A GUINEA PIG MODEL OF HUMAN RESPIRATORY SYNCYTIAL VIRUS LUNG INFECTION

by

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ABSTRACT

Children who have an episode of acute respiratory syncytial virus (RSV) bronchiolitis are at increased risk for developing asthma, a disorder characterized by reversible airway obstruction, airway hyperresponsiveness and airway inflammation. Because latent or persistent pulmonary viral infections have been implicated in the pathogenesis of the airway inflammation underlying asthma, a guinea pig model of human RSV lung infection was developed to test the hypothesis that RSV persists within the lung following resolution of acute bronchiolitis. One month old, "juvenile" guinea pigs, intranasally inoculated with human RSV, were compared on day 6 post-inoculation to two month old, "adolescent" RSV-inoculated animals and uninfected controls by clinical examination, gross lung examination and lung histopathological examination using a semi-quantitative scoring system for bronchiolar inflammation. The natural history of intrapulmonary RSV was studied in juvenile animals on days 6, 14, 60 and 125 using viral culture to test for replicating virus, transmission electron microscopy (TEM) to test for assembled virus, immunohistochemistry to test for RSV antigens and the reverse transcriptase-polymerase chain reaction (RT-PCR) to test for RSV genomic RNA. The experiments showed that juvenile guinea pigs inoculated with human RSV developed statistically significant bronchiolar inflammation by day 6 that resolved by day 14, similar to human acute bronchiolitis. Intrapulmonary RSV was documented by culture, TEM, immunohistochemistry and RT-PCR during acute infection on day 6; in the longer term studies, replicating RSV was cultured up to 14 days post-inoculation, RSV antigens were identified within alveolar macrophages up to day 60 and RSV genomic RNA was identified on day 125. In conclusion, human RSV produced a self-limited acute bronchiolitis in juvenile guinea pigs, with evidence of subsequent persistent infection of alveolar macrophages. Persistent RSV infection of alveolar macrophages may represent a mechanism by which host pulmonary defense mechanisms are compromised against other, non-specific environmental agents implicated in the pathogenesis of asthma.

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CHAPTER 1: GENERAL INTRODUCTION

According to the Canada Health Survey of 1978-79, asthma affects approximately 500,000 Canadians, one third of whom are under the age of 15 years (1). Epidemiological studies (2, 3) have reported marked increases in hospitalization rates and mortality from asthma in Canada since the early 1970s and have shown that these increases are not attributable to artifacts of diagnosis or sampling. In 1989, the latest year for which Canadian statistics are available, children under 15 spent approximately 120,000 days in hospital due to asthma (with an average hospital stay of 3.7 days) and 12 deaths attributable to asthma were reported in this age group (Dr. Y. Mao, Canadian Laboratory Centre for Disease Control, personal communication). While the costs of asthma to the Canadian health care system are unknown, in the United States, where there is also increased morbidity and mortality from asthma (4), the annual health care costs are estimated at $6 billion (5).

There is currently no satisfactory explanation for these increases in the severity of asthma. This Chapter reviews contemporary ideas about the role of airway inflammation in the pathogenesis of asthma and discusses evidence that pulmonary viral infections may stimulate the production of airway inflammation.

1.1 DEFINITION OF ASTHMA. *Asthma*, the Greek word for "panting" (6) was used as a synonym for "shortness of breath" until the late 1800s (7), when Osler (8) recommended that the term be limited to denote "bronchial asthma" in contrast to so-called "cardiac asthma" or "renal asthma" associated with pulmonary edema. The Ciba Guest Symposium of 1959 (9) used clinical criteria to classify asthma as either "extrinsic" (ostensibly related to allergy[1]) or "intrinsic" (ostensibly of non-allergic, "endogenous" origin). A poor understanding of the

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[1] In the context of bronchial asthma, "allergy" refers to so-called "atopic allergy" or "type I hypersensitivity"—immunologically defined as a predilection for localized IgE-mediated anaphylactic reactions and clinically manifested by eczema, rhinitis and positive skin tests to extrinsic allergens (10). The clinical association between allergy and many cases of asthma has been recognized for over 800 years (11). *Atopy* is defined as a familial predisposition for type I hypersensitivity and recent genetic linkage studies purport the existence of an "atopy gene" on human chromosome 11q (12, 13).

etiology and pathogenesis of asthma resulted in the inability of a second Ciba Symposium (14) to further refine the definition. The American Thoracic Society currently defines asthma as "a clinical syndrome characterized by increased responsiveness of the tracheobronchial tree to a variety of stimuli" (15): a potential pitfall of such a broad, empirical definition is that patients with causally unrelated conditions might be grouped inappropriately under the common heading, "asthma" (16). Such inappropriate grouping of patients may impede the studies needed to achieve a more precise definition of asthma based on etiology and/or pathogenesis (17). The lack of a satisfactory definition of asthma also has important practical implications: for example, current medical therapies for asthma focus on relief of symptoms rather than reversal of underlying cause(s). An improved understanding of the cause(s) and pathogenesis of asthma, even if applicable to only a subset of patients, may permit the design of new therapies for cure of disease rather than control of clinical symptoms.

1.2 CHARACTERISTICS OF ASTHMA. Clinically, an asthma "attack" is characterized by episodic cough (which may be productive of sputum), wheezing and shortness of breath that usually resolves spontaneously or following medical therapy (15). However, the wide spectrum of clinical presentations often precludes the use of clinical criteria alone to diagnose asthma[2]. Instead, the diagnosis of asthma may depend on the documentation of several "hallmarks" of the condition: reversible airflow obstruction, airway hyperresponsiveness and airway inflammation. Each of these hallmarks will be described below.

1.2.1 REVERSIBLE AIRFLOW OBSTRUCTION. Airflow obstruction is considered present when a patient forcibly exhales lower volumes of air at lower flow rates than normal subjects (15). Lung spirometry may be used to assess the extent of airflow obstruction through such pulmonary function tests as forced expiratory volume in one second (FEV_1) and forced expiratory flow rates at different lung volumes (19). Although airflow obstruction is a hallmark

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2 The heterogeneity of asthma symptoms is reflected in the classification of Bates *et al.*, where asthma is classified into five categories ranging from class I (asymptomatic, in remission) to class V (unremitting symptoms, so-called *status asthmaticus*) (18).

of all chronic obstructive pulmonary diseases (asthma, chronic bronchitis, emphysema and peripheral airways disease), asthma is distinguished by the *reversibility* of airflow obstruction (e.g., improvements in pulmonary function indices) that occurs either spontaneously or following the administration of bronchodilator agents such as salbutamol[3]. However, asthmatics "in remission" may have normal lung spirometry and further evaluation for airway hyperresponsiveness may be required for diagnosis (18).

1.2.2 AIRWAY HYPERRESPONSIVENESS. Airway hyperresponsiveness refers to the airways of asthmatic patients having a lower threshold ("increased sensitivity") and greater extent ("increased maximal response") of narrowing following exposure to a variety of physical, chemical and biological stimuli[4] (15). Airway hyperresponsiveness may be assessed in a pulmonary function laboratory where the subject is challenged with a known dose of a "provocative" bronchoconstrictor agent such as histamine or methacholine (an analog of acetylcholine) and evaluated for such indices as decreased FEV_1 or increased pulmonary resistance. Although histamine or methacholine may elicit bronchoconstriction in normal subjects, the increased sensitivity and increased maximal response of asthmatic patients to these agents over a range of doses (21, 22) produce a "left-shift" of the dose-response curve that usually permits distinction between asthmatic and normal individuals (21).

Increased airway smooth muscle contraction has long been considered to be the underlying mechanism of airway hyperresponsiveness and airflow obstruction in asthma, in part because

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3 While no single pulmonary function test is diagnostic of asthma, various guidelines have been developed to standardize the interpretation of pulmonary function tests (15). For example, a 15% improvement in FEV_1 following bronchodilator administration is generally considered to be indicative of reversible airflow obstruction (20) since improvements in FEV_1 (usually less than the 15% guideline) may also occur post-bronchodilator in the other chronic obstructive pulmonary diseases and in many "normal" subjects (15).

4 Examples of physical stimuli include cold air and exercise. Chemical stimuli include a variety of medications (e.g., ß-adrenergic receptor blockers and non-steroidal anti-inflammatory drugs), noxious gases, inhaled and systemic allergens, inflammatory mediators such as histamine and neurotransmitters such as acetylcholine. Biological stimuli include respiratory tract infections by viruses, bacteria and fungi. Acute exacerbations of asthma may also be induced by psychological factors, e.g., anxiety.

drugs that relax bronchial smooth muscle (e.g., $ß_2$-adrenergic receptor agonists, anticholinergics and methyl xanthines) are efficacious in the treatment of asthma symptoms (23). However, increased airway smooth muscle contraction may represent a sequela rather than the underlying process in the pathogenesis of asthma because several studies have failed to demonstrate differences in airway smooth muscle mechanics (either *in vitro* or *in vivo*) between asthmatic patients and normal subjects (24-26). In addition, morphometric studies (27) have suggested that the thickened submucosal layer of the airway wall in asthma potentially plays a greater role in the pathogenesis of airway narrowing than increased airway smooth muscle contraction (28, 29). The thickening of the airway wall submucosa may result from a chronic inflammatory process (30) and an improved understanding of the factor(s) which stimulate the chronic inflammation of airways may provide clues into the etiology and pathogenesis of asthma.

1.2.3 AIRWAY INFLAMMATION. Although Curschmann described asthma as "a special form of inflammation of the small bronchioles" over a century ago (8), his idea was largely forgotten until 1965, when Nadel reintroduced the concept of inflammatory injury to the airway mucosa being related to airway hyperresponsiveness (31). Two factors contributing to the delay of studying inflammatory phenomena in asthma have been the technical difficulties in tissue sampling from living asthmatic patients and the prevailing view of increased bronchial smooth muscle constriction being the underlying abnormality (23). Histological studies from postmortem (32-37), open lung biopsy (38) and transbronchial biopsy (39) specimens have described features and sequelæ of inflammation in the airway walls of asthmatic patients including cellular (eosinophilic, lymphocytic and polymorphonuclear) and fluid exudates, epithelial necrosis with repair (e.g., squamous and goblet cell metaplasia), bronchial gland enlargement with intraluminal mucus, smooth muscle hypertrophy, increased submucosal extracellular matrix and thickening of the bronchial subepithelial basement membrane. These histopathological abnormalities reflect the presence of chronic inflammation in the airways of asthmatic patients but none indicates the underlying cause(s) of the inflammatory process (30).

The cellular and biochemical events comprising the airway inflammation of asthma have been intensively studied in recent years (40, 41). The next section will describe the mechanisms of "extrinsic" asthma associated with atopic allergy (section 1.1) because atopic allergy is prevalent in children with asthma.

1.3 ATOPIC ALLERGY AND ASTHMA. Depending on the age group studied and the criteria used for diagnosis, epidemiological studies report a prevalence of atopy from 23-80% among asthmatic patients, with an especially high prevalence of atopy in asthmatic children (42). Patients with atopy develop type I hypersensitivity reactions to a variety of extrinsic antigens ("allergens") such as ragweed, various fungi and, of particular epidemiological importance in childhood asthma, the house-dust mite, *Dermatophagoides pteronyssinus* (43, 44). Type I hypersensitivity consists of an initial "sensitization" step in which a predisposed individual, upon first exposure to an allergen, produces allergen-specific IgE. Upon subsequent exposure to the allergen, complexes of allergen and allergen-specific IgE bind to mast cells (via receptors for the F_c portion of IgE) and stimulate the degranulation of mast cells. The release of chemical mediators such as histamine (section 1.2.2) stimulates the contraction of airway smooth muscle ("immediate reaction"). This reaction is followed by the "late-phase reaction", characterized by edema and inflammatory cell infiltrates (section 1.2.3). Clinically, the immediate reaction is believed to correspond to the early events in an acute exacerbation of asthma while the late-phase reaction is believed to correspond to the prolongation of clinical symptoms (45).

Type I hypersensitivity responses appear to be regulated by both genetic and environmental factors. Concerning genetic factors, further characterization of the putative atopy gene on human chromosome 11q (section 1.1) may provide new insights regarding the predilection of certain individuals to become sensitized to various allergens. Concerning environmental factors, type I hypersensitivity reactions appear to be regulated by a hierarchy of lymphocyte subsets and macrophages. For example, the so-called T_{H2} cell (46, 47), a subset of CD4+ "helper" T-lymphocytes (originally described in mice), secretes a variety of chemical mediators implicated in the pathogenesis of both immediate reactions (e.g., interleukin-4 (IL-4)) and late-

phase (e.g., IL-5, granulocyte-macrophage colony stimulating factor (GM-CSF)) reactions[5]. Lymphocytes with characteristics of T_{H2} cells are the predominant lymphocyte subset recovered at bronchoalveolar lavage (BAL) from human atopic asthmatic patients (49). A possible mechanism for the increased T_{H2} cell activity in atopic asthma is the decreased ability of alveolar macrophages to exert regulatory control on T_{H2} cells (50-52)[6]. Environmentally-induced defects of alveolar macrophage function may play a crucial role in the pathogenesis of extrinsic asthma.

The above discussion has highlighted some recent advances in our understanding of the various cellular and biochemical events underlying the pathogenesis of airway inflammation in the vast majority of children with asthma. Despite these advances, Nadel has emphasized that little is known about the factors which "signal" the production of chronic airway inflammation (54). Evidence that persistent or latent respiratory tract viral infections[7] may possibly signal the production of chronic airway inflammation in asthma is presented below. Testing this possibility is important because if respiratory tract viral infections are involved in the pathogenesis of airway inflammation in asthma (even if applicable to only a minority of patients), then treatment with anti-viral drugs such as Ribavirin (1-B-D-ribofuranosyl-1,2,4-triazole-3- carboxamide) (57) could potentially cure these patients of their disease.

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5 Among its many activities, IL-4 stimulates mast cell differentiation and promotes B cell differentiation into IgE-secreting plasma cells. IL-5 and GM-CSF stimulate the proliferation and activation of eosinophils in late phase reactions (48).

6 Alveolar macrophages phagocytose, process and present inhaled antigens to a variety of cell types, including T-cells (53). Following experimental depletion of alveolar macrophages, there is increased T_{H2} cell activity (52). The mechanisms by which alveolar macrophages interact with antigens and regulate other cell types *in vivo* are not understood.

7 For the purposes of this thesis, *persistent* infection is defined as a state in which replicating virus can be reproducibly and continuously recovered from the host well past the usual period of acute illness (with or without lysis of infected cells) and *latency* is defined as a state in which a replication-competent virus remains within the host in a reversibly nonproductive form, with replicating virus being detected only intermittently, often in association with clinical recurrence of disease (55, 56).

1.4 VIRAL RESPIRATORY TRACT INFECTIONS AS SIGNALS FOR CHRONIC AIRWAY INFLAMMATION IN ASTHMA. Although the clinical observation of upper respiratory tract infections triggering acute exacerbations of asthma had been well recognized for decades, Moll (58) postulated that the presence of microorganisms *within the lung* was responsible for the development of airway hyperresponsiveness to methacholine challenge. Subsequent efforts to confirm a possible role for various bacteria (59, 60) and fungi (61) (or the host immune response to these microorganisms) in the pathogenesis of asthma failed such that by the mid-1950s, a primary role for either bacteria or fungi was largely discounted (62, 63). The possibility that viral infections might play a role in the pathogenesis of asthma was initially based on circumstantial evidence: for example, Salk showed that the extent of apparently "allergic" responses to influenza vaccines was proportional to the concomitant amount of influenza virus within the host (64) and Stuart-Harris's group (65) observed that bacteria were rarely cultured from asthmatic patients' sputa, in marked contrast to the frequently positive sputum cultures from patients with chronic bronchitis or emphysema. In their discussion of a possible viral etiology to nasal polyps (inflammatory lesions characteristically affecting atopic individuals), Weille and Gohd speculated that "virus protein or virus infection in nasal and sinus polyps and polyposis may be important in the etiology of asthma" (66). The discovery of new respiratory tract viruses and technological improvements in laboratory methods for their detection has produced a growing body of epidemiological, clinical, physiological, histopathological and biochemical evidence implicating viral respiratory tract infections in the pathogenesis and possibly etiology of asthma.

1.4.1 EPIDEMIOLOGICAL DATA. Viral infections are implicated in asthma in at least three ways: (a) as contributors to the initial sensitization process to extrinsic allergens (section 1.3) (67); (b) as stimuli for acute exacerbations of asthma (68, 69); (b) as stimuli for ongoing airway inflammation in some asthmatic patients (69). Frick *et al.* prospectively studied 13 children with a biparental history of atopic allergy and reported that 11 of these children had a viral upper respiratory tract infection within two months of becoming sensitized to a variety of

allergens (67). In 10/11 children, the sensitization process coincided with rising titres of virus-specific IgG, and this striking correlation led the investigators to speculate that the host's humoral immune response contributed to the development of sensitization. Concerning viruses stimulating acute exacerbations of asthma, prospective studies have clearly demonstrated that antecedent upper respiratory tract infections by adenovirus (70), influenza virus (70-72), parainfluenza virus (70, 73, 74), respiratory syncytial virus (RSV) (73, 74) and rhinovirus (71, 72) provoke wheezing in many asthmatic patients. Welliver's group reported that circulating titres of RSV-specific IgE independently predict airway hyperresponsiveness in children (75) and suggested that virus-specific IgE was responsible for acute exacerbations of asthma. However, a preliminary study from a European group has not reproduced Welliver's findings (76).

