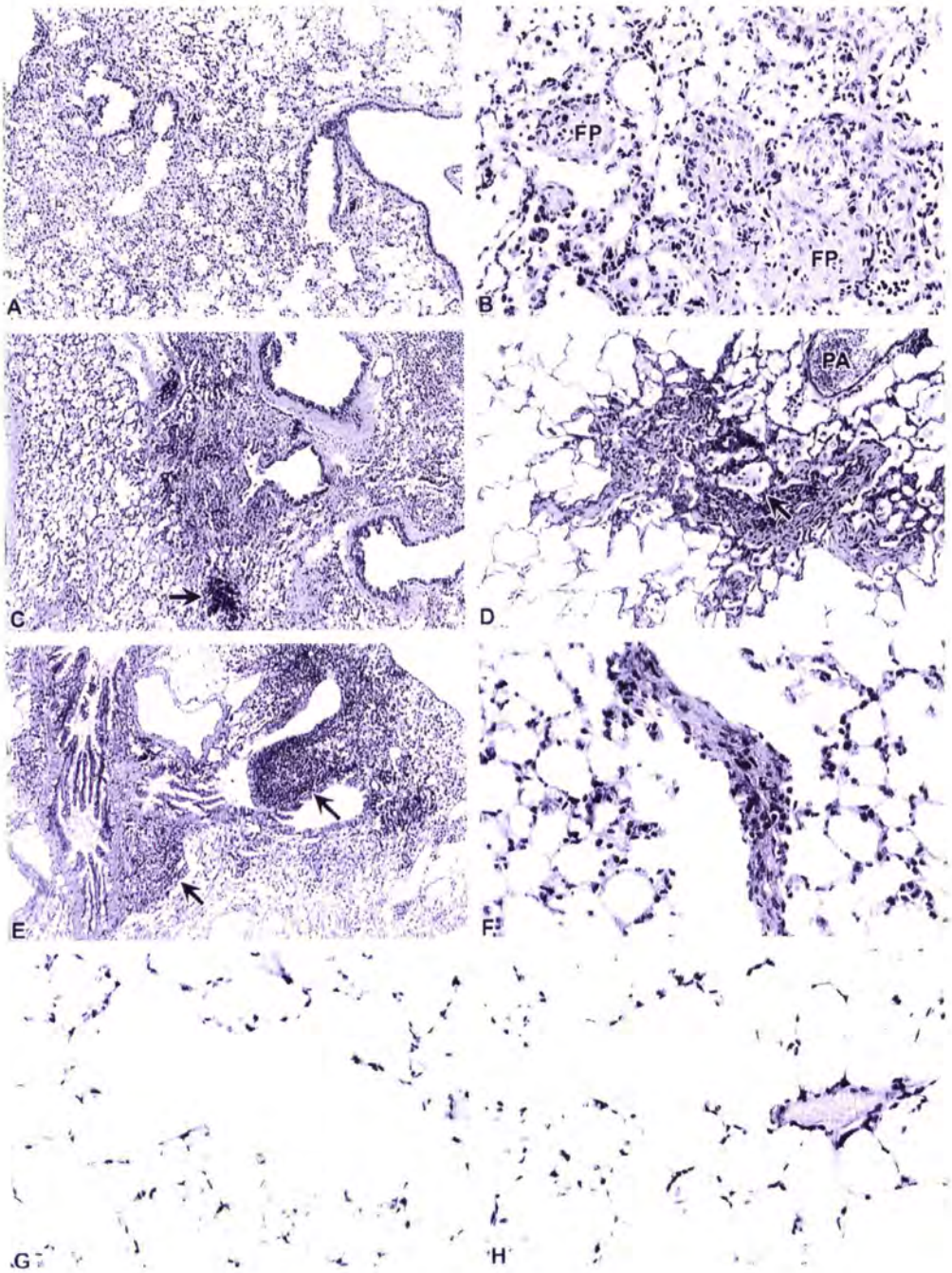

Figure 4.2. Temporal analysis of the pulmonary inflammatory response and fibrotic reaction as shown by H & E staining after i.n. inoculation with 1 x 10^7 PFU of reovirus1/L. The inflammatory mononuclear cell infiltrate, which is initially diffuse at day 7 post inoculation (**A**), is characterized by condensed lymphocytic accumulations (arrow) at day 14 (**C**), and by more prominent peribronchiolar lymphocytic proliferation (arrows) at day 21 (**E**). The stages of development of fibroblastic lesions include the association of young fibrous polyps (FP) at day 7 (**B**), the continued organization of FP in air spaces and observation of foamy macrophages (arrow) at day 14 post inoculation (**D**). The proximal acinar location of the lesion is indicated by the relationship of the lesion with a pulmonary artery (PA). At day 21 post inoculation well formed fibroblastic polyps are observed in alveolar ducts (**F**). Saline inoculated (**G**) and UV inactivated reovirus 1/L (**H**) controls are shown 7 days post inoculation. Objective magnification, X 10.(A, C, and E) and X 20 (B, D, F, G, and H).

To further characterize the nature of the cellular response in this model, Verhoeff Van Gieson (VVG) - stained, paraffin embedded lung sections were analyzed since this elastic tissue stain allows a better appreciation of the architecture of the lung. At 7 days, the cellular infiltrate, which is chiefly composed of lymphocytes and macrophages, is present in the interstitium, alveolar spaces, and alveolar ducts (Figure 4.3A). In Figure 4.3A, a cuff of mononuclear cells is observed around a pulmonary artery (PA). At day 14, fibrous tissue (F) is observed in the alveolar ducts and alveoli (Figures. 4.3B and 4.3C). When these images are viewed in color, pink staining collagen can be discerned morphologically in the fibrous tissue shown in Figure 4.3C. Elsewhere at this time the cellular accumulation has become more histiocytic and more fibrous (Figures. 4.3B and 4.3C). By day 21, prominent clustering of foamy macrophages in the air spaces associated with BOOP lesions is observed (Figure 4.3D).

A Gordon and Sweets stain for reticulin (GSR) was performed on paraffin - embedded reovirus 1/L infected lung sections to detect changes in the supporting architecture of the lung during the development of BOOP lesions (Figure 4.4). At day 14, reticulin-containing organizing fibrous tissue in alveolar ducts is observed (Figure 4.4A, arrow). In addition, reticulin fibers are observed in close association with clusters of macrophages within the airspaces of BOOP lesions (Figure 4.4B, arrow).

Hyperplasia of the lung associated lymphoid tissue is also a feature of the inflammatory response to respiratory reovirus 1/L infection. The lung associated lymphoid tissue at 7 days post reovirus 1/L inoculation is present in small amounts about a core of adipose tissue in the hilum of the lung (Figure 4.5A). By day 14, the lung associated hilar lymphoid tissue shown in figure 4.5B at the identical magnification as figure 4.5A, had undergone dramatic lymphoid hyperplasia increased in size and is densely packed with lymphocytes (Figure 4.5B). Germinal centers (GC) are also present in the lung associated lymphoid tissue at day 14 (Figure 4.5B).