Concerning viruses possibly being signals for ongoing airway inflammation in asthma, the strongest epidemiological association is the development of asthma in children who have had an episode of acute respiratory syncytial virus (RSV) bronchiolitis (68). This association was first reported in a retrospective analysis of 100 asthmatic children, of whom 32 had documented acute bronchiolitis during infancy (77). Subsequent long-term prospective studies (78-81) have confirmed the association between acute RSV bronchiolitis during early childhood and the subsequent development of asthma, with the reported incidence ranging from 21 (80) to 92 (81) per cent[8]. Although clinical, physiological, histopathological and biochemical studies suggest that RSV lung infection may play a role in the pathogenesis of asthma in these children (see below), the biological basis of this compelling epidemiological association has not been elucidated.

1.4.2 CLINICAL DATA. RSV causes over 85% of cases of acute bronchiolitis, a common pediatric disease that accounts for approximately 1% of hospital admissions during the first two years of life (82). The clinical illness shares several features with an acute exacerbation of

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8 For comparison, the estimated prevalence of asthma is 2-5% of the general population (1, 4).

asthma, including abrupt onset of cough, dyspnea, wheezing and chest wall retractions (83). Ventilatory support may be required in severely affected patients and the case fatality rate is approximately 1 per cent. While most patients recover within 10-14 days, RSV shedding may continue for up to 27 days in immunocompetent patients (84) and for up to 47 days in immunocompromised patients (85). Intriguingly, "acute bronchiolitis" is rarely diagnosed in patients over 4 years of age (86) and given the clinical similarities between an episode of acute viral bronchiolitis and an acute exacerbation of asthma, investigators have recently speculated that the distinction between the two conditions may reflect diagnostic bias on the basis of the patient's age rather than differences in etiology or pathogenesis (7, 54, 87).

1.4.3 PHYSIOLOGICAL DATA. Numerous studies in humans (88-93) and experimental animals (94-100) have shown that viral respiratory tract infections provoke airway hyperresponsiveness. Pulmonary function testing of children with acute RSV bronchiolitis has revealed decreased forced expiratory flow rates and increased respiratory resistance (90, 91) which are reversible following administration of bronchodilator drugs (91). These abnormalities of pulmonary function are consistent with reversible airflow obstruction, a hallmark of asthma (section 1.2.1). Furthermore, these abnormalities have persisted for up to eight months following clinical resolution of acute RSV bronchiolitis (92). While bronchoconstrictor challenge testing for airway hyperresponsiveness is unethical in human infants with acute RSV bronchiolitis, bronchoconstrictor challenges of RSV-infected sheep (100) and parainfluenza virus-infected rats (99) have documented both acute and persistent (over weeks to months) airway hyperresponsiveness.

1.4.4 HISTOPATHOLOGICAL DATA. The histopathological features of acute bronchiolitis have been primarily determined from autopsy material (101-104) because it is not feasible to biopsy infants and young children with acute bronchiolitis. The histopathological features of fatal cases of acute bronchiolitis are common to all lower respiratory tract viral pathogens and include: (a) necrosis and sloughing of bronchiolar epithelium; (b) peribronchiolar mononuclear infiltrates consisting primarily of small lymphocytes; (c) edema of the bronchiolar submucosa

and adventitia; (d) mucus and necrotic cellular debris within the airway lumen; (e) goblet cell metaplasia of the bronchiolar epithelium; (f) in rare instances, intracellular viral inclusions. Increased amounts of bronchiolar smooth muscle and thickening of the subepithelial basement membrane have also been observed in a case of fatal acute RSV bronchiolitis (87). Epithelial necrosis, airway wall inflammation and edema, intraluminal mucus and metaplastic epithelial repair are also histopathological features of asthma (section 1.2.3) and the histopathological differences between acute bronchiolitis and asthma (e.g., prominent eosinophilic infiltrates, smooth muscle hypertrophy and subepithelial basement membrane thickening in asthma) may reflect the chronicity of the inflammatory process in asthma.

1.4.5 BIOCHEMICAL DATA. There are at least three biochemical mechanisms by which respiratory viruses possibly contribute to the pathogenesis of asthma: (a) increased constriction of airway smooth muscle in response to inflammatory mediators; (b) enhanced cellular secretion of inflammatory mediators; (c) alterations of pulmonary defense mechanisms to non-specific environmental agents that trigger acute exacerbations of asthma. With respect to viruses increasing the constriction of airway smooth muscle in response to inflammatory mediators, Saban *et al.* (96) demonstrated that guinea pigs infected with parainfluenza virus had increased bronchial smooth muscle contraction to substance P, a neuropeptide secreted by intrinsic nerves within airway smooth muscle and implicated as a bronchoconstrictor in human asthma (105). Busse has speculated that the damaged airway epithelium in asthma results in exposure of nerve fibres in the airway wall to non-specific environmental factors that stimulate substance P production (68).

With respect to viruses enhancing the secretion of inflammatory mediators, Lin *et al.* have documented increased IL-2 production *in vitro* from peripheral blood lymphocytes of asthmatic children who have concurrent influenza A viral upper respiratory tract infections (106). IL-2 promotes the proliferation of T-lymphocytes and these cells may secrete additional mediators (e.g., IL-4, IL-5) that stimulate the recruitment and activation of other effector cells such as mast cells and eosinophils (48, 107). Alternatively, enhanced secretion of histamine-releasing

factor (HRF), a cytokine that induces mast cell degranulation and histamine release (108, 109), has been described in cultured mononuclear cells infected with either influenza virus (110) or RSV (111). A third possibility is the binding of virus-IgE complexes to induce histamine release, as shown by Ida *et al.* for adenovirus and influenza virus (112). Busse's group has extended these observations by showing that influenza virus induces histamine release in cultured human leukocytes via both IgE-dependent and IgE-independent mechanisms (113). These investigators also have documented a synergistic effect on histamine release from concomitant virus infection and ragweed antigen exposure, consistent with a virus-allergen interaction.

With respect to viruses altering pulmonary defense mechanisms to non-specific environmental agents, Slauson *et al.* have shown that parainfluenza virus infection of alveolar macrophages decreases the ability of these cells to phagocytose inhaled cobalt oxide particles (114). Witten *et al.* (115) have speculated that airway inflammation may result from the delayed phagocytosis and clearance of non-specific environmental agents by virus-infected alveolar macrophages. Furthermore, since RSV-infected macrophages *in vitro* display defects of immunological function such as fewer cell membrane receptors for the F_c portion of immunoglobulin (116) and increased production of an inhibitor to IL-1 (117), these defects might also compromise pulmonary defense mechanisms against non-specific environmental agents (115).Whether virus infection of alveolar macrophages results in increased T_{H2} cell activity (section 1.3) is unknown.

1.5 SUMMARY. Asthma is a condition characterized by reversible airflow obstruction, airway hyperresponsiveness and chronic inflammation of airways. Epidemiological data suggest that the morbidity and mortality of asthma have increased in Canada over the last two decades, especially in children. There is increasing evidence that chronic inflammation of airways is the primary abnormality responsible for the production of reversible airflow obstruction and airway hyperresponsiveness. While much is known about the various inflammatory cell types and biochemical mediators involved in the pathogenesis of asthma, much less is understood

about the "signals" which stimulate chronic inflammation of airways. Persistent or latent viral pulmonary infections possibly signal this chronic inflammatory process, based on epidemiological, clinical, physiological, histopathological and biochemical studies. Of particular epidemiological importance is the strong association between acute RSV bronchiolitis and the development of asthma in children, a situation where persistent RSV lung infection may play a role in the pathogenesis of disease.

CHAPTER 2: RESPIRATORY SYNCYTIAL VIRUS

2.1 HISTORICAL BACKGROUND. In 1956, a novel viral pathogen, the "chimpanzee coryza agent", was discovered by serendipity at the Walter Reed Army Medical Center in Washington, D.C., when a group of laboratory chimpanzees developed a cluster of upper respiratory tract infections (118). The following year, Chanock and associates (119) isolated an antigenically identical virus from two infants[9] with lower respiratory tract infection and the name "respiratory syncytial virus" (RSV) reflected the virus's tendency to produce fusion of infected cells into syncytia *in vitro*. Subsequent epidemiological studies demonstrated that RSV was the infectious agent most frequently isolated from children with acute bronchiolitis and pneumonia (120-122) and today RSV is regarded as "the most important respiratory pathogen of infancy and early childhood" (83).

2.2 CLASSIFICATION OF RSV. Human RSV, bovine RSV and pneumonia virus of mice comprise the genus, *Pneumovirus* within the family, *Paramyxoviridæ* (123). RSV lung infection has also been described in wild mountain goats (124) and sheep (125) but these so-called "caprine" (126) and "ovine" (100) variants of RSV have not been well characterized. The *Paramyxoviridæ* family also includes the *Paramyxoviruses* (e.g., parainfluenza and mumps viruses) and *Morbilliviruses* (e.g., measles virus) and the common features of these viruses are: (a) single-stranded, negative polarity RNA genome; (b) assembled virions 80-500 nm in diameter; (c) 12-21 nm diameter nucleocapsids with helical symmetry; (d) assembly of nucleocapsids in the cytoplasm of infected cells (measles virus can also assemble in the nucleus); (e) envelopment of virus on the plasma membrane of infected cells (127). RSV is distinguished from the other *Paramyxoviridæ* by its smaller diameter (12 nm) nucleocapsid and propensity for infection limited to the respiratory tract and middle ear.

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[9] The so-called "Long" strain of RSV was one of Chanock's original isolates and named after the patient from whom it was recovered. The Long strain is the prototypic "type A" RSV (section 2.3).

2.3 STRUCTURE OF RSV. The single-stranded, non-segmented RNA of RSV encodes ten genes, all of which have been cloned and sequenced (128, 129). Table 1 lists the 3'-5' transcription order of these ten genes and the known properties of the gene products (123, 128, 130). Two subtypes of human RSV (A and B) (131) have been distinguished by antigenic differences between the F, G and P proteins (132) and type A virus is considered to produce more severe clinical disease (133). In contrast to influenza virus, the genome of human RSV is relatively stable (83, 123)[10].

2.3.1 NUCLEOCAPSID (N) PROTEIN. The RSV N protein is a 42 kD product of a gene 1197 bases long. Between subtypes A and B of human RSV, there is 86% sequence identity at the nucleotide level and 96% identity at the amino acid level (134). The N protein is closely associated with the viral genomic RNA (Table 1), has a regulatory role in viral replication (section 2.4) and is antigenic in terms of the host humoral and cellular immune responses (section 2.7). These structural and functional aspects of the RSV N protein make it an attractive target for the study of the natural history of RSV following acute lung infection.

2.4 LIFE CYCLE OF RSV. The natural hosts of RSV are humans, cows and chimpanzees (83). Virus-containing droplets or fomites enter the host via the nasal, or ocular routes. Adsorption of RSV occurs via binding of the viral G protein to a receptor on the host cell membrane (as yet uncharacterized), with 60-90% of virus being adsorbed by 30 minutes at 37°C. Following cleavage of the viral F protein into F1 and F2 fragments by a host-derived protease, an exposed hydrophobic region on the F2 fragment produces holes in the viral envelope and cell membrane to result in fusion and release of free viral nucleocapsid into the host cell's cytoplasm.

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10 The further subclassification of RSV (e.g., A1, A2 strains within type A) likely reflects antigenic drift (i.e., point mutations of genomic RNA). The major differences in several proteins between type A and type B RSV are speculated to represent antigenic drift (i.e., exchanges of portions of genomic RNA between distinct viruses) (131, 132).

TABLE 1: HUMAN RSV GENES AND GENE PRODUCTS

GENE	# RNA BASES	# AMINO ACIDS	MW (kD)	LOCATION	FUNCTION/PROPERTIES OF GENE PRODUCT
1C (NS1)	528	139	15.6	not known	non-structural; acidic protein
1B (NS2)	499	124	14.7	not known	non-structural; basic protein
N	1197	391	42	nucleocapsid	encases RNA genome in a flexible helix
P	907	241	27.1	nucleocapsid	? component of polymerase; phosphorylated
M	952	256	28.7	inner aspect of viral envelope	contacts N protein and internal end of viral G protein; hydrophobic, basic protein
SH (1A, NS3)	405	64	7.5	viral envelope	non-structural; binds antibody and CD4+ T-lymphocytes; small hydrophobic protein
G	918	298	32.6	full thickness of viral envelope	attachment protein of virus to host cell membrane receptor; extensive post-translational glycosylation (MW 84-90 kD post-glycosylation)
F	1899	574	63.5	full thickness of viral envelope	fusion of viral envelope to host cell membrane; ? causes formation of syncytia. The F1 subunit has MW ~20 kD and the F2 subunit has MW ~43 kD.
M2	957	194	22.2	inner aspect of viral envelope	matrix protein of unknown function; basic protein
L	~6500	?	~200	nucleocapsid	viral RNA polymerase

A viral-associated RNA polymerase modulates replication into progeny viruses within the host cell's cytoplasm. The RNA polymerase has two distinct activities: (a) transcription of 10 individual *m*RNAs and translation into the corresponding proteins for viral assembly; (b) transcription of the complementary *m*RNA of the entire RSV genome (i.e., full length transcript) which serves as a template for the further full length transcription into progeny RNA genomes by the same polymerase. Which of these two activities predominates at any given time depends on the relative cytoplasmic concentrations full length *m*RNA transcripts — N protein complexes vs. free N protein. For example, if there is a relatively high cytoplasmic concentration of these complexes, the polymerase will preferentially transcribe ten individual *m*RNAs for translation into proteins and thus increase the cytoplasmic concentration of free N protein. Conversely, if there is a low cytoplasmic concentration of these complexes, the polymerase will preferentially transcribe more full length complementary *m*RNA. Assembled RSV matures by budding through the host cell membrane to produce cell-to-cell spread of infectious virus. Cellular infection with RSV produces characteristic effects *in vitro* and *in vivo* as will be discussed below.

2.5 RSV INFECTION *IN VITRO*.

2.5.1 LYTIC INFECTION. In the diagnostic virology laboratory, RSV is propagated on permissive cell lines derived from human epithelial malignant neoplasms such as HEp-2 cells and HeLa cells (135). The characteristic "cytopathic effect" (CPE) of RSV infection *in vitro* is the formation of syncytial giant cells produced by fusion of cell membranes from adjacent infected cells. Cell lysis occurs when the replication of progeny viruses overwhelms the infected cell's capacity for homeostasis.

Lytic RSV infection *in vitro* has recently been described in primary cultures of adult human nasal epithelial cells, bronchial epithelial cells and alveolar macrophages (136), three cell types susceptible to RSV infection *in vivo* (104). Of the three cell types, nasal epithelial cells were most susceptible to RSV infection *in vitro*, underwent the greatest amount of cell lysis and released the most virus into culture supernatants; alveolar macrophages were least susceptible

to infection, underwent the least amount of cell lysis and retained the most virus intracellularly. These experiments suggest that the release of RSV from nasal epithelial cells allows viral spread to neighboring cells to produce a clinical upper respiratory tract infection (section 2.6) while the retention of RSV by alveolar macrophages without cell death may result in persistent RSV infection *in vivo*.

2.5.2 NON-LYTIC INFECTION. Acute RSV infection of alveolar macrophages *in vitro* has been studied as a model of *acute* non-lytic infection (136, 137), with descriptions of virus-induced alterations in cell structure and function (section 1.3.5). In addition to acute non-lytic RSV cellular infections, several empirical studies have implicated the existence of cellular and viral factors that permit *persistent* non-lytic RSV infections *in vitro*. Concerning cellular factors, persistent non-lytic RSV infections have been described in cultures of HEp-2 cells at high passage number (138) and in cultures of a cell line derived from the Balb/c mouse embryo (139). Concerning viral factors, persistent non-lytic infection has been described when temperature sensitive (*ts*) mutants of RSV are cultured in a variety of cell types (140). For example, at 31°C, three epithelial cell types[11] infected with *ts* mutants of RSV expressed viral antigens on the cell membrane and had ultrastructural features of cultured cells transformed by oncogenic nuclear viruses, including overlapping of adjacent cells and agglutination by a low concentration of concanavalin A (141). When the temperature was increased to 39°C, RSV antigen expression was confined to the cytoplasm and the "pseudo-transformed" phenotype was reversed. These results showed that RSV, a cytoplasmic virus, apparently did not have to integrate into the host cell's genome to produce persistent cellular infection or induce a transformed phenotype. The presence of viral antigens on the host cell membrane was associated with an altered cellular phenotype but the molecular mechanisms of these changes have not been elucidated[12].