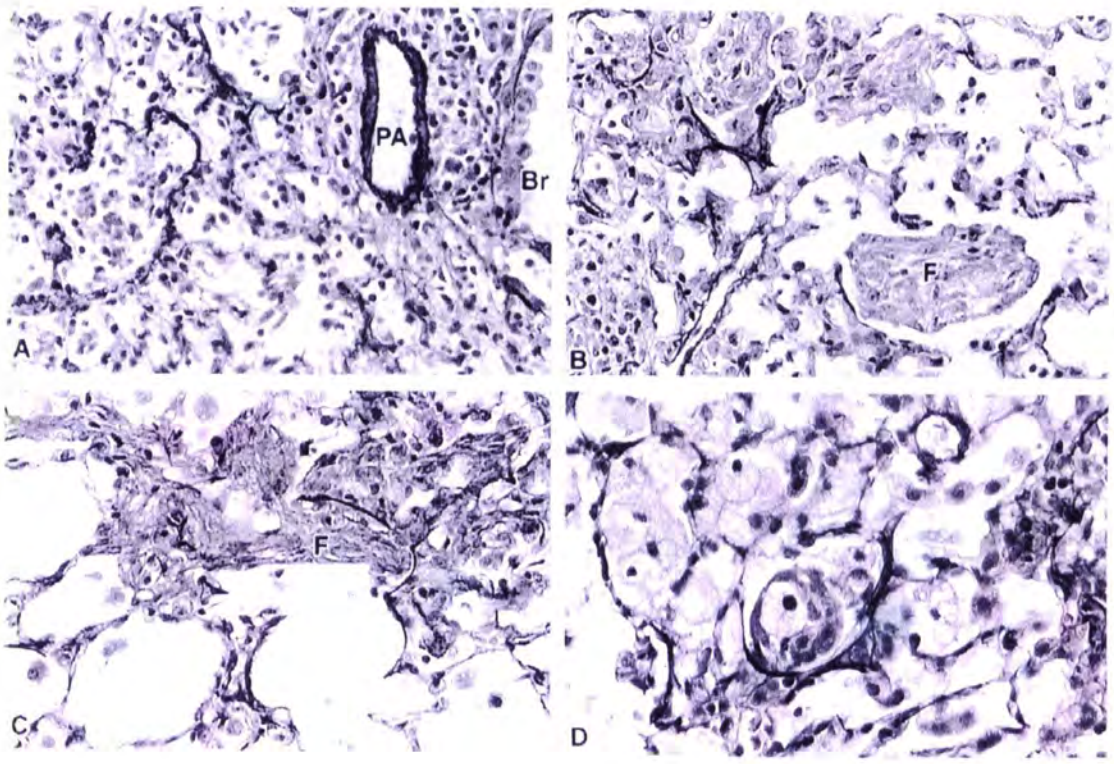

Figure 4.3 Further histologic characterization of the cellular response in CBA/J mice induced by 1 x 10^7 PFU of reovirus-1/L. VVG staining shows a cuff of mononuclear cells around a pulmonary artery (PA) adjacent to a bronchiole (Br) at day 7 post inoculation (**A**). Fibrous tissue (F) at day 14 (**B**) and fibrous tissue with collagen also observed at day 14 (**C**) are found in alveolar ducts and alveoli. Clustering of foamy macrophages in air spaces associated with BOOP lesions at day 21 (**D**).
Objective magnification, X 40.

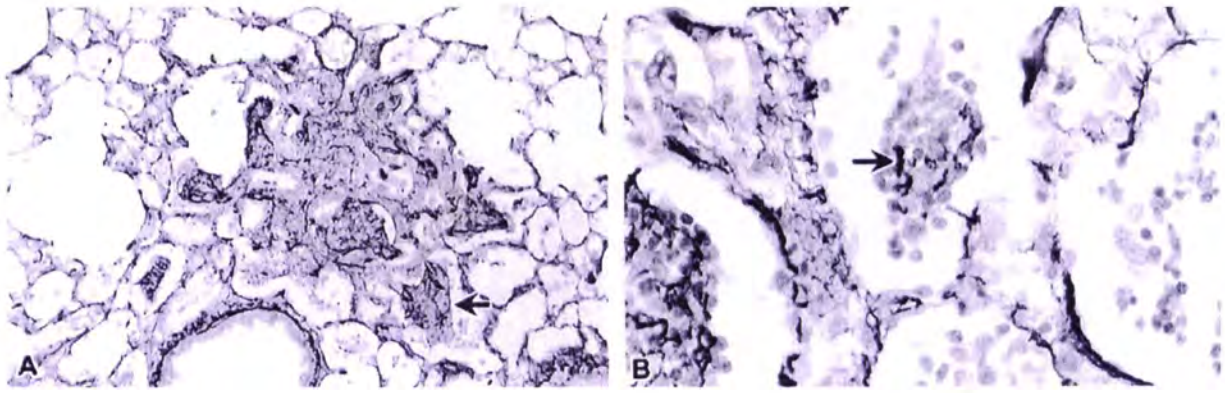

Figure 4.4. Histologic characterization of alterations in lung architecture in CBA/J mice after inoculation with 1 x 10^7 PFU of reovirus 1/L. GSR staining at day 14 post inoculation shows reticulin containing fibrous tissue (arrow) in alveolar ducts (**A**) and a close association of reticulin fibers (arrow) with macrophages (**B**).
Objective magnification, X 10 (A) and X 64 (B).

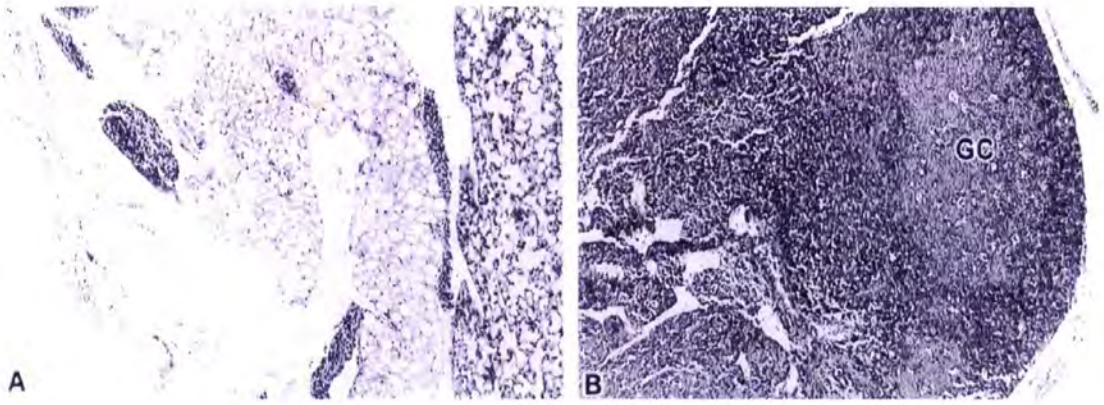

Figure 4.5. Histologic characterization of lung associated lymphoid tissue hyperplasia frpm a CBA/J mouse after inoculation with 1 x 10^7 PFU of reovirus 1/L. H & E staining of hilar lymphoid tissue at day 7 post inoculation (**A**) and at day 14 with a germinal center (GC) shown (**B**).

Ultrastructural Characterization of BOOP Lesions

Transmission electron microscopy (TEM) was used to examine BOOP lesions in the lungs of CBA/J mice 19 days post reovirus inoculation (Figure 4.6). The electron microscopic view of the BOOP lesion in Figure 4.6A depicts the extension of a collagenous lesion (Col) into the air space, which is defined by an epithelial type I cell (E, center of field) and a type II cell (II, left center). The cellular processes of fibroblasts (F) that have interdigitated into the collagen bundles can also be observed in the lesion (Figure 4.6A). A pulmonary epithelial cell (E) lines the alveolar extension of the fibrotic (BOOP) lesion and an alveolar macrophage (M) is also present (Figure 4.6A). Figure 4.7B shows another ultra structural feature of a BOOP lesion. In this electron micrograph, long strands of young fibroblast processes can be seen embedded in a matrix of glycosaminoglycans (GAG) (Figure 4.6B). Macrophages are also present in close association with fibroblasts and the extracellular material of this lesion (Figure 4.6B).