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11 The cell lines were derived from human embryonic lung (MRC-5), feline embryo (FEA) and mink lung (Mvi Lu).

12 Although persistent RSV infection has not been described *in vivo*, there are well documented examples in which persistent non-lytic infection by RNA viruses produces

2.6 RSV INFECTION *IN VIVO*. In contrast to RSV infection *in vitro*, where acute lytic, acute non-lytic and persistent non-lytic infections have been described, only acute lytic RSV infections are known to occur *in vivo*. Acute lytic RSV infections produce a spectrum of clinical diseases ranging from the trivial common cold to the potentially life-threatening condition, acute bronchiolitis. Children under the age of two years are at particular risk for developing acute RSV bronchiolitis (123) and may go on to become asthmatic (Chapter 1). In adults, RSV infection is usually limited to the nasopharynx as a common cold (145) but may cause pneumonia in patients who are immunocompromised, institutionalized or who have underlying chronic obstructive pulmonary disease (146). Several possibilities for the apparent predilection of RSV for the lower respiratory tract of children are discussed below.

2.6.1 PREDILECTION OF RSV FOR THE LOWER RESPIRATORY TRACT IN CHILDREN. There is experimental evidence for at least three factors to account for the apparent predilection of RSV to the lower respiratory tract of children: (a) anatomical and functional characteristics peculiar to the airways of children; (b) "intrinsic" cellular factors imparting variable susceptibilities to RSV infection in different cell types; (c) immunological factors related to viral clearance and/or the possibility that the host immune response is responsible for the pathogenesis of clinical disease. Concerning anatomical and functional characteristics of children's airways, Hogg *et al.* (147) used a retrograde catheter technique (148) to show that the peripheral airways resistance is much higher in children than in adults, implying that a similar amount of bronchiolar inflammation would produce clinical symptoms in children but not in adults. Furthermore, Reid has speculated that the small dimensions and

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deleterious effects on infected cells without known integration into host genome. For example, infection of the mouse pituitary gland with lymphocytic choriomeningitis virus (LCMV), a single-stranded RNA *Arenavirus*, produces growth retardation without evidence of cell injury or inflammation (142, 143). Mumps virus, like RSV a member of the family, *Paramyxoviridæ,* may cause persistent infection within the central nervous system (144). In contrast, latent infections appear to be a property of a variety of DNA viruses (e.g., herpes viruses) that can integrate into the host's genomic DNA, although so-called "preintegration" latency (i.e., before reverse transcription into DNA provirus) has been described for human immunodeficiency virus, an RNA retrovirus (56). In the absence of definitive experimental data supporting the occurrence of latent RSV infection, only the possibility of persistent RSV infection was considered in this thesis.

physical geometry of the children's airways facilitate the spread of viruses to the bronchioles (149). The combination of high baseline peripheral airways resistance with lung anatomy conducive to viral spread places children at particular risk for developing acute RSV bronchiolitis.

Concerning "intrinsic" cellular factors conferring varying susceptibilities to RSV infection in different cell types, empirical observations have confirmed that RSV preferentially infects epithelial cell lines *in vitro* (135). A recent report (136) has extended these observations by showing a differential susceptibility to RSV infection between various types of epithelial cells from the same person: cultured nasal epithelial cells were readily infected by RSV infection compared to cultured bronchial epithelial cells. No studies to date have specifically examined whether respiratory epithelial cells from children have a greater susceptibility to RSV infection than similar cells from adults. The failure to characterize the nature and distribution of the cellular receptor(s) for RSV has greatly impeded this area of study.

Concerning the role of the host immune response to RSV, it is controversial whether RSV or the host immune response to RSV is responsible for the pathogenesis of clinical disease. For example, congenitally athymic mice and irradiated mice (150) and cotton rats rendered immunodeficient by cyclophosphamide treatment (151) have been used as animal models of persistent, *lytic* RSV pulmonary infection and these models suggest that the virus itself is responsible for the pathogenesis of inflammatory lesions. In contrast, early field trials of potential RSV vaccines (derived from formalin-inactivated virus) showed that, upon subsequent natural RSV infection, *vaccinated* persons developed more severe pulmonary disease than unvaccinated control subjects (152-154), suggestive of an exaggerated host immune response being responsible for the production of clinical disease[13]. The role of the

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13 Several deaths occurred in vaccinated subjects. These findings have subsequently been confirmed in RSV-inoculated cotton rats (155-157) in which vaccinated animals developed more extensive pulmonary inflammation than controls. Although the mechanism for the untoward effects of vaccination remains unexplained, the process of formalin inactivation may have altered viral antigens to elicit the production of antibodies deleterious to the host (152). No safe, effective RSV vaccine is currently available.

host immune response to the eradication of RSV and in the possible pathogenesis of RSV-induced disease is discussed further in section 2.7.

2.7 IMMUNITY TO RSV INFECTION.

2.7.1 HUMORAL IMMUNITY. In humans, numerous RSV-specific antibodies of different classes (IgM, IgG, IgE and IgA) to various RSV surface (F, G, 1A) (158-160) and structural (N, P, L, M) (161) proteins have been identified but none of these antibodies shows a reproducible correlation between circulating titres and the neutralization, clearance and protective immunity against RSV (162, 163). Similarly, studies of humoral immunity to RSV in experimental animals (161, 164-172) have been inconclusive. As mentioned in section 2.6.1, the unfortunate clinical experience of early RSV vaccines suggests that host-derived antibodies may be involved in the pathogenesis of acute bronchiolitis. However, this contention has been disputed for several reasons: for example, there is no epidemiological or clinical evidence of previous RSV sensitization in children who develop acute RSV bronchiolitis and, in general, successive RSV infections tend to produce increasingly milder symptoms in the host (162, 173).

2.7.2 CELL-MEDIATED IMMUNITY. Studies in humans (163, 168, 174, 175) and experimental animals (150, 160, 169, 176-178) suggest that virus-specific cytotoxic T-lymphocytes (CTL) play a crucial role in the host immune response to RSV[14]. Exogenously administered CTL can eradicate pulmonary RSV infection in both normal (178) and immunodeficient mice (150) but giving a large number (3×10^6) virus-specific CTL to RSV-infected mice produces more extensive airway inflammation, increases the severity of clinical symptoms and may be fatal (181).

In summary, there appears to be a precarious balance in the host immune response to RSV between eradication of the virus and exacerbation of disease. One possibility to explain whether the immune response is beneficial or deleterious to the host is the contribution of concomitant

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14 There are descriptions of CTL which recognize the RSV surface 1A protein (160, 179) and structural N protein (180) but their importance *in vivo* is not known.

viral factors to the host-virus system: in studies of immunity to RSV, comparatively little attention has been paid to documenting or localizing RSV itself (section 1.4). The technical and ethical considerations of tissue sampling in humans has limited the ability of investigators to test for the presence of persistent RSV lung infection. Consequently, RSV has been experimentally inoculated into a number of animal species to address questions that would not be possible in humans.

2.8 ANIMAL MODELS OF RSV LUNG INFECTION. Inoculation of RSV into various experimental animals has been attempted for over thirty years (182). Chimpanzee and monkey are the only known "natural hosts" (besides humans) in which human RSV produces acute lower respiratory tract illness and bronchiolar inflammation (183-185). Inoculation of bovine RSV into cows also produces clinically significant symptoms and bronchiolar inflammation (186-188). Lambs inoculated with bovine RSV (94, 189-192) or so-called "ovine" RSV (100, 193) develop minimal clinical signs of lower respiratory tract illness and mild bronchiolar inflammation but may develop persistent airway hyperresponsiveness (100). The high costs and specialized facilities required to maintain these large species have limited their widespread use (194).

Smaller laboratory animals, including ferrets (195), mice (196-198) and cotton rats (199), given up to 10^7 plaque forming units (pfu) of human RSV, do not develop acute lower respiratory tract disease or histologically impressive bronchiolitis. Furthermore, these species do not model the increased susceptibility of human infants to acute RSV bronchiolitis because pulmonary infection occurs only in mature mice (198) while cotton rats of all ages are equally susceptible (199). Despite these limitations, the cotton rat and the mouse have been favored in studies of humoral and cell mediated immunity to RSV (150, 168, 169, 172, 176, 179, 181, 200, 201) and in animal trials of new vaccines (156, 157, 202, 203).

To circumvent the difficulties of establishing small animal models of acute RSV bronchiolitis, some investigators have opted to infect experimental animals with other viruses that produce clinical disease in the host: for example, canine parainfluenza virus type 2 (97) and

canine adenovirus (98) infection of beagle puppies and parainfluenza type I (Sendai) virus infection of rats (99). While the production of acute respiratory disease and airway hyperresponsiveness have been reported in these animal models, their major limitation is that the human counterparts of the animal parainfluenza and adenoviruses are epidemiologically of minor significance (82). One small laboratory animal that has not been used as a model of acute RSV bronchiolitis is the guinea pig, a species that potentially has advantages over the cotton rat and the mouse (section 2.9).

2.9 THE GUINEA PIG AS A POSSIBLE ANIMAL MODEL OF HUMAN ACUTE RSV BRONCHIOLITIS. Previous studies of guinea pigs challenged with human RSV have documented the development of otitis media following instillation into the middle ear (204) and production of RSV-specific neutralizing and complement-fixing antibodies following intranasal instillation (164, 182). Review of *Index Medicus* dating from the discovery of the chimpanzee coryza agent until the present time fails to reveal any reports examining for pulmonary lesions in guinea pigs inoculated with human RSV; however, the guinea pig has been used as a model of virus-induced airway hyperreactivity to another *Paramyxovirus*, parainfluenza type 3 virus (95, 205).

Compared to other small laboratory animals, the guinea pig particularly resembles the human in terms of its reproductive physiology, corticosteroid metabolism and type I (immediate hypersensitivity) and type IV (delayed hypersensitivity) immune responses (206). Because of its well characterized airway physiology, the guinea pig has long been favored as an animal model of asthma (207)[15]. Factors that potentially limit the guinea pig as an animal model of acute RSV bronchiolitis, a pediatric disease, include the "precociousness" of newborn animals (fully furred, teethed and open-eyed), early weaning by postnatal day 33 and early sexual maturity at approximately 60-90 days of age (body mass > 450 g) (209). Secondly,

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15 With the knowledge that no species other than humans spontaneously develops an illness resembling asthma, some investigators prefer to use the expression, "experimentally-induced airway hyperresponsiveness" in reference to animal models of asthma (208).

although guinea pigs produce IgE (210), type I hypersensitivity is primarily mediated by IgG_1. A third limitation is the relatively small number (compared to the mouse) of leukocyte antigens characterized in guinea pigs (211), thus rendering difficult studies of host lymphocyte responses to RSV. In addition, although guinea pigs are not overly susceptible to viral lung infections, they spontaneously develop lung infections to such bacterial pathogens as *Streptococcus pneumoniæ* and *Bordetella* species (213). While one may be confident that a guinea pig inoculated with human RSV will probably not develop a lung infection from an unrelated virus or transmit RSV to another guinea pig, the presence of bacterial lung infection could confound the interpretation of results. Precautions to avoid undesired infections in experimental animals (without having to resort to so-called "pathogen-free" animals (214) requiring specialized breeding, transport and housing conditions) include randomly assigning guinea pigs into RSV-inoculated and control groups, housing animals in closed rooms with no other traffic and investigators employing isolation procedures (e.g., gowns, masks, hats, gloves and shoe covers) during animal handling. With these potential advantages and limitations in mind, in this thesis the guinea pig was used as a new animal model of acute RSV bronchiolitis to test for the possibility of persistent RSV infection *in vivo* within a genetically heterogeneous population.

2.10 SUMMARY. Human RSV is a member of the *Pneumovirus* genus within the family, *Paramyxoviridæ*. Although its life cycle, ultrastructure, proteins and nucleic acid sequences have been well characterized, there is no unifying explanation for the propensity of children to develop lower respiratory tract infections and only limited understanding of the host immune response to RSV. Other than studies of severely immunodeficient animals, investigators have paid little attention to the possibility of persistent RSV lung infections *in vivo*. Animal models of acute RSV bronchiolitis in species that are not natural hosts are limited by such factors as different age susceptibilities to lung infection compared to humans, absence of clinical features of acute lower respiratory tract disease and development of mild bronchiolar inflammation despite inoculation with large amounts of virus. Previous experiments in guinea pigs have

shown that human RSV produces otitis media and a virus-specific humoral immune response: this thesis examined whether human RSV produces acute bronchiolitis and/or persistent lung infection in guinea pigs.

CHAPTER 3: WORKING HYPOTHESIS, SPECIFIC AIMS AND STRATEGY

The first two Chapters of this thesis provided the background and rationale for the working hypothesis and experimental portion. In Chapter 1, the concept of asthma as an inflammatory airway disease was discussed and evidence for latent or persistent viral pulmonary infections contributing to this chronic airway inflammation was presented from the level of epidemiology to biochemistry. In Chapter 2, the biology of RSV was discussed in terms of the pathogenesis of acute bronchiolitis and the circumstances under which persistent cellular infection may occur. This Chapter also presented the available information concerning current animal models of RSV lung infection and discussed the potential advantages and limitations of using the guinea pig as a new animal model to test the working hypothesis of this thesis.

3.1 WORKING HYPOTHESIS.

The working hypothesis of this thesis was based primarily on the epidemiological association between acute RSV bronchiolitis and subsequent asthma in children and previous descriptions of persistent RSV cellular infection *in vitro*:

> Persistent cellular RSV infection may occur within the lung following resolution of acute bronchiolitis and this chronic persistence of virus places the host at risk for developing chronic inflammation of airways, a hallmark of asthma.

The significance of this working hypothesis was that, for the first time, an attempt was made to examine a plausible biological basis for the epidemiological association between acute RSV bronchiolitis and asthma.

3.2 SPECIFIC AIMS.

(1) To test whether human RSV produces acute bronchiolitis in the guinea pig;

(2) To document the natural history of RSV in the guinea pig lung following primary infection.

3.3 STRATEGY.

Specific Aim 1 was a study of the comparative biology between acute RSV lung infection of humans and guinea pigs. Outbred guinea pigs (modeling a genetically heterogeneous pediatric human population) were evaluated by clinical and pathological criteria based on human acute RSV lung infection. Specific Aim 2 was a study of the natural history of

intrapulmonary RSV from the time of a known primary infection. Because of possible differences between the human and guinea pig immune response to RSV (section 2.9), we used methods that tested *directly* for evidence of intrapulmonary RSV. Table 2 summarizes the techniques used in this thesis and refers to representative previous studies of these techniques for the study of RSV infection.

Clinical-pathological evaluation consisted of clinical examination, gross and histological lung examination that permitted comparison of the guinea pig model to human disease (103) and the cotton rat (199) and mouse (198) models of acute RSV bronchiolitis. In addition, this thesis tested for a possible age susceptibility of guinea pigs to develop acute RSV bronchiolitis, examined the extent of airway inflammation following infection with a larger amount of virus and examined for long-term clinical or pathological sequelæ of primary RSV lung infection.

The documentation of intrapulmonary RSV included viral culture to test for replicating virus, transmission electron microscopy (TEM) to test for assembled viral particles and viral nucleocapsids, immunohistochemistry to test for viral proteins and the reverse transcriptase polymerase chain reaction (RT-PCR) to test for viral genomic RNA. Viral culture on permissive cell lines *in vitro* is widely considered to be the "gold standard" of viral diagnosis because the identification of a characteristic cytopathic effect (CPE) *in vitro* confirms the viability of the virus (135). However, the absence of CPE does not imply absence of virus because persistent non-lytic infections (section 2.5) may produce "negative" cultures due to intracellular retention of virus (144).

Transmission electron microscopy (TEM) is considered to be a specific technique (191, 222) to document intracellular virus particles and nucleocapsids but technical aspects of tissue sampling (a typical "ultrathin" TEM section is a 60-100 nm cut through a 1 mm^3 specimen) limit the sensitivity of TEM to only 5-30% (228-231). Recently, the specificity of TEM in the identification of *Paramyxoviruses* has been questioned (232) because these viruses tend to assemble incompletely (220) and their tubular and filamentous nucleocapsids (127) might be confused with normal cellular structures such as intermediate filaments and microtubules.

TABLE 2: TECHNIQUES FOR THE GUINEA PIG MODEL OF RSV LUNG INFECTION

SPECIFIC AIM (1) CLINICAL-PATHOLOGICAL EVALUATION			
TECHNIQUE	PURPOSE	PREVIOUS APPLICATIONS	REFERENCE(S)
clinical examination (coryza, cough, tachypnea, ruffled fur, poor weight gain, decreased activity)	evidence of acute respiratory disease and "failure to thrive"	cotton rat and mouse models of acute RSV bronchiolitis	(198, 199)
gross lung examination (wet weight, gross appearance)	evidence of lung consolidation or edema	human autopsy lungs	(215, 216)
airway histological scoring system	evaluate extent of airway inflammation in RSV-challenged animals vs. controls	human chronic obstructive lung disease	(217, 218)
SPECIFIC AIM (2) DOCUMENTATION OF INTRAPULMONARY RSV			
TECHNIQUE	PURPOSE	PREVIOUS APPLICATIONS	REFERENCE(S)
viral culture	documentation of replicating viruses	- human nasopharyngeal cells - human autopsy lungs - experimental animal lungs	(219) (104) (198, 199)
transmission electron microscopy (TEM)	documentation of assembled viral particles and viral nucleocapsids	- ultrastructure of human RSV - ultrastructure of bovine RSV	(220, 221) (191, 222-224)
immunohistochemistry	documentation of viral antigens	- human nasopharyngeal cells - human autopsy lungs	(219, 225) (104)
reverse transcriptase - polymerase chain reaction (RT-PCR)	documentation of viral nucleic acid sequences	- human otitis media - human nasopharyngeal cells	(226) (227)

In response to these concerns, Phillips has used the term "virus-related particles" to specify structures that have features of *Paramyxoviruses* but are not completely assembled (233). With these technical limitations in mind, TEM was used as an ancillary method to examine for evidence of viral assembly within the lung.

Studies employing immunohistochemistry to diagnose human acute RSV infection report a sensitivity of anti-RSV antibodies from 67 to 76 percent and a specificity approaching 100% compared to viral culture (104, 219, 225). Since the working hypothesis of this thesis included the possibility of persistent, non-lytic RSV lung infection with potentially negative cultures, immunohistochemistry was particularly important to document evidence of intrapulmonary RSV after resolution of acute bronchiolitis.

An advantage of the reverse transcriptase polymerase chain reaction (RT-PCR) is the potential to "amplify" cDNA (following reverse transcription of genomic RNA) to detectable levels in specimens containing insufficient target RNA to be detected by conventional Northern blotting (234). To date, RT-PCR for RSV has been limited to specimens from acute human infections: middle ear effusions from patients with otitis media (226) and nasopharyngeal aspirates from children with acute RSV bronchiolitis (227). In nasopharyngeal aspirates, RT-PCR had a sensitivity of 94% and a specificity of 97% compared to viral culture (227) but the sensitivity of RT-PCR to detect a known quantity of RSV RNA was not tested. While the utility of RT-PCR has been established for acute RSV infections, there are no published reports of RT-PCR in the study of persistent RSV infections.

3.4 SUMMARY. The guinea pig was used as a new animal model of human RSV lung infection to test the working hypothesis that RSV persists within the lung following resolution of acute bronchiolitis. The susceptibility of the guinea pig for human RSV infection was assessed by clinical examination for evidence of acute respiratory disease, gross lung examination for evidence of pulmonary consolidation or edema and histopathological examination for features of human acute bronchiolitis. The natural history of intrapulmonary

RSV was assessed by viral culture, TEM, immunohistochemistry and RT-PCR to document structural aspects of RSV from the level of replicating virus to the viral genome.

CHAPTER 4: MATERIALS AND METHODS

4.1 PRELIMINARY STUDY. Figure 1 summarizes the protocol of a preliminary study relating to Specific Aim 1 that was designed to evaluate whether human RSV produces acute bronchiolitis in guinea pigs.

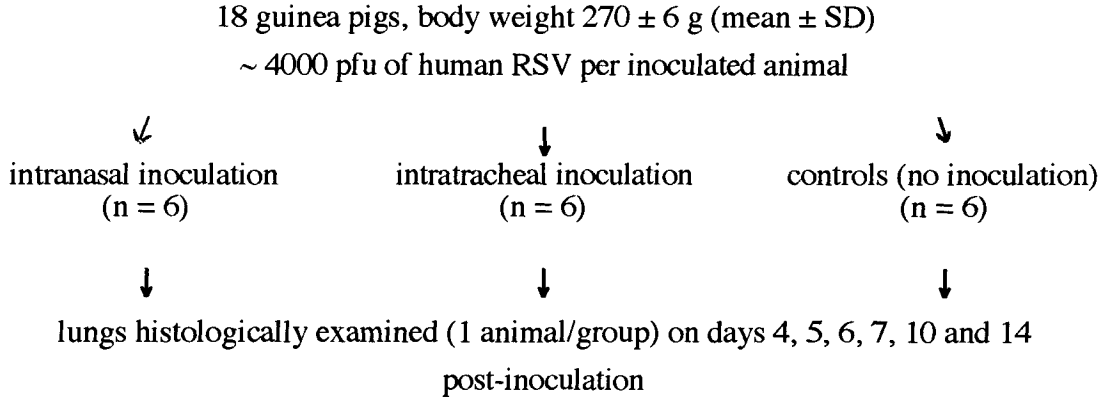

Figure 1. Preliminary study of guinea pig inoculation with human RSV (Experiment #1742). The details of guinea pig handling, virus preparation and lung tissue processing for histological examination are given below.

Based on the results of this preliminary study, Specific Aim 1 was completed by using the intranasal route of RSV inoculation for the protocol outlined in Table 3.

For the selection of study days post-RSV inoculation, day 6 was chosen because the greatest amount of bronchiolar inflammation was observed on this day in the preliminary study. Final studies on day 6 also included evaluation of a possible age susceptibility of guinea pigs to human RSV lung infection and clinical-pathological evaluation of juvenile guinea pigs given sevenfold more RSV. Day 14 was chosen as the "convalescent" phase of acute RSV bronchiolitis analogous to human infection. For chronic studies, day 60 was chosen because it is approximately twice the greatest reported time that RSV has been cultured from an immunocompetent human (84, 235) and day 125 was chosen because it is more than

TABLE 3: FINAL PROTOCOL FOR INOCULATION OF HUMAN RSV INTO GUINEA PIGS

STUDY (EXPT #)*	RSV (n)	CONTROL (n)	GUINEA PIG AGE**	RSV DOSE ***	CULT	TEM	IMM	RT-PCR
DAY 6 (1755)	10	10	juvenile	low	yes	yes	yes	no
DAY 6 (1791)	10	10	juvenile	high	yes	no	yes	no
DAY 6 (1830)	10	9	adolescent	low	yes	no	yes	no
DAY 6 RT-PCR (1979)	4	2	juvenile	low	yes	yes	yes	yes
DAY 14 (1779)	10	9	juvenile	low	yes	yes	yes	no
DAY 60 (1889)	10	11	juvenile	low	yes	no	yes	no
DAY 125 (1950)	4	1	juvenile	low	yes	no	yes	yes

* "EXPT #" refers to the U.B.C. Pulmonary Research Laboratory Experiment Number.

** "Juvenile" guinea pigs had body weights < 450 g (22-29 days old) when inoculated with human RSV while "adolescent" guinea pigs had body weights > 450 g (64-70 days old) at the time of RSV inoculation, based on published values for these age groups (209, 236).

*** "Low dose" RSV refers to inoculation of guinea pigs with $3.9 \pm 0.3 \times 10^3$ pfu Long strain human RSV (mean ± SD); "high dose" RSV refers to inoculation of guinea pigs with $2.8 \pm 0.4 \times 10^4$ pfu Long strain human RSV.

Abbreviations: CULT = viral culture of lung digests on HEp-2 cells; TEM = transmission electron microscopy of ultrathin sections of lung; IMM = immunohistochemistry on lung tissue sections; RT-PCR = reverse transcriptase polymerase chain reaction on homogenized lung

twice the greatest reported time that RSV has been cultured from an immunosuppressed human (85). The control animals in the final protocol received uninfected cell culture supernatant (section 4.4) in order to make the results comparable to the cotton rat and mouse models of acute RSV bronchiolitis (198, 199).

All animals had clinical-pathological evaluation (Specific Aim 1) consisting of clinical examination, gross lung examination and histological lung examination. For the documentation

of the natural history of intrapulmonary RSV (Specific Aim 2), animals were studied as summarized in Table 3.

4.2 SPECIES. Female Cam Hartley guinea pigs (Charles River Laboratories, Montreal, PQ) were randomly assigned into either RSV-inoculated or control groups and housed in separate rooms under identical conditions of plastic cages containing Bed o'Cobs® corn cob bedding (The Andersons, Industrial Products Division, Maumee, OH), access to Purina guinea pig chow (Ralston Purina Company, St. Louis, MO), alfalfa hay cubes and water and 12 hour alternating light-dark cycles.

4.3 VIRUS. All virus handling occurred in a Biohood Model 1148 biological safety cabinet (Forma Scientific, Marietta, OH). The Long strain of subgroup A human RSV (American Type Culture Collection, Rockville, MD) was propagated at multiplicities of infection from 0.01-0.1 (237) on HEp-2 cell monolayers (gift of U.B.C. Virology Laboratory) in 75 cm^3 plastic flasks (Corning Laboratory Science Products, Mississauga, ON) at 34°C in a Fisher Model CO_2 610 incubator (Fisher Scientific, Napean, ON) containing humidified air with 5% CO_2. The cell culture medium was Dulbecco's minimal essential medium (MEM) (Grand Island Biological Company, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS, heat-treated (56°C for 30 minutes) to inactivate complement) (GIBCO), 0.292 mg/mL L-glutamine (GIBCO), vitamins (GIBCO), 100 U/mL penicillin G (Sigma Chemicals, St. Louis, MO), 100 μg/mL streptomycin (Sigma) and 10 μg/mL amphotericin B (GIBCO)[16]. To passage virus, RSV-inoculated HEp-2 cell monolayers were disrupted into 20 mL of cell culture medium by the addition of autoclaved 3 mm diameter solid glass beads (Propper Mfg. Co. Inc., Germany) to the flask and placing the flask on a high speed Genie-2 vortex (Fisher Scientific) for 10 seconds. One and one-half mL of this cell lysate were added to a 75 cm^3 flask of subconfluent HEp-2 cells in 18.5 mL of fresh cell culture medium at 34°C. Formation of small syncytia, the

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16 For studies of days 60 and 125 post-inoculation and day 6 for RT-PCR, antibiotics and amphotericin B were not used in the cell culture medium.

characteristic RSV cytopathic effect (CPE) (Figure 2), was typically observed within 5-6 days under a Nikon TMS-F inverted microscope.

4.4 INOCULATION PROCEDURE. On the day of inoculation of guinea pigs (designated study day 0), RSV-infected HEp-2 cell monolayers showing extensive CPE were disrupted as described above. Lysed cell suspensions underwent centrifugation at 800 x *g* on a benchtop centrifuge (International Equipment Company (IEC) Model Centra-8, Needham Heights, MA) at 4°C for 4 minutes and the supernatant was transferred to a sterile Falcon 2057 tube (Becton Dickinson, Lincoln Park, NJ) using an autoclaved cotton-plugged Pasteur pipette (Kimble, Toledo, OH). Supernatant from disrupted uninfected HEp-2 cells was obtained in a similar manner.

Guinea pigs were anesthetized with 3-5% halothane (Ayerst Laboratories, Montreal, PQ) in oxygen delivered by a Medishield Inhalational Anesthetic Machine (Ohio Medical, Rexdale, ON). Depending on the group to which they were stratified, anesthetized animals received either 100 μL of RSV-containing supernatant or 100 μL uninfected supernatant delivered intranasally using an Eppendorf pipetter with autoclaved 0-100 μL tips (Elkay Products, Inc., Shrewsbury, MA)[17].

4.5 VIRAL PLAQUE ASSAY. In order to quantify the amount of viable RSV delivered intranasally to each animal, viral plaque assays were performed in duplicate as described by Lennette and Schmidt for the identification of RSV syncytia in infected HEp-2 cells (238). Supernatants remaining from the inoculation of guinea pigs were serially diluted (1:10^3 to 1:10^5) in MEM/5% FBS and 0.5 mL of these diluents were added to confluent HEp-2 cells in Falcon 3046 six well culture plates (Becton Dickinson). After allowing 90 minutes for viral adsorption at 37°C, the diluent was removed and replaced by

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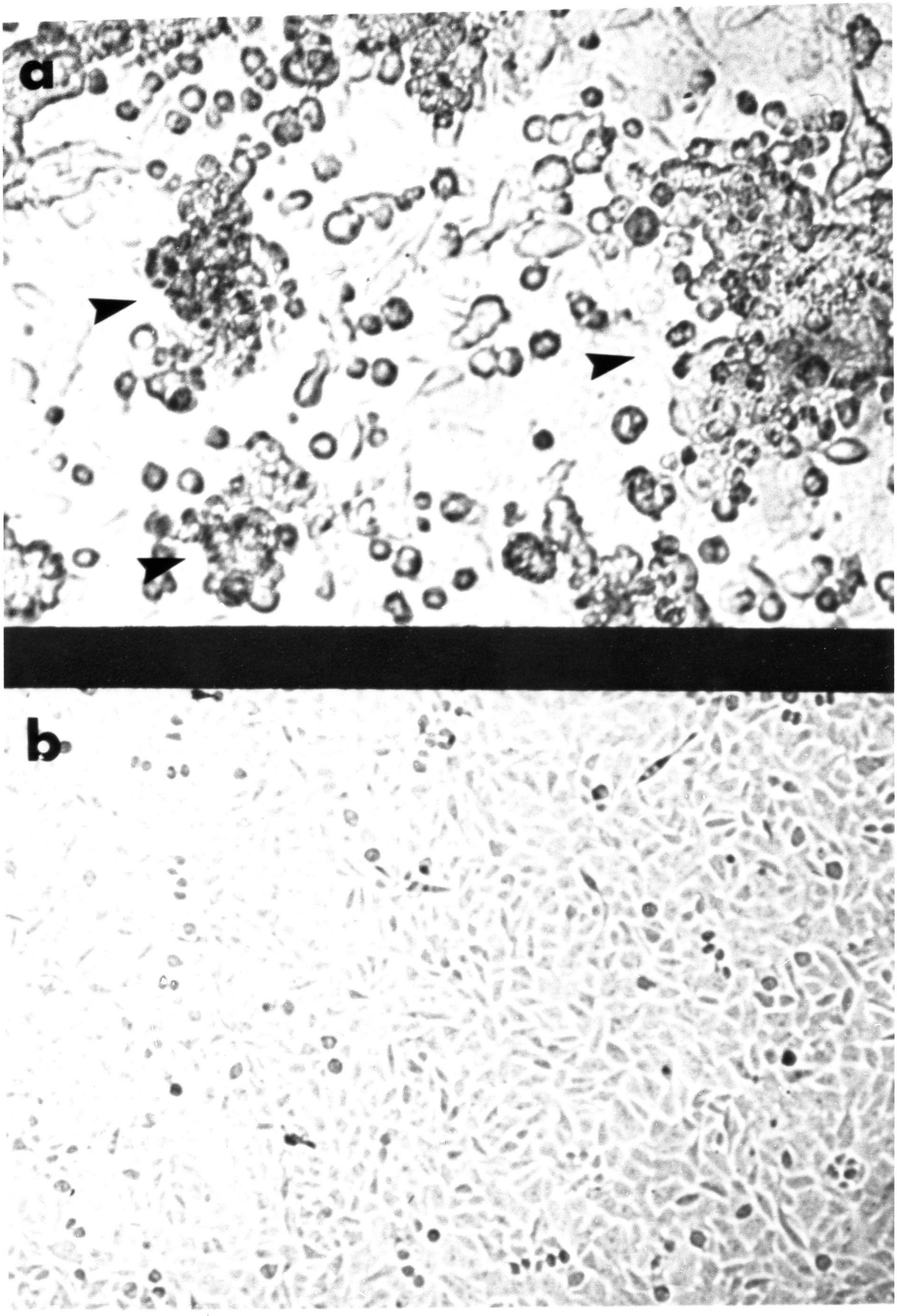
17 In the preliminary experiments, RSV was also instilled intratracheally to 6 guinea pigs via a flexible plastic tube attached to a 1 mL tuberculin syringe (Becton Dickinson).

Figure 2. Photomicrographs of HEp-2 cell monolayers (inverted microscope). Panel **a** shows RSV-infected Hep-2 cells with the formation of syncytia (arrowheads) characteristic of RSV cytopathic effect (final magnification: x208). Panel **b** shows uninfected HEp-2 cells with the normal "cobblestone" pattern *in vitro* (final magnification: x104).


a
b

2.5 mL of a liquid mixture of 2 parts "double strength" dsMEM (i.e. MEM prepared in half the volume of dH_2O)/10% FBS to 3 parts autoclaved 1% agarose (GIBCO BRL, Gaithersburg, MD) in dH_2O. After the dsMEM-agarose mixture hardened (usually within one minute), the culture plates were transferred to the CO_2 incubator at 34°C and kept for 5-7 days until syncytia could be discerned under a Nikon TMS-F inverted microscope.