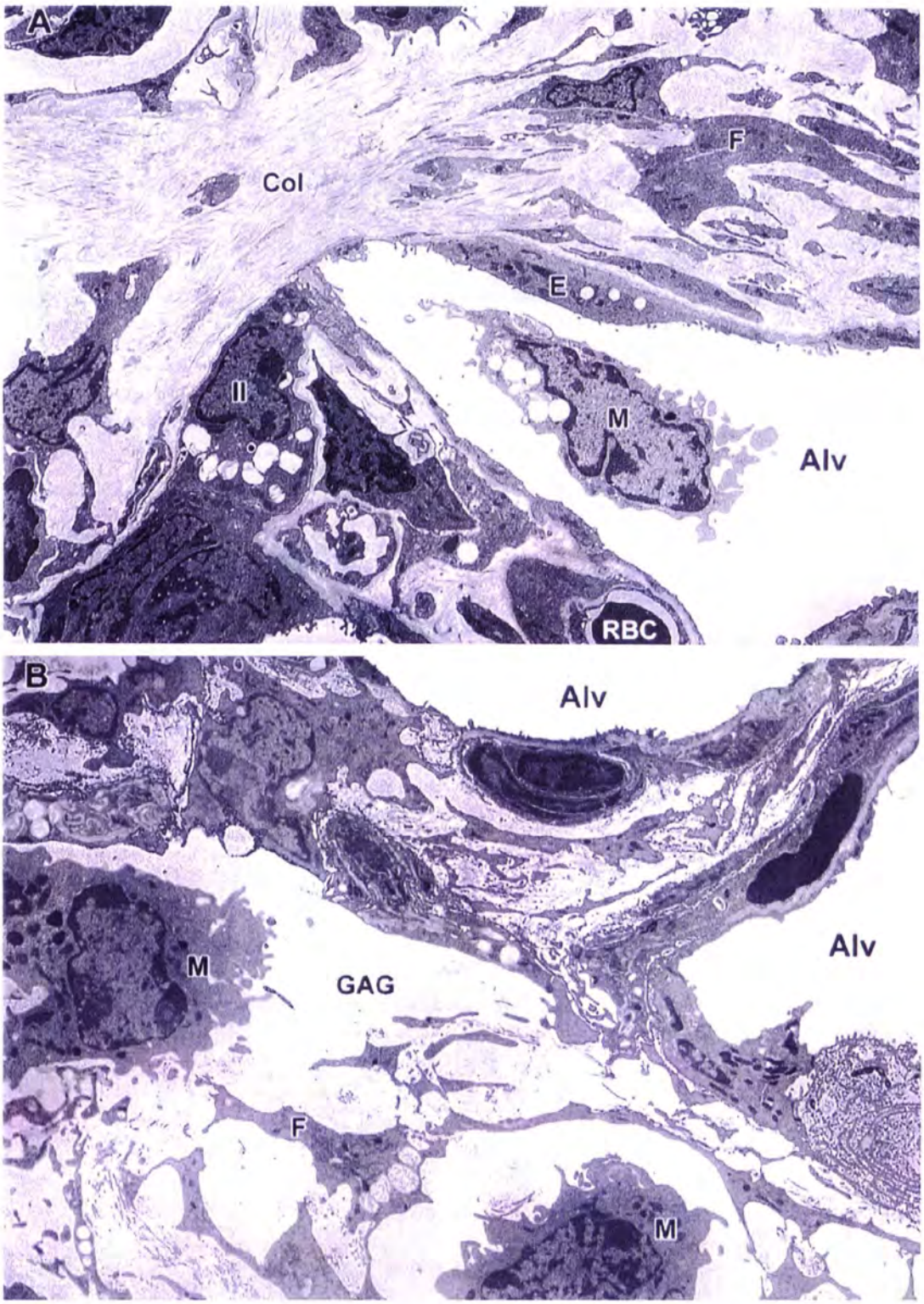

Figure 4.6. Ultrastructural characterization of a BOOP lesions. At 19 days after inoculation with 1 x 10^7 PFU of reovirus 1/L transmission electron microscopy shows a lesion composed of collagen (Col) and fibroblasts (F) in close association with alveolar epithelial type I (E) and type II (II) cells, extending into an alveolar space (Alv), (**A**). A macrophage (M) and red blood cell (RBC) are also observed. Macrophages (M) and cellular processes of fibroblasts (F) are found embedded in a matrix of glycosaminoglycans (GAG), (**B**).
Magnification, X 6250.

Immunohistochemical Localization of Reovirus Associated Proteins in Reovirus 1/L - infected Lungs

Immunohistochemistry was used for the *in situ* identification of reovirus-encoded proteins in this model. At day 1, a patchy distribution of reovirus-encoded proteins is observed as a diffuse focus of deposition at low magnification (Figure 4.7A). Higher magnification of the same lesion demonstrates positive staining for reovirus proteins in a large number of cells; however, the precise nature of the cells cannot be determined because of the intensity of the staining (Figure 4.7B). At day 7, the immunoperoxidase product is still present on cells that appeared to be macrophages and alveolar epithelial cells (Figure 4.7C). Control lungs stained at 7 days after inoculation with UV - inactivated reovirus 1/L were negative for reovirus associated proteins (Fig. 4.7D).

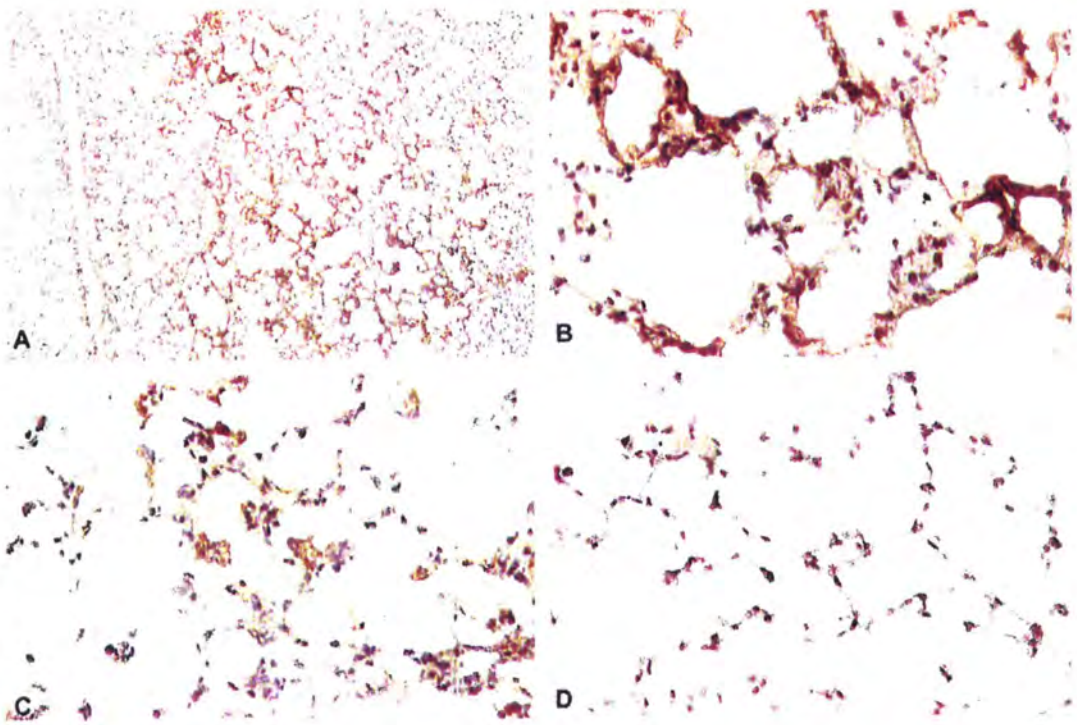

Figure 4.7. Immunohistochemical analysis of reovirus - encoded proteins in CBA/J mice after i.n. inoculation with 1 x 10^6 PFU of reovirus1/L. Anti-reovirus staining was performed on frozen sections prepared on day 1 post inoculation, which is shown at low magnification (**A**) and higher magnification (**B**). Staining was also performed at day 7 (**C**) and on control lungs at day 7 post inoculation with UV inactivated reovirus 1/L (**D**).

Clearance of Reovirus 1/L from the Lungs of Infected Mice

The clearance of virus from the lungs of mice infected with reovirus 1/L was assessed by a standard plaque assay of lungs homogenized by sonication (Figure 4.8). At day 1 of the infection the average titer of infectious virions is 6.5 x 10^6 PFU/lung representing an approximately 65% increase from the initial viral inoculum of 1 x 10^6 PFU The titer increases to 7.5 x 10^6 PFU/lung on day 3 and drops to 2.7 x 10^6 PFU/lung on day 5. However, at day 7 a 1.5 log decrease of infectious virions is observed (1.2 x 10^5 PFU/lung) as compared to the titer of infectious virus present on day 5. By day 21, infectious virions are not detected within the limits of the assay. Infectious virions are not observed in homogenized, saline inoculated control lung tissues (data not shown). Additionally, in a separate experiment reovirus 1/L dissemination to tissues other than the lung also assessed by a standard plaque assay of homogenized spleen and Peyer's Patches (PP) during a one week time course after i.n. inoculation with 1 x 10^7 PFU of reovirus 1/L. At day 1 of the infection the average titer of infectious virions in the lung, spleen, and PP was 2.1 x 10^8 PFU, 1.7 x 10^2 PFU and 3.5 x 10^3 PFU respectively. In lungs the titer increases slightly to 2.3 x 10^8 PFU/lung on day 3 and drops to 6.8 x 10^6 PFU/lung on day 7. Spleen and PP demonstrate approximately a ten fold increase in infectious virus titer from day 1 to day 3 after inoculation (spleen, 4 x 10^3 PFU and PP, 1.5 x 10^4 PFU), but then on day 7 (spleen, 3.3 x 10^2 PFU and PP, 5 x 10^2 PFU) both of these tissues show a return to titers similar to those observed on day 1.

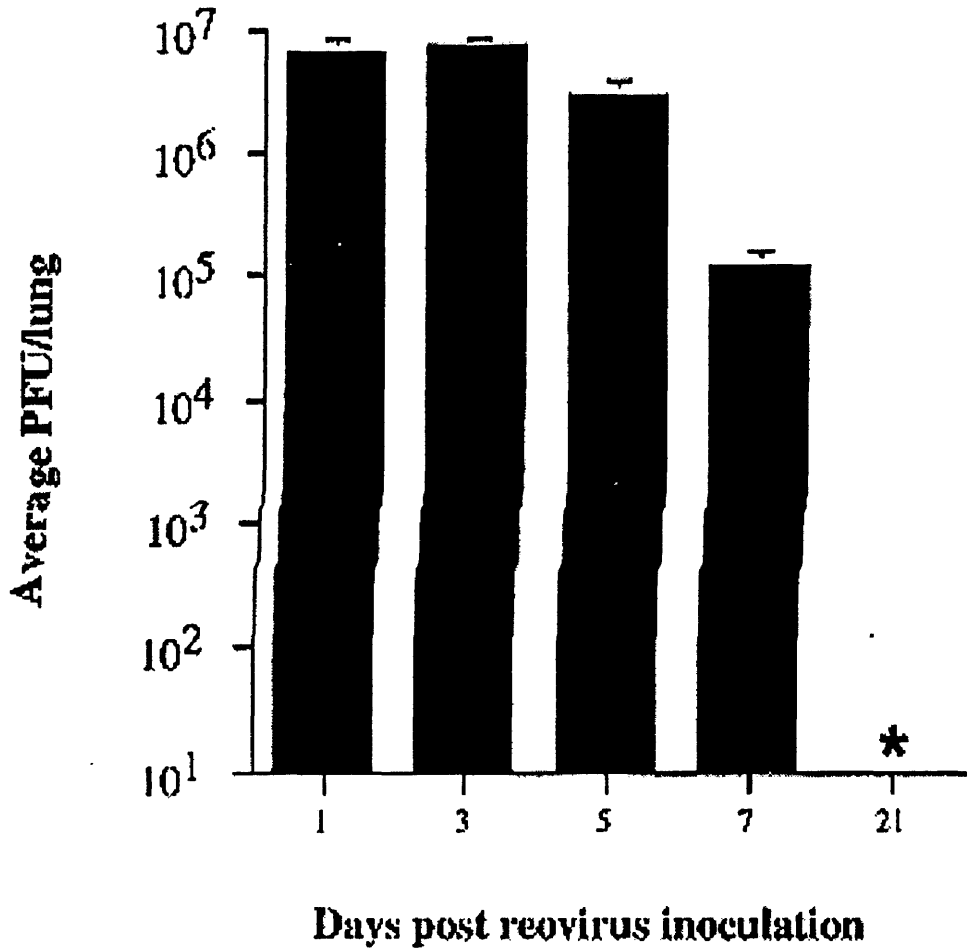

Figure 4.8. Infectious virus titers of the lungs of reovirus 1/L-infected mice. The average ± SE number of PFU per whole lung was determined from the lungs of three mice per timepoint. *No plaques were detected at the limit of detection (50 PFU/lung) from the lungs of saline-inoculated controls or from the lungs of reovirus 1/L-inoculated mice 21 days after infection.

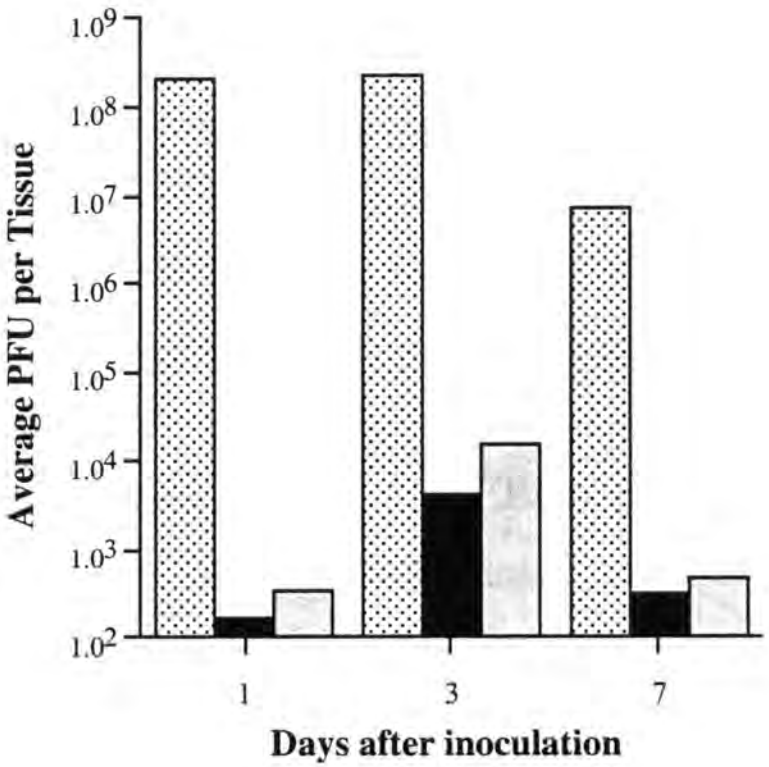

Figure 4.9. Infectious titers of the lungs (⬚), spleen (■), and Peyer's patches (□). The average number of plaque forming units (PFU) per whole lung was determined from the lungs of three mice per timepoint.

Discussion

BOOP belongs to a group of lung disorders whose response to acute lung injury is primarily characterized by fibroblast proliferation within the lumina rather than the interstitial deposition of collagen observed in other fibrotic lung diseases (Myers, *et al.* 1994, Katzenstein and Askin 1990). However, unlike related fibroblast proliferative disorders such as the interstitial pneumonias and bronchiolitis obliterans, a constrictive bronchiolar disease, BOOP is distinguished from these by the following features: (i) the formation of connective tissue polyps in the lumina of distal air spaces; (ii) fibrinous exudates; (iii) the collection of foamy macrophages in the alveoli; (iv) the infiltration of alveolar walls with lymphocytes; (v) plugs of myxoid connective tissue in airspace lumina; (vi) the maintenance of lung architecture (Epler 1992). The current understanding regarding the development of BOOP is based largely on histopathologic and radiological observations and on pre-existing knowledge of mechanisms of fibrosis. BOOP is usually detected in humans as it approaches end stage (Epler, *et al.* 1985). In this setting, the cellular and molecular factors that were involved in the formation of BOOP can no longer be identified. While there are existing models of BOOP in the literature, they are either not practical because the use of large animals is involved, or they fail to accurately parallel the histopathologic observations of BOOP in humans (Castleman 1985; Fukuda, *et al.* 1985; Rhodes, *et al.* 1989). Since many cases of idiopathic BOOP in humans are believed to be the sequelae of undocumented viral infections (Kuwano, *et al.* 1990; Epler 1992), the development of a small animal model of virus-induced BOOP would aid investigations concerning the temporal development and resolution of the intraluminal fibroblastic lesions.