Cells were fixed over 30 minutes at 34°C by the diffusion of 2 mL 10% neutral buffered formalin (BDH Chemicals, Toronto, ON) through the dsMEM-agarose. The outer aspect of the dsMEM-agarose was then cut with a spatula blade and removed (taking care to avoid damage to underlying cells). Cells were stained with 0.5% neutral red (Fisher Scientific) for 1 minute and gently rinsed several times in tap water. The number of "plaques" (syncytia) were counted in each well with the mean being expressed as the number of plaque forming units (pfu) per 100 μL of original supernatant given to guinea pigs.

4.6 CLINICAL EVALUATION. For the day 6 and 14 studies, guinea pigs were examined daily for signs of respiratory disease (coryza, coughing or tachypnea) and general signs of illness (ruffled fur, decreased activity) (198). Body weights were recorded on the applicable study day. For several studies (Appendix A), animals had "initial" (day of inoculation) and "final" (study day) body weights recorded to evaluate changes in body weight over the study interval.

4.7 GROSS LUNG EXAMINATION AND TISSUE PROCESSING. Guinea pigs were anesthetized by intraperitoneal injection with 0.5 mL euthanyl forte® (MTC Pharmaceuticals, Cambridge, ON) and exsanguinated via right ventricular needle puncture. The chest was opened and the heart and lungs were removed *en bloc*. Following suture ligation of the right mainstem bronchus, the right lung was isolated and weighed in a sterile vessel.

The left lung was inflated through the trachea with 5 mL of 4% paraformaldehyde (BDH Chemicals) in phosphate buffered saline (PBS) (Oxoid, Basingstoke, UK) containing 0.1% diethylpyrocarbonate (DEPC) (Aldrich Chemical Company, Milwaukee, WI). After overnight

fixation at 4°C, serial 3 mm sagittal sections of the left lung were examined for gross evidence of consolidation.

Midsagittal slices (239) of paraformaldehyde-fixed lungs were processed in the usual way for paraffin embedding (240) by an Histomatic Automated Tissue Processor Model 266 MP (Fisher Scientific). Serial 4 μL sections were prepared by Histology Laboratory staff at St. Paul's Hospital and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stains.

The right middle lobe was dissected and processed for viral culture and TEM (sections 4.9 and 4.10). For day 6, day 14 and day 60 studies, the remainder of the right lung was frozen in liquid nitrogen (with or without inflation with cryoprotective O.C.T. Compound (Miles Scientific, Elkhart, IN) and stored at -70°C in an UT 1786 freezer (Revco Scientific Inc., Asheville, NC). In the day 125 study, a portion of the right middle lobe was processed for viral culture (section 4.9), the remainder of the right lung was inflated with 4% phosphate buffered paraformaldehyde through the pleura as described by Churg (241) and processed for light microscopy while the left lung was prepared for RT-PCR (section 4.12).

4.8 LIGHT MICROSCOPY. The variability of bronchiolar inflammation induced by intranasal inoculation of RSV to that induced by uninfected cell culture supernatant was compared using a semi-quantitative scoring system based on previous reports of RSV infection in experimental animals (145, 192) and human chronic obstructive pulmonary disease (217, 218). Bronchioles were scored for six histological features including epithelial necrosis, mononuclear cell infiltrates and edema because they are features of human acute viral bronchiolitis (103); polymorphonuclear (PMN) cell infiltration as an index of concomitant acute inflammatory phenomena (242); hyperplasia of bronchus-associated lymphoid tissue (BALT) as an index of the mononuclear cell response (171) and goblet cell metaplasia as an index of respiratory epithelial repair (243). For a given airway, each feature was scored from 0 (normal) to 2 (moderate to severe changes) by comparison to photomicrographs of guinea pig airways (Figure 3). Ten bronchioles covering all regions of the lung section were examined per slide

and the observed score for each histological feature was expressed as the sum of individual airway scores (maximum score of 2 x 10 = 20 per parameter). The criteria for selection of airways to be scored included: (a) evaluation limited to membranous bronchioles (i.e. muscular, non-cartilaginous airways); (b) scoring a given airway only once; (c) avoidance of nearby cuts of an already scored airway.

A Zeiss Photomicroscope II was used for scoring. Slides were coded such that the origin of a particular section (control or RSV-inoculated animal) was not known to the microscopist. Upon completion of all scoring, the code was broken and statistical analysis performed (section 4.14).

4.9 VIRAL CULTURE. One half of the fresh right middle lobe was minced into fine pieces with a sterile razor blade, transferred to a 120 mL sterile specimen jar (Fisher Scientific) containing an autoclaved magnetic stir bar and digested into single cells by addition of 10 mL filtered 0.25% trypsin (GIBCO) in PBS and placing the jar on a Corning Model PC353 magnetic stirrer (Corning, NY) for 90 minutes at 37°C. The single cell suspension was transferred to a sterile Falcon 2057 tube and centrifuged at 800 x *g* (IEC Model Centra-8) at room temperature for 4 minutes. Following centrifugation, the supernatant was removed and replaced with 1 mL sterile cell culture medium. The resulting suspension was added to subconfluent HEp-2 cell monolayers growing in 25 mL Corning flasks containing 4 mL cell culture medium at 34°C (135). Flasks were examined daily under the Nikon inverted microscope for signs of RSV cytopathic effect (CPE). Cells which did not show CPE were passaged at weekly intervals for up to one month using the protocol described above for the passage of uninfected HEp-2 cells. A culture was classified as "positive" when CPE was observed, regardless of the number of syncytia. A culture was classified as "negative" when no CPE was observed over the one month interval.

To quantify the number of replicating viruses in the guinea pig lung parenchyma at day 6 post-inoculation, viral plaque assays (section 4.5) were performed using trypsin-digested

Figure 3. RSV bronchiolitis scoring system: standard photomicrographs. Unless otherwise specified, all panels are hematoxylin and eosin stained sections of guinea pig lung with final magnification: x208.

Panel **a**: Grade 0 (normal) membranous bronchiole.

Panel **b**: Grade 1 respiratory epithelial cell necrosis. Some epithelial cells are sloughed into the airway lumen.

Panel **c**: Grade 2 respiratory epithelial cell necrosis. There is more extensive epithelial cell sloughing with intraluminal necrotic debris.

Panel **d**: Grade 1 mononuclear cell infiltrates. Mononuclear cells have a peri-bronchiolar distribution.

Panel **e**: Grade 2 mononuclear cell infiltrates. Mononuclear cells extend through the bronchiolar smooth muscle into the subepithelial space.

Panel **f**: Grade 1 polymorphonuclear (PMN) cell infiltrates. PMNs have predominantly a peri-bronchiolar distribution with occasional cells in smooth muscle and subepithelial space (final magnification: x260).

Panel **g**: Grade 2 PMN infiltrates. There is more extensive PMN infiltration into smooth muscle and subepithelial space than in Grade 1 (final magnification: x260).

Panel **h**: Grade 1 airway wall edema. There is mild dilatation of the adventitia.

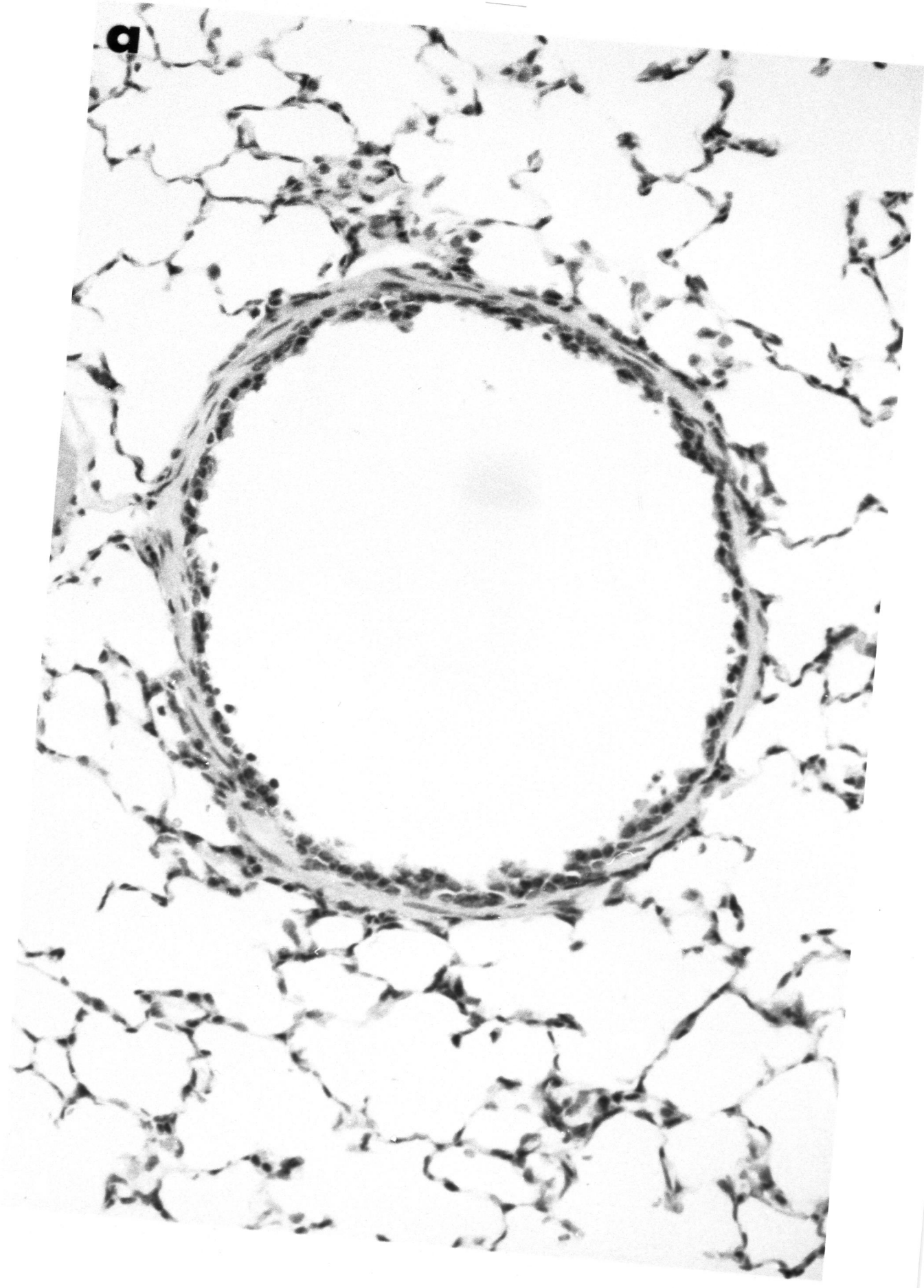
Panel **i**: Grade 2 airway wall edema. Congestion of adventitial vessels is apparent.

Panel **j**: Grade 1 BALT. The bronchiole contains a small proportion of BALT.

Panel **k**: Grade 2 BALT. The bronchiole has prominent BALT.

Panel **l**: Grade 1 goblet cell metaplasia. PAS-positive goblet cells constitute 25-50% of bronchiolar epithelial cells (PAS stain).