A temporal analysis of respiratory reovirus 1/L infection in CBA/J mice demonstrated that the formation of BOOP lesions is preceded first by a patchy distribution of reovirus protein expression which is followed by a similar patchy distribution of inflammatory mononuclear cell infiltrates. These patchy inflammatory infiltrates have a

proximal acinar location and are initially loosely organized into the typical foci of an active viral pneumonia. During the three weeks following reovirus 1/L inoculation, the initial mononuclear cell infiltrate condenses into prominent peri-bronchiolar lymphoid accumulations. However, in CBA/J mice, a second process occurs in which a subset of lesions become infiltrated with fibroblasts and progress over the three week period into discrete BOOP lesions. The pattern of mononuclear cell organization without the involvement of intraluminal fibrosis, which is observed in CD-1 and Balb/c mice, results in lesions histopathologically consistent with the non - fibrotic human syndrome termed follicular bronchiolitis (FB) (Myers and Colby 1993; Wells and du Bois 1993). Since CBA/J mice develop similar foci of mononuclear cell organization, our studies reveal that BOOP lesions occur within a spectrum of inflammatory lung diseases that occur in the distal air spaces. Our studies with respiratory reovirus infection in different strains of mice suggest that genetic host factors are critical determinants of the degree of progression of a pulmonary insult from a primarily inflammatory response (FB) toward a fibrotic response (BOOP).

Since infiltrating lymphocytes seem to be involved with the initiation of BOOP lesions (Mukae, *et al.* 1995; Costabel, *et al.* 1992), it is possible that these cells play an active role in the progression of inflammatory foci into lesions which are progressively dominated by fibroblasts. The hyperplastic, reactive hilar lymphoid tissue and organizing lymphocytic peri-bronchiolar accumulations observed after reovirus infection suggest that a vigorous immune response is elicited in the lung and draining lymph nodes. We have previously demonstrated that the organizing lymphocytic peribronchiolar accumulations consisted of both $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, and increased numbers of macrophages in CD-1 and Balb/c mice (Bellum, *et al.* 1996; Thompson, *et al.* 1996). In Balb/c mice, there is a dominance of $CD8^+$ T lymphocytes in the lung interstitium and alveolar spaces after respiratory reovirus 1/L infection (Thompson, *et al.* 1996). Lung and hilar lymph node lymphocyte populations contain virus-specific cytotoxic T lymphocytes

A comprehensive review of BOOP suggested that BOOP "is a nonspecific mode of repair of parenchymal structures after acute or subacute injury, and thus cannot be dissociated from the spectrum of inflammatory reactions that have both alveolar epithelial injury and fibrinous intra-alveolar deposition as a common denominator " (Cordier, *et al.* 1994). These results describing the comparative differences in the respiratory response to reovirus infection among strains of mice are in agreement with this model. Thus, it is our contention that respiratory reovirus infection provides a small animal model to investigate the relationship of BOOP as a continuum of inflammatory lung disease.

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CHAPTER 5

Respiratory Reovirus 1/L-Induced Acute Respiratory Distress Syndrome: A Model for the Study of the Immunopathogenesis of Acute Bronchoalveolar Inflammation

Introduction

Acute respiratory distress syndrome (ARDS) is a significantly important acute lung disorder that is characterized by the most severe inflammatory reaction known to occur in the lungs (Luce 1998). The mortality rate exceeds 50% and generally ARDS is not responsive to corticosteroid treatment as is BOOP and currently only supportive therapy such as mechanical ventilation is available to treat ARDS (Rubin and Farber 1994). One of the most prominent pathological indicators of ARDS is the formation of hyaline membranes along the basal lamina between the vascular endothelium and the alveolar epithelium (Luce 1998). The formation of these membranes results from the deposition of fibrin, albumin, cellular debris, and other material that is associated with widespread damage to the endothelial and epithelial surfaces (Luce 1998). The collapse of physical barriers between the intraluminal compartment of the alveoli and the vasculature leads to significant edema, which is a leading cause of mortality among ARDS cases (Luce 1998). Histopathological of observations of ARDS development in humans and in a rat model of ARDS demonstrate that the destruction of the vascular endothelium is preceded by damage to alveolar type I cells (Lamy 1976, Bachofen 1977). The pathogenesis of ARDS also includes infiltration of lymphocytes into the lung (Luce 1998). However, unlike BOOP and other lung disorders characterized by a foundation of organizing pneumonia with a patchy distribution in the lung, the distribution of infiltrating lymphocytes in ARDS is widespread (Luce 1998).

The histopathological alterations that occur in the lung during the development of ARDS appear to be a generalized acute response to significant destruction of the alveolar epithelium. This suggestion is indicated by the observation that many of the common causes of ARDS, including sepsis syndrome (systemic bacterial infection), aspiration of gastric contents, pulmonary contusions, and near drownings all generate similar pulmonary pathological changes preceding ARDS (Hudson 1995). Currently one of the major obstacles in providing improved treatment modalities for ARDS is the lack of information

regarding the inflammatory cell populations that mediate the acute inflammatory response in the lung following alveolar epithelial cell damage (Baughman 1996). T-lymphocyte responses have long been observed to be associated with inflammatory responses in the lung, including ARDS, that are initiated by a wide variety of pathogenic, environmental, and autoimmune related causes (Kradin, *et al.* 1986; Costabel, *et al.* 1987; Hunninghake, *et al.* 1980; Dail 1992). However, the role of T-lymphoctes in acute inflammatory reactions in the lung have not been clearly defined (Kumar and Lykke 1995). Our laboratory has characterized a model of ARDS in CBA/J mice following respiratory reovirus 1/L infection. In this model approximately 40% of the infected animals develop an acute pulmonary inflammatory reaction during the second week of infection that recapitulates the histopathological features of ARDS described in humans. The mortality rate of CBA/J mice that develop ARDS in this model is 100%. Those animals that do not develop ARDS survive and develop BOOP lesions. The contribution of T lymphocytes in the pathogenesis of ARDS in this model was examined by inducing a respiratory reovirus 1/L infection in CBA/J mice that were rendered T lymphocyte deficient by neonatal thymectomization. This approach demonstrated that none of the neonatally thymectomized (NTx) CBA/J mice experienced an acute and deadly pulmonary inflammatory response to reovirus that is associated with ARDS. However, Ntx CBA/J mice still developed the intraluminal fibrosis that typifies BOOP lesion formation

RESULTS

Respiratory reovirus 1/L infection alternatively induces ARDS in a subset of CBA/J mice

Histological characterization was performed on H&E stained formalin-fixed paraffin-embedded lung sections from CBA/J mice that died of ARDS related causes 12 days post reovirus 1/L infection (Figure 5.1). The reovirus-induced pulmonary cellular infiltrate was chiefly composed of mononuclear cells and contained relatively few macrophages or PMNs. Overall, the cellular infiltrate was diffuse, but had demonstrated a degree of peri-acinar organization (near bronchiolar divisions) (Figure 5.2A). Evidence of impaired circulation as a potential consequence of vascular damage included the observation of dense collections of compacted red blood cells (RBCs) (stained bright red) in the lung capillaries (Figure 5.2A). Higher magnification of the lung vasculature demonstrated the formation of hyaline membranes on damaged alveolar walls (Figure 5.2B). Typically hyaline membranes are composed of dead cellular debris and serum proteins like albumin that are all joined together in a fibrin matrix (Katzenstein, et al. 1976). Their presence is considered to be a hallmark histopathological feature of ARDS (Katzenstein and Askin 1990).

ARDS development in CBA/J mice following reovirus inoculation is dose dependent

Since an inoculum dose of 1 x 10^7 PFU of reovirus 1/L resulted in a high mortality rate from ARDS in CBA/J, we examined the effect of decreasing inoculum doses of reovirus 1/L on the generation of BOOP lesions in additional experiments. In these studies we found that if a 1 log decrease in the viral inoculum dose was used, the percentage of CBA/J mice that died from ARDS-related causes dropped from 90% to 40% (Table 5.1). All of the surviving mice still developed BOOP lesions that were indistinguishable from those observed in mice inoculated with 1 X 10^7 PFU. Mice inoculated with 1 X 10^5 PFU or

1 X 10^4 PFU of reovirus had a 100% survival rate but did not generate severe inflammatory lesions nor did they generate pronounced fibrotic BOOP lesions (Table 5.1).

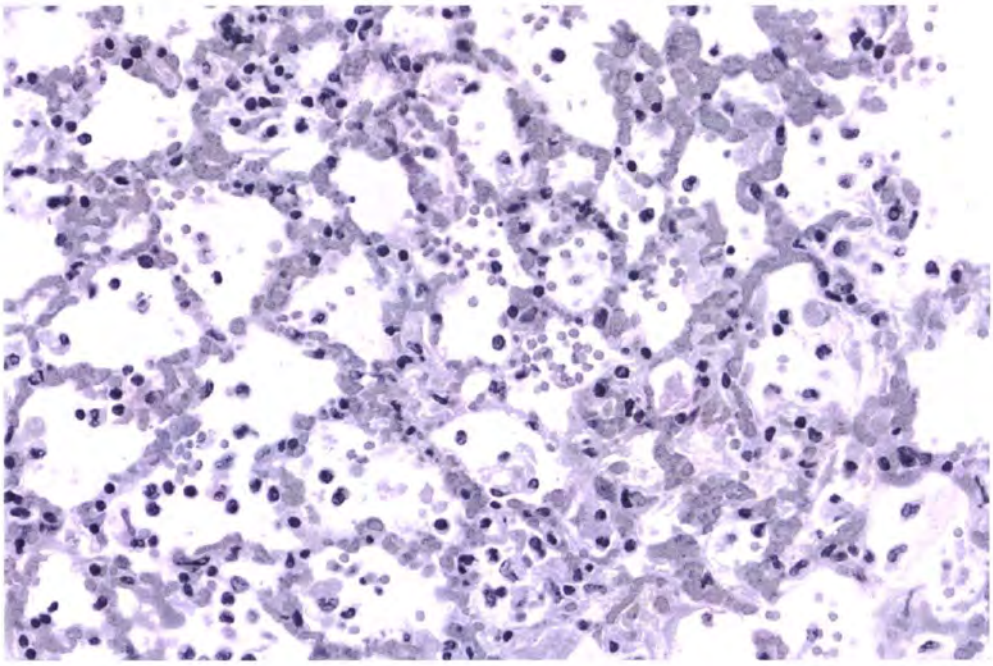
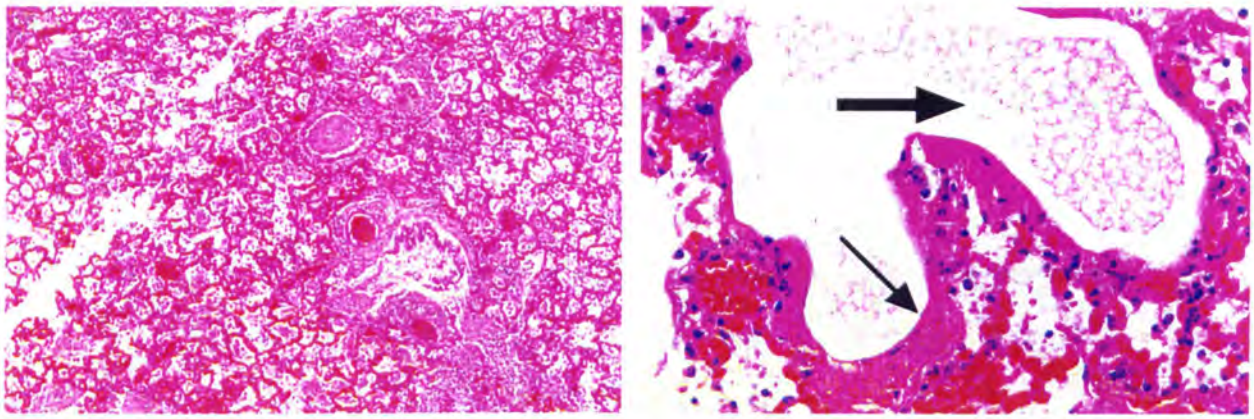


Figure 5.1. Respiratory reovirus 1/L infection induces ARDS in a subset of CBA/J mice. H & E staining shows accumulation of mononuclear cells and PMNs in the interstitial and alveolar tissue
Objective magnification, X 20.



A **B**

Figure 5.2 . *Histologic characterization of acute respiratory distress syndrome (ARDS) in the subset of CBA/J mice that do not develop BOOP following respiratory reovirus 1/L infection.* H & E staining shows the over-accumulation of red blood cells (RBCs) in the capillaries of the lung (**A**, red staining). Higher magnification demonstrates the presence of proteinaceous edema in the vasculature (**B**, large arrow) and the development of thick hyaline membranes along the walls of the lung vasculature (**B**, small arrow).
Objective magnification, X 10 (A) and X 20 (B).

Table I

Inoculum (PFU reovirus)	% Survivors
10^7	10
10^6	60
10^5	100
10^4	100

Histopathological characterization of the lungs of neonatally thymectomized CBA/J mice following respiratory reovirus infection

Confirmation of the T-lymphocyte deficiency generated by neonatal thymectomy was obtained by utilizing flow cytometry analysis to compare the expression of the pan T-cell surface marker, CD3, on peripheral lymphocytes (PLNs) isolated from NTx CBA/J to the expression of CD3 on PLNs from normal age matched nonthymectomized CBA/Js (Figure 5.3). This analysis demonstrated that only 14 % of PLN lymphocytes from NTx CBA/J mice were positive for CD3 as compared to the 69% of PLN lymphocytes that were positive for CD3 nonthymectomized CBA/J mice (Figure 5.3).

Histological observations of reovirus 1/L infected or saline inoculated control NTx CBA/J lungs were made on H&E stained paraffin embedded lung sections. By day 3 post reovirus inoculation, a mixed inflammatory infiltrate composed primarily of macrophages in addition to smaller subsets of polymorphonuclear (PMN) and mononuclear cells had gathered diffusely in the alveolar spaces and the interstitium of the lung (Figure 5.4B). In contrast, a cellular infiltrate was not observed in control lungs (Figure 5.4A). At day 7 post reovirus inoculation the cellular infiltrate had become less diffuse, and more organized around the bronchioles (Figure 5.4.C). The bronchoalveolar association of the cellular infiltrate became more noticeable at two weeks after reovirus inoculation (Figure 5.4D). Potential damage to the lung vasculature was also evident at two weeks after reovirus inoculation by the presence of edema in the alveolar spaces associated with the cellular infiltrate (Figure 5.4D, arrow). Higher magnification of the lesion two weeks after reovirus inoculation further revealed the thickening of alveolar walls, indicating alterations to the architecture of the lung (Figure 5.5A, large arrow). Additionally, at day 14 post reovirus infection fibrous polyps of collagenous tissue had developed in the alveolar spaces affected by the cellular infiltrate (Figure 5.5A, small arrow). As demonstrated in nonthymectomized CBA/J mice the fibroblastic polyps in NTx CBA/J mice could be observed in terminal bronchioles, alveolar ducts and alveoli. The continued development of these fibroblastic

polyps resulted in the formation of mature BOOP lesions by 21 after reovirus inoculation (Figure 5.5B). These BOOP lesions were patchily distributed in the lung and were associated with prominent clusters of intra-alveolar populations of foamy macrophages (Figure 5.5B, arrow).

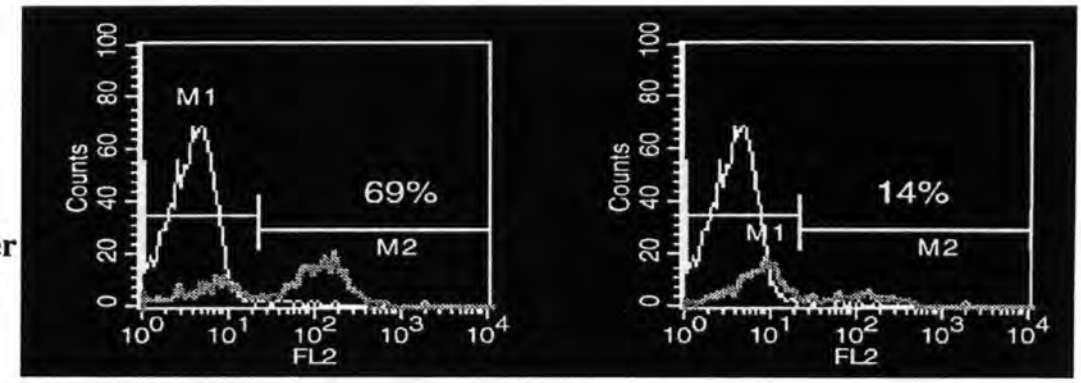


Figure 5.3. Flow cytometric analysis of splenic populations for the pa-T lymphocyte marker CD3 from normal and neonatally thymectomized (NTx) CBA/J mice.