Panel **m**: Grade 2 goblet cell metaplasia. PAS-positive goblet cells constitute over 50% of bronchiolar epithelial cells (PAS stain).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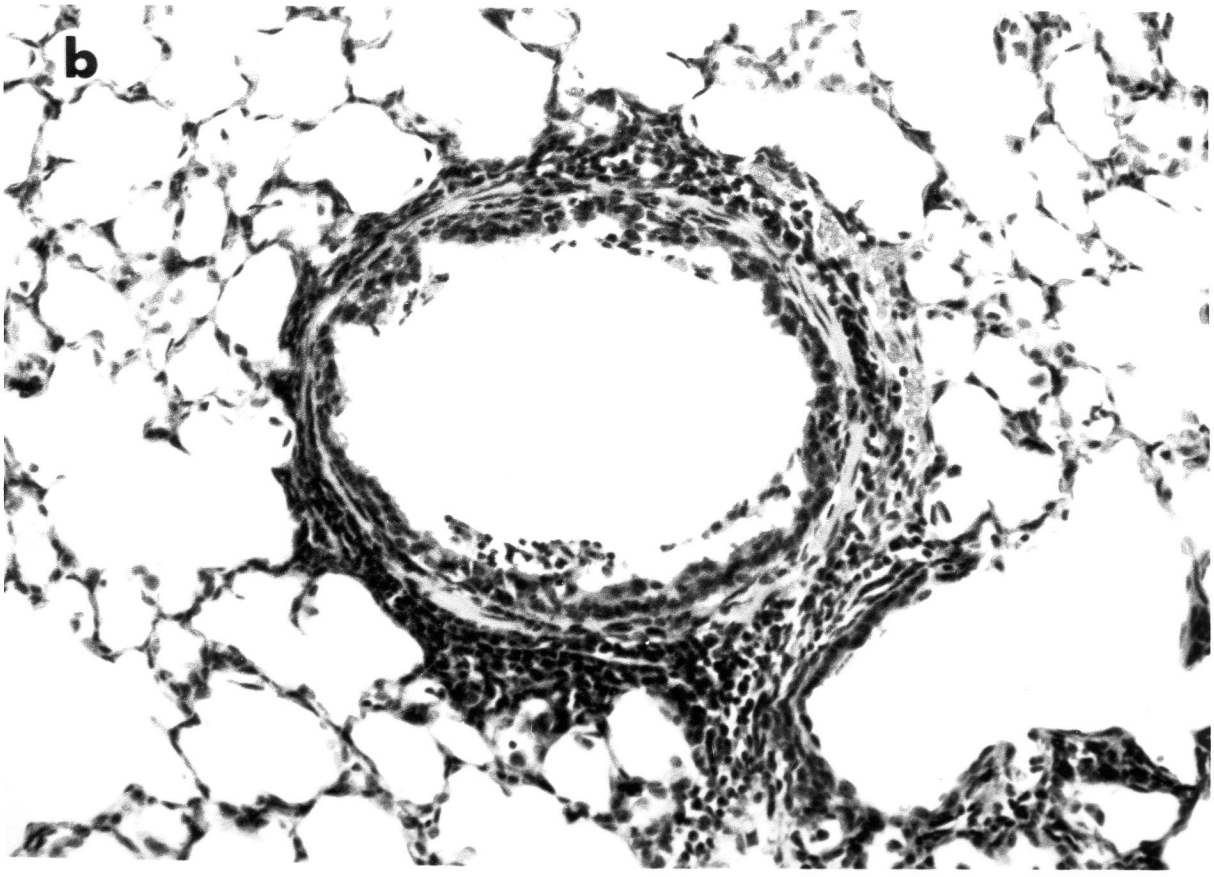
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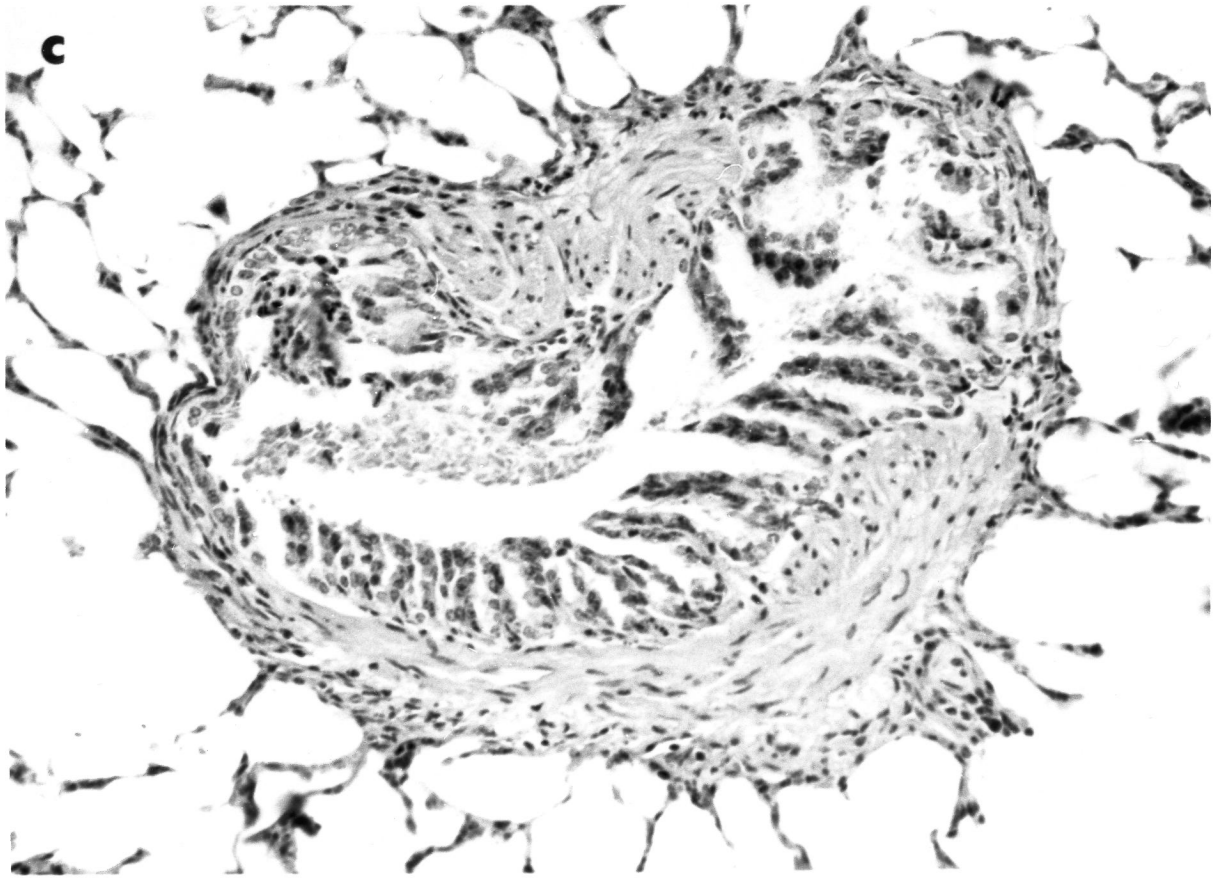
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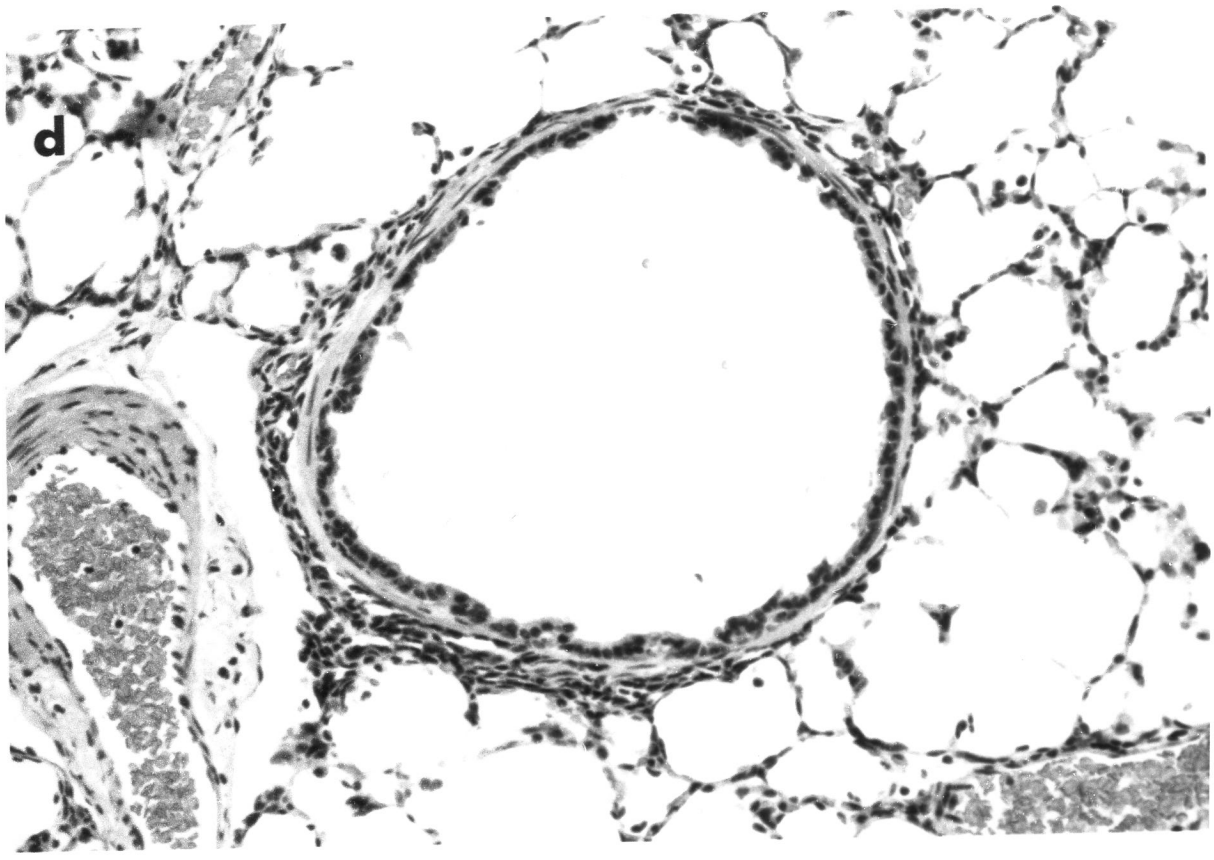
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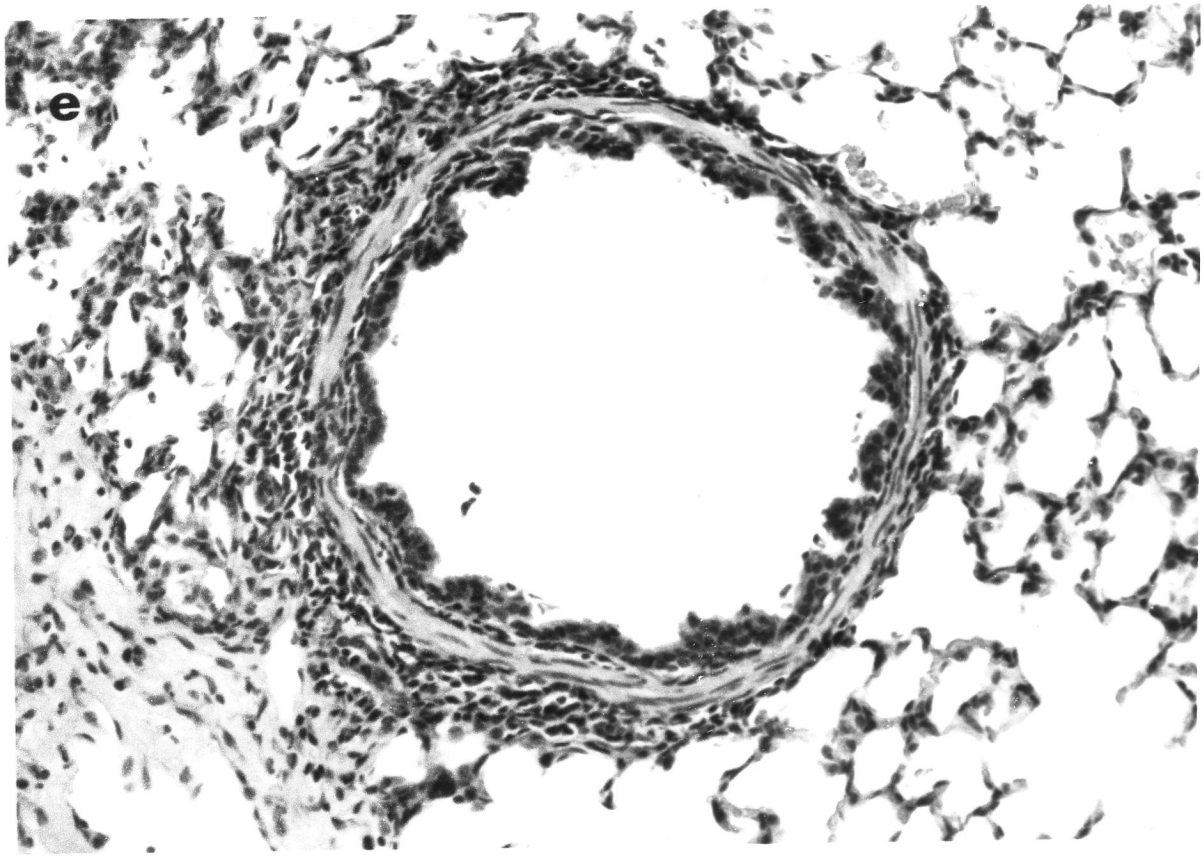
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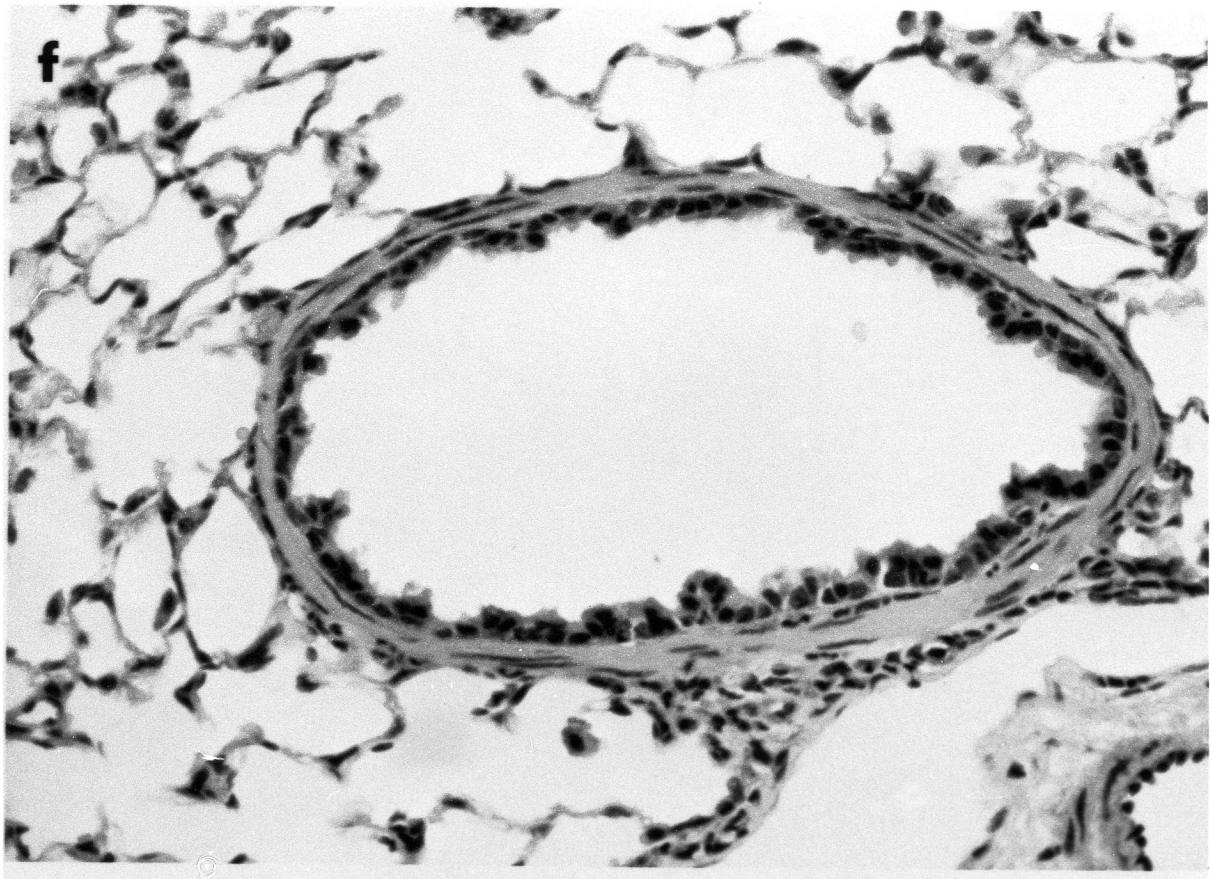
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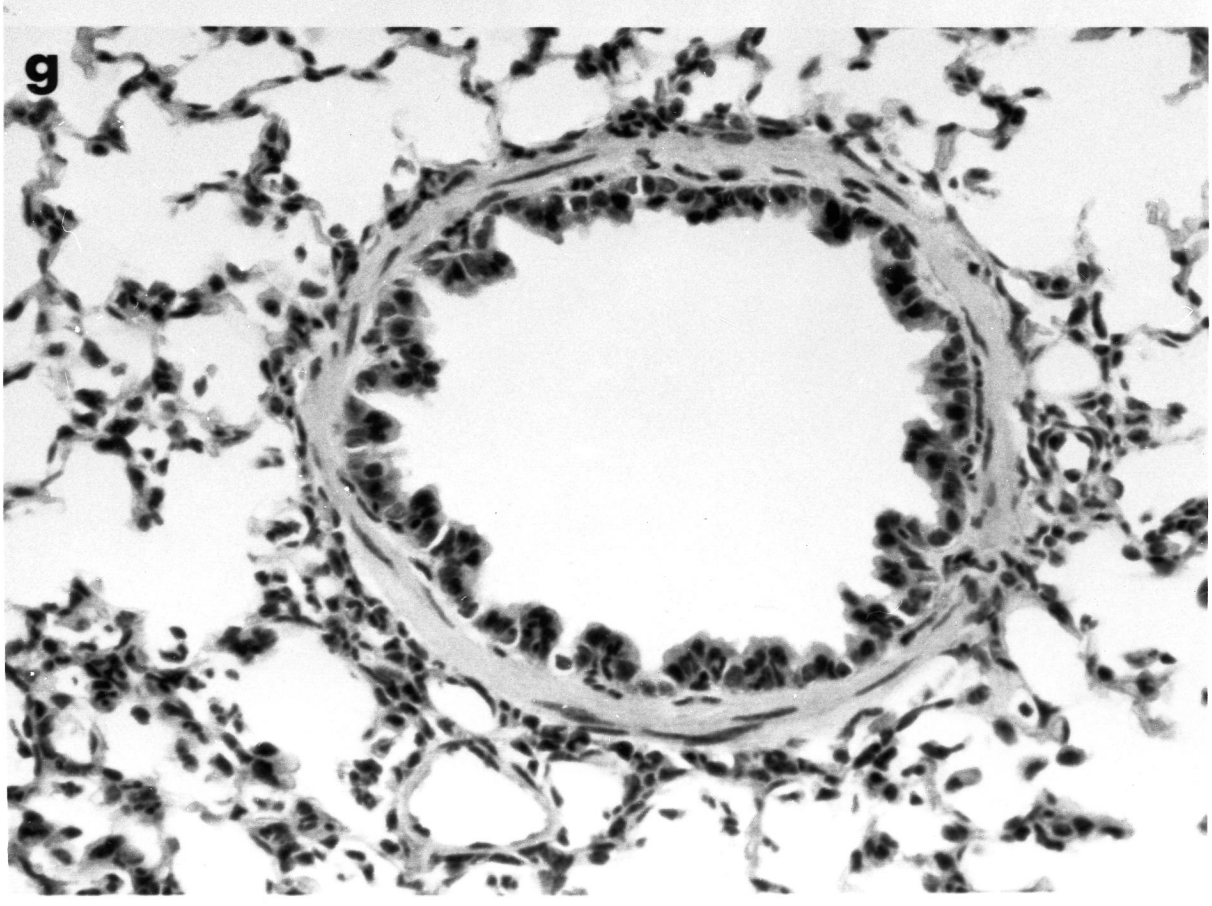
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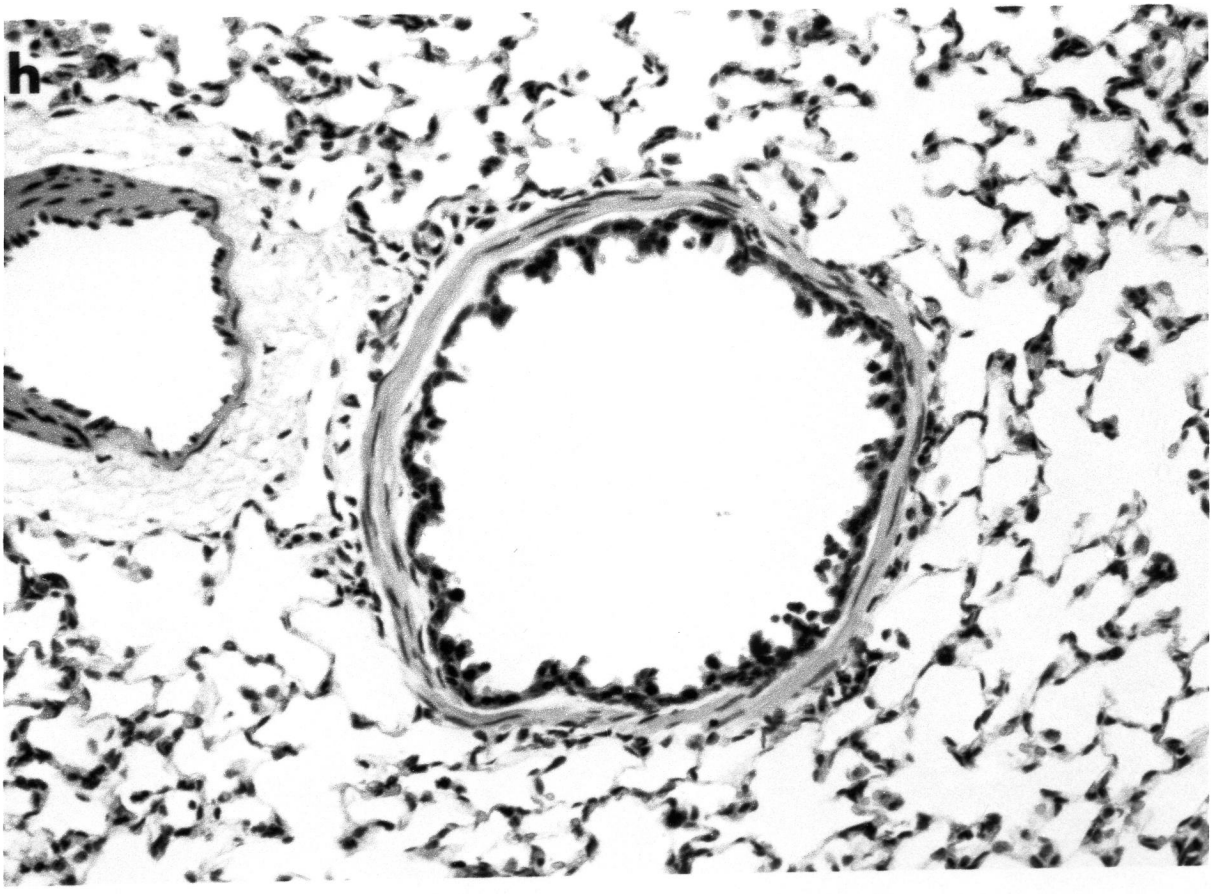
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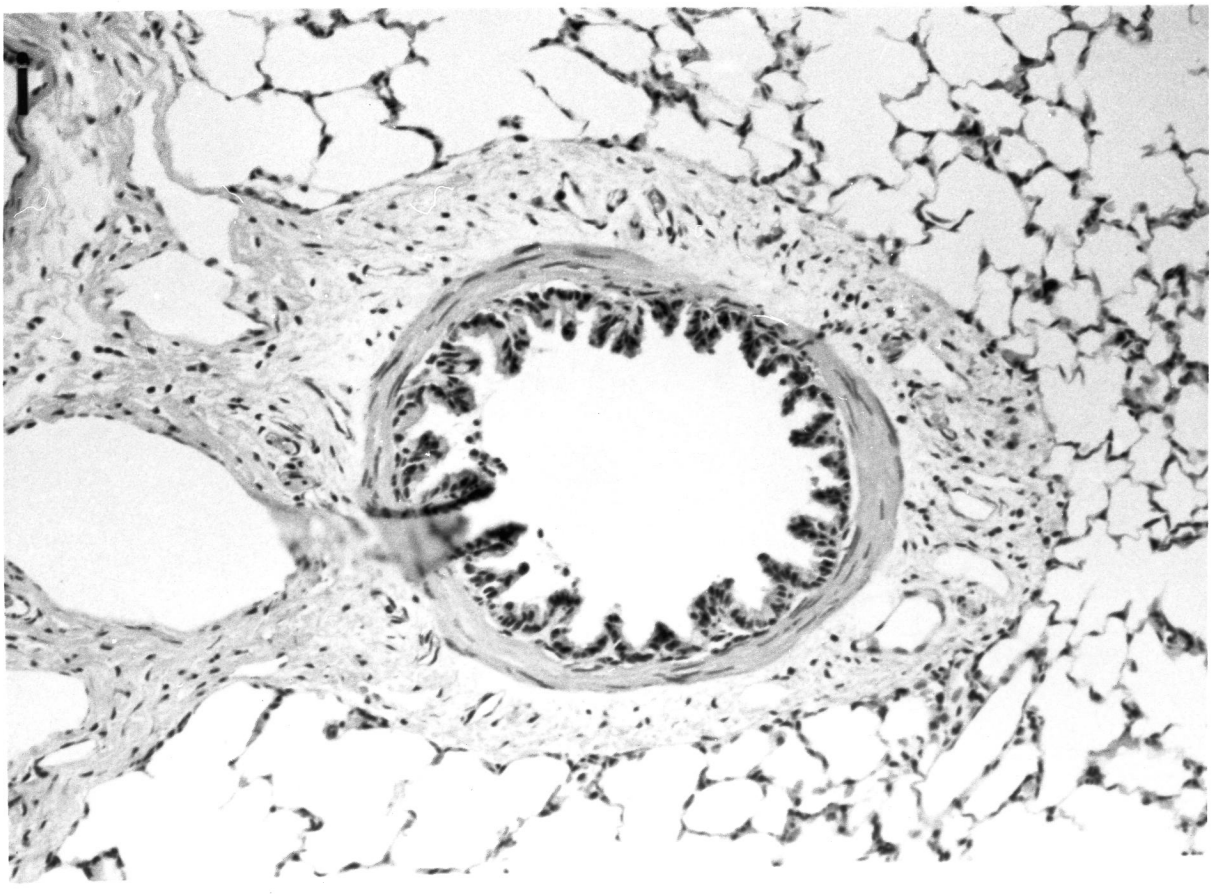
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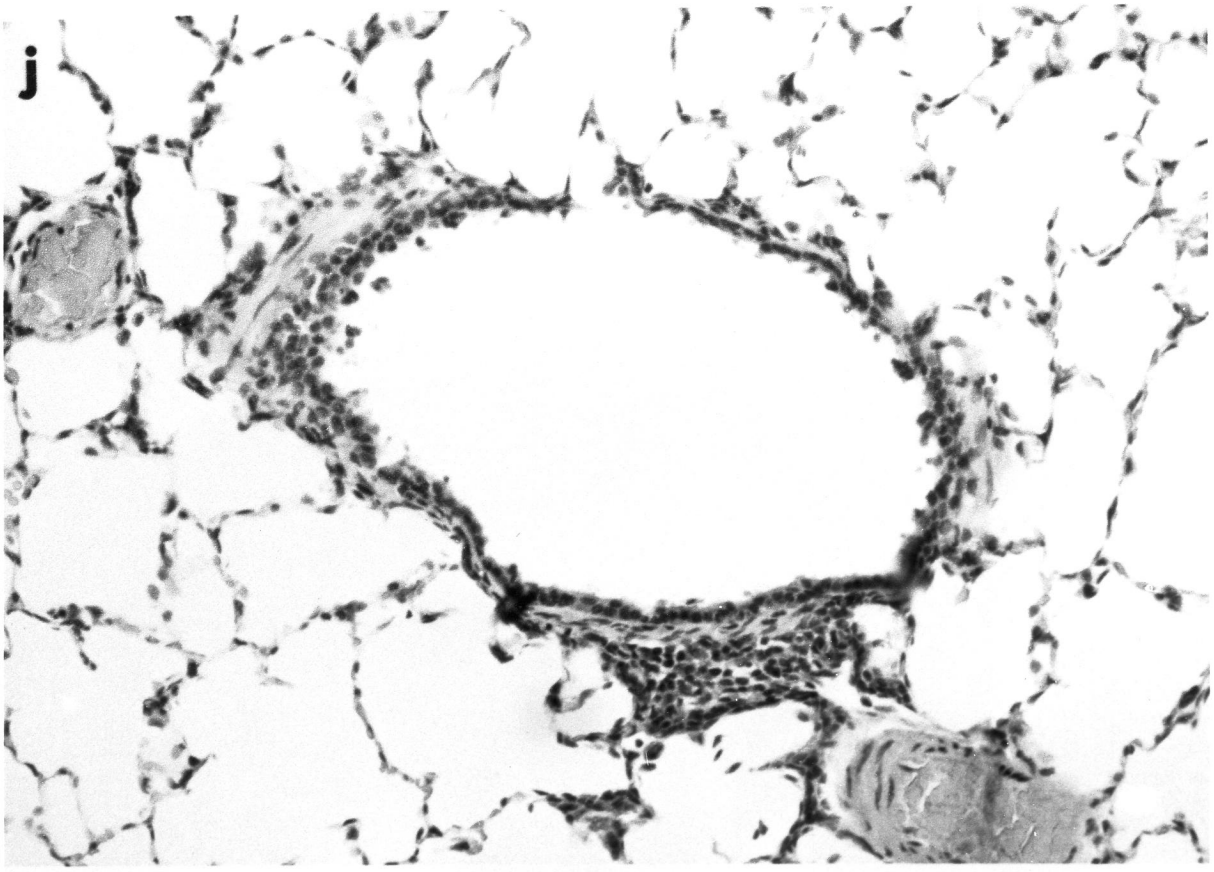
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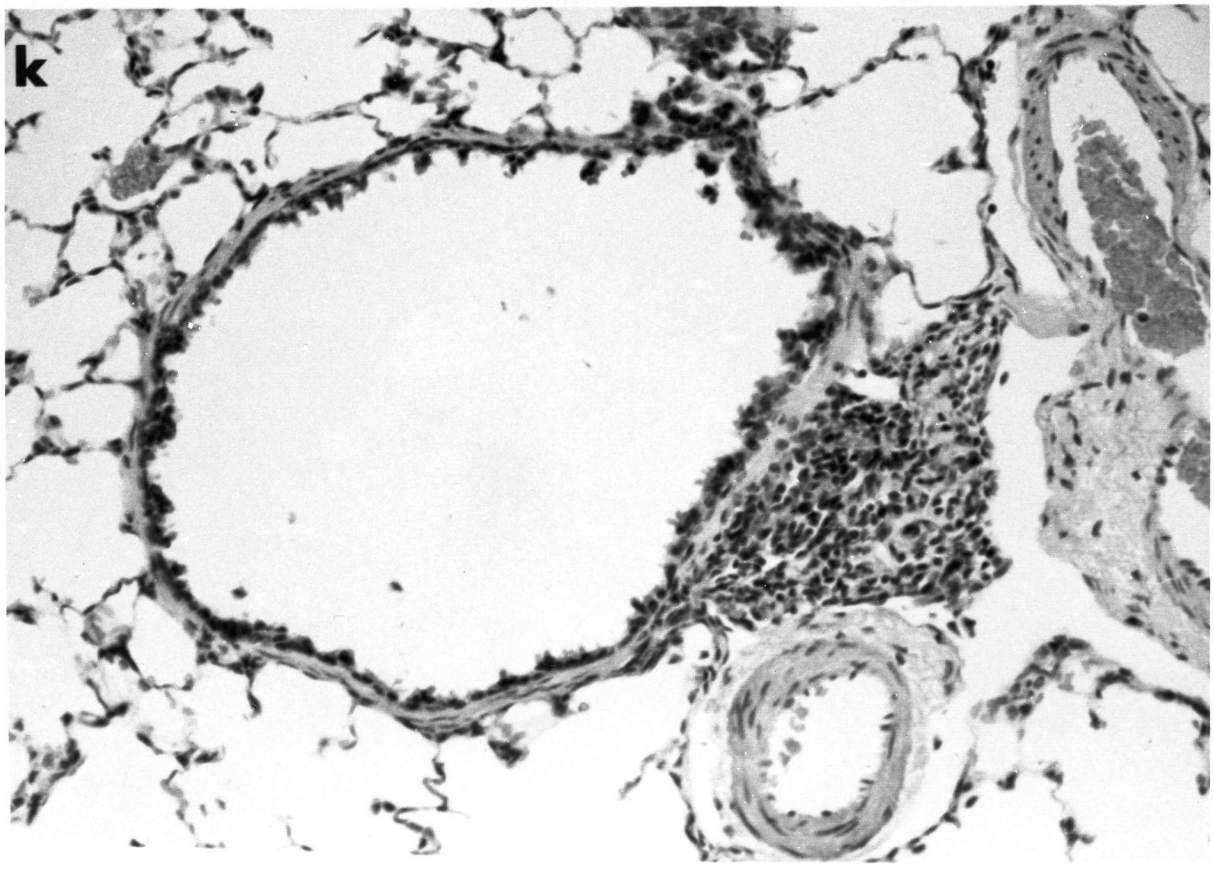
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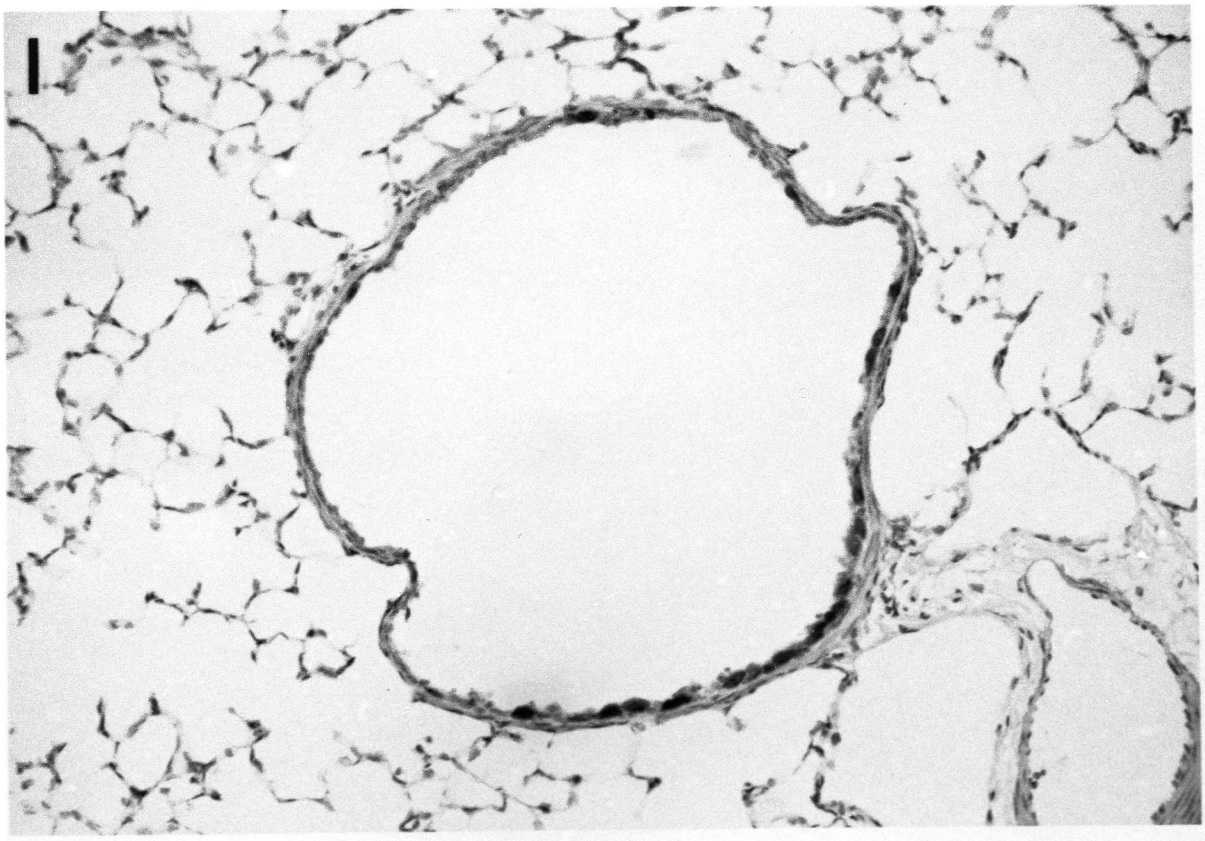
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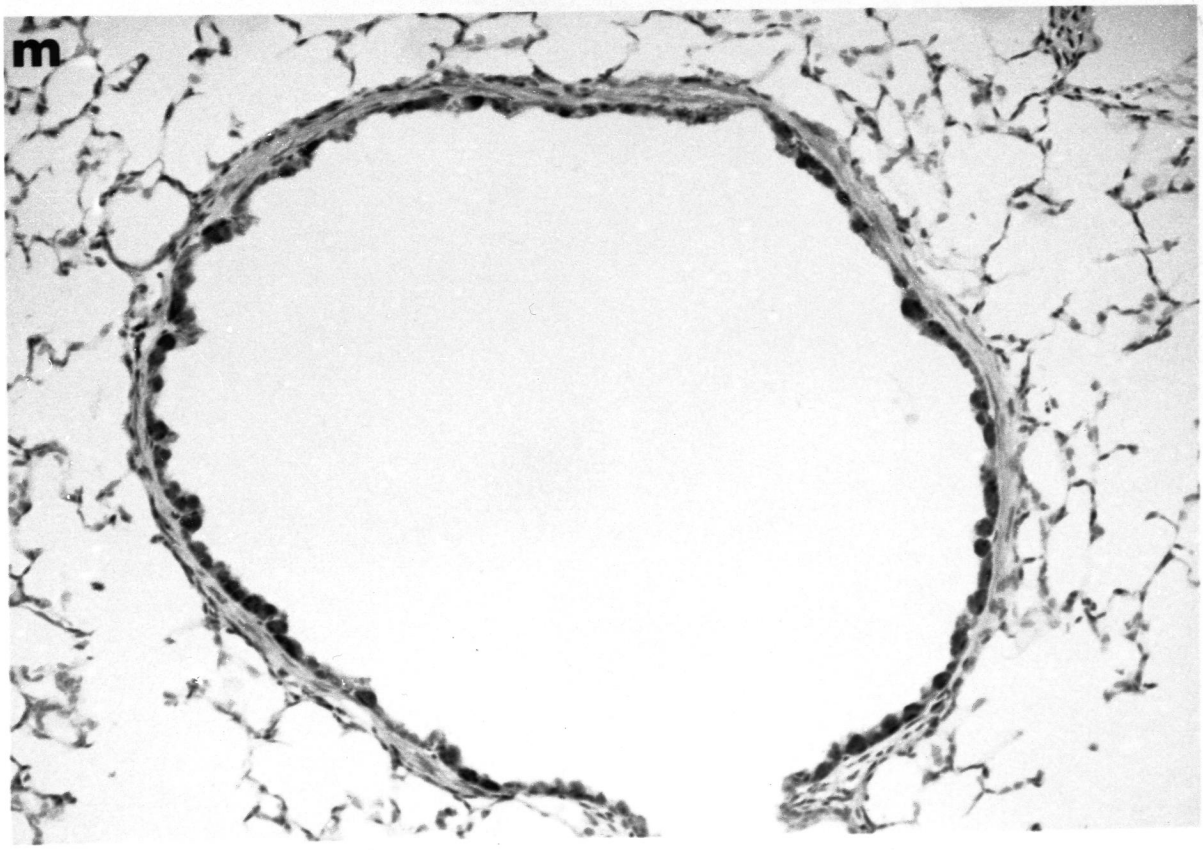
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lung homogenates on 4 RSV-inoculated guinea pigs and 2 controls from the day 6 RT-PCR study. The results were expressed as the number of pfu/g wet weight of fresh lung.