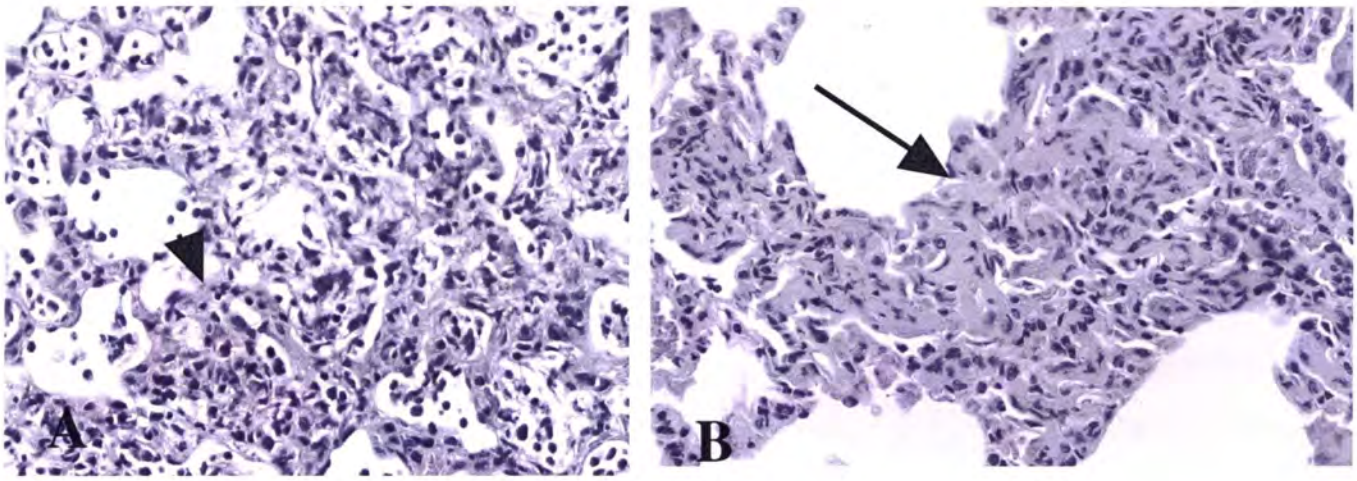


Figure 5.5. Histological characterization of BOOP in NTx CBA/J mice. Day 14 after reovirus 1/L infection (A). Day 21 after reovirus infection (B).
Magnification, X 40

Discussion

ARDS, the clinical diagnosis of the most severe form of lung inflammation known to occur, affects between 1.5% and 4% of the population each year and has a mortality rate of more than 50% (Webster, *et al.* 1988; Thomsen, *et al.* 1995; Lewandowski, *et al.* 1995). Any pathogenic, endogenous, physical, or chemical agent that is capable of causing widespread destruction of the alveolar epithelium can induce the pathology and gas exchange abnormalities associated with ARDS (Luce 1998, Hudson 1995). Although the development of ARDS seems to involve a generalized acute inflammatory response to extensive bronchoalveolar injury, the cellular and molecular mechanisms of this response have not been elucidated (Luce 1998). The participation of lymphocytic populations in acute inflammatory lung disorders like ARDS has been well documented (Schrier, *et al.* 1983; Dail and Hammar 1994). Infiltrating lymphocytic populations are also described as prominent cellular components of the pulmonary inflammatory response in reovirus-induced experimental models of follicular bronchiolitis (FB) in CD-1 mice and bronchiolitis obliterans organizing pneumonia (BOOP) in CBA/J mice (Bellum *et al.* 1996; Bellum *et al.* 1997). FB is characterized by the infiltration of CD4+ and CD8+ T-lymphocytes into the alveolar air spaces and interstitium (Bellum *et al.* 1996). BOOP, a disorder with a similar underlying pathology as FB, is further defined by the development of bronchoalveolar intraluminal fibrotic lesions (Bellum *et al.* 1997). Another aspect of the reovirus-induced response in the lungs of CBA/J mice is the development of an acute inflammatory reaction that recapitulates the histopathology of ARDS in humans. The development of ARDS in CBA/J mice occurs during the second week of infection in approximately 40% of CBA/J mice intranasally inoculated with 10^6 PFU of reovirus 1/L. The remaining 60% develop the intraluminal bronchoalveolar fibrotic lesions associated with BOOP, but do not develop any of the histopathological hallmarks of ARDS such as the deposition of hyaline membranes, vascular congestion, and widespread edema. The relationship between ARDS and reovirus

appears to be dose dependent relationship since a ten-fold increase in the titer of the viral inoculum results in 90% of the CBA/J mice developing ARDS and 10% developing BOOP. It is difficult to determine if those CBA/J mice that develop ARDS following reovirus inoculation could also develop BOOP since edema, RBC congestion, and hyaline membrane formation precede BOOP lesion development by 2 to 3 days. It is during this interim that the CBA/J mice that develop ARDS die of ARDS-related causes, before the time at which BOOP lesion formation is most likely to occur. It is possible, however, that ARDS represents a more acute form of the inflammatory reaction that results in the development of BOOP lesions. In humans histopathological observations of acute inflammatory responses in the lower respiratory tract suggest that ARDS is representative of a more severe form of the same type of inflammatory reaction that precedes BOOP formation (Wright, *et al.* 1992). Generally, among those individuals that survive ARDS, most will also develop BOOP (Kuhn, *et al.* 1989).

The participation of T-lymphocytes in the development of the acute inflammatory reaction in the lungs of CBA/J mice following respiratory reovirus infection is suggested by a marked reduction in the severity of the reovirus-induced pulmonary inflammatory response in T-lymphocyte-deficient neonatally thymectomized (NTx) CBA/J mice. However, NTx CBA/J mice still develop BOOP lesions following reovirus infection, even though the reovirus-induced cellular infiltrate is dominated by macrophages and significantly less lymphocytic than observed in nonthymectomized CBA/J mice following respiratory reovirus infection. These observations suggest the possibility that two separate components of the reovirus-induced inflammatory response can be identified in CBA/J mice. The first aspect of the pulmonary inflammatory response to reovirus in CBA/J mice could be a T-cell independent process that is primarily mediated by resident parenchymal cells and results in the development of intraluminal fibrotic lesions. A second component of the reovirus-induced pulmonary inflammatory response that is characterized by the development of ARDS may be a T-lymphocyte mediated progression of the inflammatory

reaction to an acute stage defined by the widespread vascular endothelial and alveolar epithelial destruction.

CHAPTER 6

Final Discussion

Mechanisms that mediate the pathogenesis of inflammatory and fibrotic disorders of the bronchoalveolar compartment of the lungs remain ill-defined even though these disorders are becoming increasingly recognized as important secondary complications of connective tissue disorders, bone and lung transplantation, toxic fume exposure, and viral and bacterial infection (Epler 1992, Wright, *et al.* 1992, Wells and du Bois 1993, Myers and Colby 1993, Kanda, *et al.* 1997). Numerous observations of inflammatory diseases of the small airways by pathologists and morphologists have resulted in well-established histopathological descriptions of these disorders (Kuhn, *et al.* 1989, Wright, *et al.* 1992, Wells and du Bois 1993, Myers, *et al.* 1994). Such accounts of inflammation in the lung have resulted in the suggestion that many small airway inflammatory and fibrotic disorders belong to a continuum of lung disease, unified by a foundation of lymphocyte accumulation in the alveolar and interstitial compartments of the lung (Wright, *et al.* 1992, Myers and Colby 1993). However, the cellular and molecular events that orchestrate the regulation of pulmonary inflammation and fibrosis have not been well-delineated. The reovirus 1/L-induced model of respiratory inflammation strongly suggests that the genetic backgrounds of different strains of mice contribute to the biological mechanisms that control the pulmonary inflammatory response to a single pathogenic agent. In this model system respiratory reovirus-1/L infection can be used in CBA/J mice to generate the acute small airway disorders BOOP and ARDS. In contrast, respiratory reovirus 1/L infection of CD-1, Balb/C, and C3H mice generates FB, a significantly less intense inflammatory reaction that is not accompanied by fibrosis or severe parenchymal cell damage.