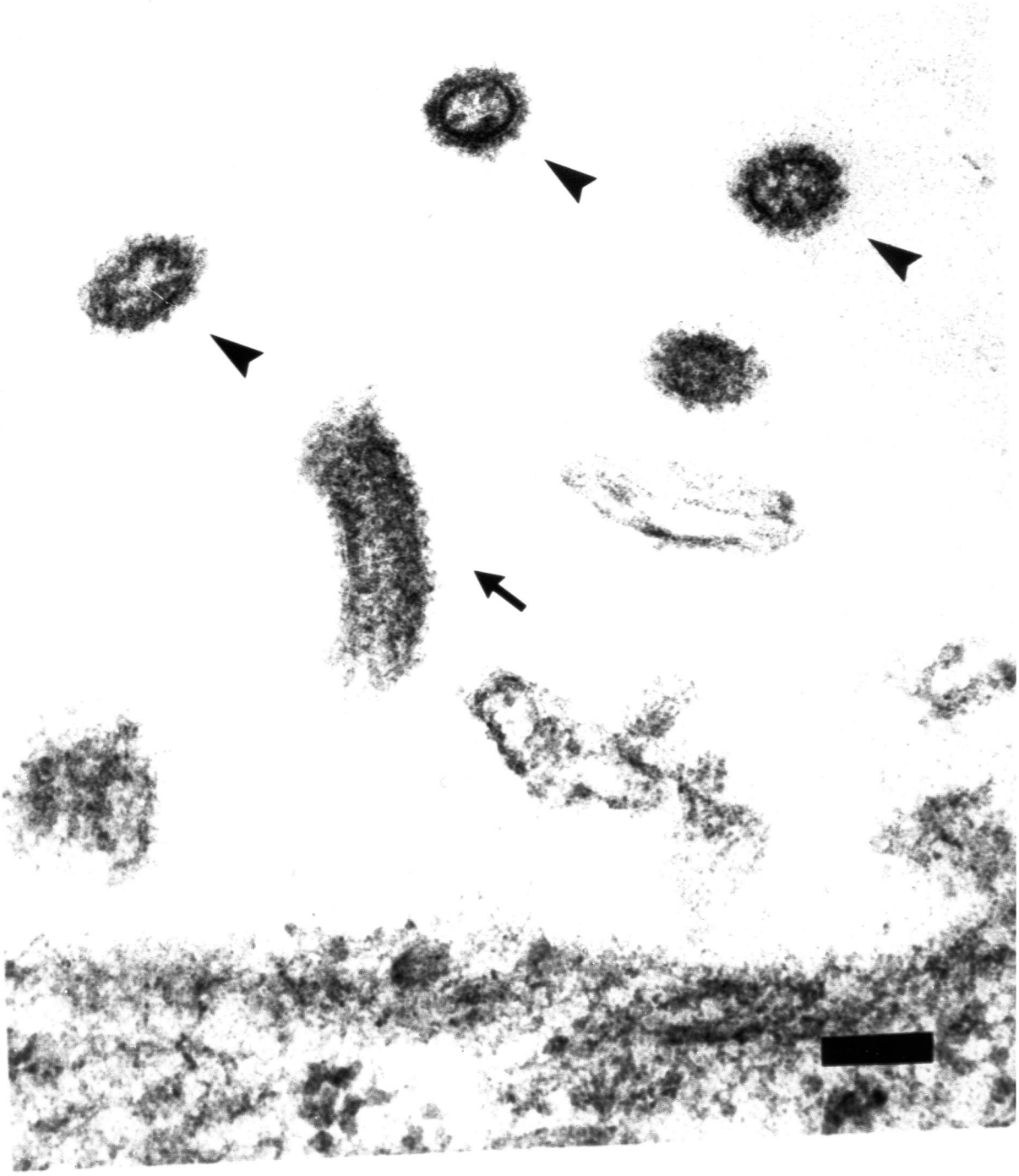
4.10 TRANSMISSION ELECTRON MICROSCOPY (TEM). The remaining half of the right middle lobe was inflated with 2% glutaraldehyde (Electron Microscopy Services (EMS), Fort Washington, PA) in 0.1 M sodium cacodylate buffer (EMS) through a 25 gauge needle (Becton Dickinson) as previously described (241) and immersed in this fixative for 2 hours at 4°C. Fixed lung tissue was cut with a razor blade into 1 mm^3 cubes, washed three times in 0.1 M cacodylate buffer, postfixed for 1 hour in 1% OsO_4 (EMS) at room temperature, washed for 15 minutes in dH_2O, dehydrated by serial 10 minute immersions in 30%, 50%, 70%, 90% and 100% ethanol and infiltrated with LR White (Polysciences, Warrington, PA). Specimens were placed into plastic beam capsules containing LR White which polymerized overnight at 65°C.