Several observations suggest that the variability in lung pathology among these mouse strains following reovirus infection is more related to the regulatory events of the inflammatory response to reovirus than to just simply the susceptibility of each strain to reovirus-mediated lytic cell damage. These include: (i) the titer of the inoculum of reovirus 1/L used to infect CBA/J mice (BOOP and ARDS) is ten-fold lower than that used to infect

CD-1, Balb/C, and C3H mice (FB); (ii) the clearance of infectious reovirus-1/L from the lungs CBA/J mice and CD-1 mice occurs with similar kinetics; and (iii) reovirus-1/L replication ceases in the lungs of CBA/J mice and CD-1 mice with similar kinetics.

Normally, following injury to the alveolar epithelium, the lung generates proteolytic enzymes to reduce the presence of fibrin and other ECM proteins in the air spaces (Simon and Paine 1995). In particular, the activity of the urokinase-type plasminogen activator has been implicated as the major fibrinolytic factor in the lung (Idell S, *et al.* 1988, 1989; Bertozzi, *et. al.* 1990). In both animal models and in humans decreased activity of urokinase has been associated with the development of ARDS (Idell *et al.* 1989; Bertozzi *et* al. 1990). Therefore, it is possible that regulatory differences in ECM proteolytic mechanisms between CBA/J mice and other mouse strains may also contribute to the acute pulmonary inflammatory response to reovirus in CBA/J mice that is manifested by BOOP and ARDS. In the experimental model of interstitial lung inflammation and fibrosis induced by the toxic chematheraputic, bleomycin the lungs of C57BL/6N mice (H-2^b) are exquisitely sensitive to bleomycin and respond to both i.t. and i.v. administration of bleomycin with intense lung inflammation and interstitial fibrosis (Baecher-Allan and Barth 1993). However, BALB/c mice (H-2^d) are generally considered to be resistant to the inflammatory and fibrotic events occurring after bleomycin treatment (Janick-Buckner, *et al.* 1989). The initial nuclear damage mediated by bleomycin to the bronchiolar and alveolar epithelium was equivalent in C57BL/6N and BALB/c mice; however, C57BL/6N mice demonstrated lower levels of hydrolase (enzyme that breaks down bleomycin) and increased levels of poly ADP-ribose polymerase (mediates cell damage by depleting NAD^+) in comparison to BALB/c (Rossi, *et al.* 1987). These results are evidence of the strong role cellular enzymes may have in determining the susceptibility to pulmonary fibrosis.

An association between expression of the murine major histocompatability complex (MHC) haplotype H-2^b, which is expressed by C57BL/6N mice, and acute lung inflammation and fibrosis has also been demonstrated in the bleomycin model (Janick-

Buckner, *et al.* 1989). Whereas, mouse strains expressing H-2^a, H-2^d, and H-2^k haplotypes are generally low responders to bleomycin. When the H-2 haplotype of a low responder strain like Balb/C is inserted to a high responder background like C57BL/6N, there is considerable decrease in pulmonary fibrosis following i.t. bleomycin treatment (Rossi, *et al.* 1987). Likewise the movement of a high responder H-2 haplotype to a low responder background can result in an increase in intensity of the fibrotic response to bleomycin (Rossi, *et al.* 1987). An analogous link between MHC background and an acute inflammatory response to respiratory reovirus infection was not demonstrated between CBA/J and the other mouse strains screened with reovirus. The haplotype H-2^k is shared by CBA/J and C3H mice, yet in response to respiratory reovirus infection CBA/J mice develop BOOP or ARDS, but the reovirus induced response in C3H mice is characterized by a relatively mild lymphocytic infiltration into the interstitial and alveolar spaces of the lung.

In both clinical cases and in animal models, bleomycin induced lung injury is accompanied by an inflammatory infiltrate (Janick-Buckner, *et al.* 1989). The inflammatory infiltrate is typically characterized by PMNs and lymphocytes (Janick-Buckner, *et al.* 1989). The infiltration of PMNs only occurs in the early stages of the response to bleomycin, but the lymphocytes persist throughout the fibrosis (Janick-Buckner, *et al.* 1989). Most of the lymphocytes are T cells and only 10% of the lymphocytes are B cells (Janick-Buckner, *et al.* 1989). When the bleomycin fibrosis model is moved from C57BL/6 mice to outbred nude athymic C57BL/6N mice, which lack a normal T-lymphocyte component of the immune response, the resulting pulmonary fibrosis is similar between the normal and nude animals, which suggests that T-lymphocyte independent mechanisms may be involved in the pulmonary fibrotic process in response to bleomycin (Szapiel, *et al.* 1979). Also, *in vivo* antibody depletion of $CD4^+$ or $CD8^+$ populations does not appear to alter the course of pulmonary fibrosis in C57BL/6J mice; however, the role of

T -lymphocytes in the tissue remodeling that occurs late in the fibrotic process has not been investigated (Janick-Buckner, *et al.* 1989).

The prominence of infiltrating lymphocyte populations in the CD-1 model of FB and of the CBA/J model of BOOP/ARDS (Bellum, *et al.* 1996, Bellum, *et al.* 1997) reflect the association of lymphocytic infiltrates with human FB, BOOP, and ARDS (Yousem, *et al.* 1985, Wright, *et al.* 1992, Kuhn, *et al.* 1989). T-lymphocytes have also been identified as primary mediators of inflammation and fibrosis in models of interstitial pulmonary fibrosis (Suzuki, *et al.* 1996, Sharma, *et al.* 1996, Zhu, *et al.* 1996). In the lungs of T-lymphocyte deficient NTx CBA/J mice the reovirus-induced cellular infiltrate is not characterized by a prominent lymphocytic component, but is instead largely composed of macrophages and PMNs. Despite the decreased presence of T-lymphocytes in the reovirus-induce inflammatory infiltrate Ntx CBA/J mice continue to develop characteristic BOOP lesions, but do not develop the widespread vascular damage associated with ARDS. These observations suggest that the initiating event for BOOP and ARDS may be related to the reaction of the lung parenchyma to reovirus infection; however, the pathogenesis of ARDS may involve a T-lymphocyte mediated mechanism.

Clearly, lung inflammation and fibrosis involves a complex network of interactions between a large number of cell types. Infection of resident pulmonary populations of cells by reovirus may initiate the production of a unique pattern of pro-inflammatory cytokines or non-peptide mediators which contribute to the characteristic pathologies observed in CBA/J mice versus mouse strains that do not develop an acute pulmonary inflammatory response to reovirus 1/L. Investigations utilizing reovirus 1/L infection of mice will provide a model to systematically investigate the cellular and molecular mechanisms that lead to the development as well as the resolution of acute inflammatory diseases like BOOP and ARDS. Such studies are of significance not only for understanding the pathophysiology of FB, BOOP and ARDS, but are of broader significance since it is likely that a large proportion of inflammatory disorders of the distal regions of the lung exist undetected in

the population until they become symptomatic or life-threatening. Consequently, an improved understanding of the development and resolution of reovirus 1/L-induced inflammatory lung lesions may give great insight into the natural course of common lung diseases.

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BIOGRAPHY

Stephen C. Bellum
Department of Microbiology & Immunology
Medical University of South Carolina
171 Ashley Avenue
Charleston, SC 29425

Date of Birth: June 20, 1970

Place of Birth: Tampa, Florida

Education: 1988-1992 B.S. Microbiology, Clemson University, Clemson, South Carolina

1992-Present (Ph.D. expected Spring 1998) Doctoral Student, Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC.

Extracurricular: 1995-1996 Treasurer of the Student Government Association, Medical University of South Carolina

1995-1996 Service Chairman of the Student Government Association, Medical University of South Carolina

1995-1996 Student Representative for the Department of Microbiology and Immunology to the Graduate Student Association, Medical University of South Carolina

Abstracts and Presentations:

S. D. London, **S.C. Bellum**, A.H. Thompson, L. London, and D. Hamamdzic; Reovirus as a probe of mucosal immune responses, The 5th Immunobiology Vaccine Center MIRG Mini Symposium, Birmingham Alabama. July 29, 1994.

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