Multiple semi-thin (0.5 μm) sections were prepared, stained with toluidine blue (Fisher Scientific) and those sections containing foci of bronchiolar inflammation were selected for preparation of ultrathin (60-80 nm thickness) sections using an ultramicrotome (Ultracut, Reichert, Austria). Ultrathin sections were mounted on copper grids (EMS) and examined under a Philips 400 transmission electron microscope.

Lung sections from 14 RSV-inoculated guinea pigs at day 6 and 2 RSV-inoculated guinea pigs at day 14 were examined by TEM. Ultrathin sections from RSV-infected HEp-2 cells were positive controls (Figure 4) and sections from uninfected HEp-2 cells were negative controls.

4.11 IMMUNOHISTOCHEMISTRY. The protocol was adapted from Neilson *et al.*, who documented RSV antigens in paraffin-embedded sections of human lung obtained at autopsy (104). Five μm thick sections of paraformaldehyde-fixed, paraffin-embedded guinea pig lung were incubated with 0.1% protease, type XIV (Sigma) in 0.5 M TBS, pH 7.6 (TBS) (BDH Chemicals) at 37°C for 10 minutes to disrupt protein crosslinks induced by fixation. Following brief rinses in tap water and 95% EtOH, sections were incubated with 0.9% H_2O_2 (BDH

Figure 4. Electron micrograph of RSV-infected HEp-2 cells. There are completely assembled RSV seen in cross-section (arrowheads) and in longitudinal section (arrow). Cross-sections show the trilaminar membrane with projections ("fuzzy" coat) and centrally located electron dense nucleocapsids (bar represents 100 nm).



Chemicals) in methanol (Baxter Health Care Corporation, Muskegan, MI) for 25 minutes at room temperature to eliminate endogenous peroxidase activity. This incubation was followed by a tap water rinse and 5 minute wash in TBS at room temperature.

To prevent non-specific IgG binding, sections were preincubated in normal swine serum (DAKO) diluted 1:20 in primary antibody diluting buffer (Biomeda) for 30 minutes at room temperature. Incubation with primary rabbit anti-RSV antibody B344 (DAKO, Denmark)[18] diluted 1:300 in TBS/2% bovine serum albumin fraction V (BSA) (BDH Chemicals)/1% human AB serum was performed for 90 minutes at room temperature. Negative control slides were incubated in parallel with TBS/2% BSA/1% human AB serum in the absence of anti-RSV antibody. Following incubation with primary antibody, sections were washed in TBS for 5 minutes at room temperature.

Sections were next incubated with biotinylated swine anti-rabbit secondary antibody (DAKO) diluted 1:300 in TBS/2% BSA/1% human AB serum for 45 minutes at room temperature, followed by a 5 minute wash in TBS. A 45 minute incubation at room temperature in peroxidase-conjugated streptavidin (DAKO) diluted 1:600 in TBS/2% BSA/1% human AB serum was followed by a 10 minute wash in TBS.

The colorimetric peroxidase reaction consisted of developing sections in 20 μL working AEC solution (1 drop 3-amino-9-ethylcarbazole (Sigma), 1 drop 3% H_2O_2 in 3 mL 0.1M sodium acetate, pH 5.2 (Fisher Scientific)) for 15 minutes at room temperature. Following a rinse in dH_2O, sections were counterstained with Mayer's hematoxylin for 1 minute at room temperature and rinsed with dH_2O. Coverslips were mounted using Immu-Mount aqueous mounting medium (Shandon Labs, Pittsburgh, PA).

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18 The polyclonal rabbit anti-RSV antibody recognizes epitopes from several viral proteins, including the surface F protein and the nucleocapsid N protein (P.C. Grauballe, DAKO Patts, Denmark, personal communication). A mouse monoclonal anti-RSV N protein antibody (SeroTec MCA 491, Oxford, UK) cross-reacted with normal guinea pig lung parenchyma and was therefore not used.

Formalin-fixed, paraffin-embedded lung tissue from two fatal cases of human acute RSV bronchiolitis (courtesy of Dr. J. Dimmick, B.C. Children's Hospital and Dr. L. Holloway, New Zealand) were used as positive controls and a formalin-fixed, paraffin-embedded block from an adult autopsy lung (St. Paul's Hospital, Vancouver, BC) was used as a negative control.

4.12 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR).

4.12.1 PRELIMINARY STUDIES OF RT-PCR. The RT-PCR method was initially developed using total cellular RNA extracted from RSV-infected HEp-2 cells because these cells were a known source of viral genomic RNA. The sensitivity of the PCR amplification step was tested by using serial dilutions of a known amount of plasmid DNA (pGEM 3) containing the full length cDNA of the RSV N gene (generous gift of Dr. P.L. Collins, National Institutes of Health, Bethesda, MD). The specificity of RT-PCR was tested by using several controls: (a) no nucleic acid template; (b) uninfected HEp-2 cells; (c) RSV-infected HEp-2 cells which did not undergo reverse transcription prior to PCR; (d) RSV-infected HEp-2 cells which underwent pretreatment with 5 units RNase A (Sigma) at 37°C for 30 minutes prior to reverse transcription. The first control tested whether solutions were contaminated by either RSV or plasmid DNA. Uninfected HEp-2 cells were a source of non-specific total cellular RNA and the two positive controls were a source of viral genomic RNA. Once the optimal conditions for RT-PCR were established, the protocol was used in two studies of juvenile guinea pigs inoculated with low dose RSV: day 6 (n = 4 RSV-inoculated, 2 controls) and day 125 (n= 4 RSV-inoculated, 1 control).

4.12.2 SELECTION OF OLIGONUCLEOTIDES. For reasons given in section 2.3.1, the 1197 base N gene of human RSV (129) was chosen as a target for PCR amplification. Three oligonucleotides were synthesized at the DNA Synthesis Laboratory, University of Calgary:

(1) 5' GCG ATG TCT AGG TTA GGA AGA A 3' (bases 223 to 244 of the N gene *m*RNA sense strand);
(2) 5' GCT ATG TCC TTG GGT AGT AAG CCT 3' (the complement of bases 632 to 609 of the N gene *m*RNA sense strand);
(3) 5' TAG CTC CAG AAT ACA GGC ATG ACT C 3' (bases 449 to 473 of the N gene *m*RNA sense strand) .

The first two oligonucleotides were used as flanking PCR primers and the third was used as a probe of an internal sequence of the predicted PCR product. The oligonucleotides were chosen by the following criteria: (a) sequences toward the "middle" of the target N gene; (b) length greater than 20 bases (to ensure high specificity); (c) numerical balance between A+T (double hydrogen bonds) and G+C (triple hydrogen bonds); (d) no predicted secondary structure that would result in significant "folding" of the oligonucleotide onto its own complementary base sequences; (e) no significant sequence homology to the other two oligonucleotides or to other known viral, human or rodent genomic sequences available on the Genbank® database; (f) sufficient distance between the flanking primers to yield a reasonably-sized PCR product of 410 bp.

4.12.3 PURIFICATION OF OLIGONUCLEOTIDES. Crude oligonucleotide pellets were dissolved in 1.5 mL 0.5 M ammonium acetate (BDH Chemicals). Sep-Pak C18 cartridges (Millipore Corporation, Milford, MA) were prepared for chromatography by passing 10 mL 100% acetonitrile (Fisher Scientific) through the cartridge, followed by passage of 10 mL dH_2O. The dissolved oligonucleotides were transferred to a sterile 3 mL syringe (Becton Dickinson) and injected into the Sep-Pak cartridge. Ten millilitres of dH_2O were put through the cartridge and equal fractions were collected in seven 1.5 mL microfuge tubes. One millilitre of room air was then injected into the cartridge through a syringe to remove remaining dH_2O.

Three millilitres of 20% acetonitrile were passed through the cartridge and equal 1 mL fractions were collected in separate microfuge tubes. The DNA content of each fraction was estimated by measuring the optical density at a wavelength of 260 nm with a Perkin Elmer UV/VIS Lambda 2 Spectrophotometer (Uberlinger, Germany) and Hellma quartz glass cuvettes (Concord, ON). Oligonucleotides were dried by evaporation of 20% acetonitrile on a rotary Speedvac SC 100 with refrigeration unit RT 100 and vacuum pump VP 100 (Savant Instruments Inc., Farmingdale, NY) over 2 hours at 65°C and the pellets were resuspended in autoclaved dH_2O to a final concentration of 20 μM and stored at -70°C.

4.12.4 TOTAL CELLULAR RNA EXTRACTION[19]. Total cellular RNA from guinea pig lung was extracted by a modification of the method of Chomczynski and Sacchi (244). Three mL of solution D (4M guanidinium isothiocyanate (Sigma); 25 mM sodium citrate, pH 7 (BDH Chemicals); 0.5% sarcosyl (Sigma); 0.1 M 2-mercaptoethanol (Sigma)) was instilled into the guinea pig lung via a plastic tube inserted through a tracheostomy. The lung was excised, frozen in liquid nitrogen for 30 minutes, transferred to an autoclaved 50 mL Oakridge centrifuge tube (Nalge Company, Rochester, NY) filled with 2 mL solution D. The lung was homogenized into solution D by three 15 seconds pulses on a Polytron (Kinematic GMBH, Lucerne, Switzerland) set at full speed. To the homogenized lung was added 0.5 mL 2 M sodium acetate, pH 4.1 (BDH Chemicals), 5 mL water-saturated molecular biology grade phenol (Bethesda Research Laboratories (BRL), Gaithersburg, MD) and 1 mL of a 24:1 mixture of chloroform (BDH Chemicals) and isoamyl alcohol (Sigma). After vigorously agitating the lung homogenate, the Oakridge tube was placed on ice for 15 minutes and underwent centrifugation at 10000 *g* (Beckman Model J 21-C) at 4°C for 20 minutes. Five hundred microlitre aliquots of the upper aqueous phase (containing the RNA fraction) were aspirated with an Eppendorf pipetter using autoclaved aerosol-resistant tips (Continental Laboratory Products, Seattle, WA) and transferred to autoclaved 1.5 mL microfuge tubes (National Scientific Supply Company, Inc., San Rafael, CA). An equal volume of isopropanol (BDH Chemicals) was added to each aliquot and the RNA precipitated over one hour at -20°C in an Amana Model 17 freezer (not frost-free).

A pellet of RNA was obtained by centrifugation at 10000 *g* (MSE MicroCentaur, Johns Scientific, UK) at 4°C for 10 minutes and the supernatant was aspirated with an aerosol resistant pipette tip as described above. The RNA pellet was dissolved in 0.3 mL of solution D

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19 For cultured cells grown in 75 cm^3 Corning flasks, the volumes of all solutions were reduced by 80%. Cell monolayers were washed with sterile PBS. PBS was aspirated out of the flask with an autoclaved cotton-plugged Pasteur pipette, solution D was poured directly onto cell monolayers and the mixture was transferred into an autoclaved Oakridge tube. The Polytron was not used.

and precipitated with 0.3 mL isopropanol at -20°C over one hour. A pellet of RNA was obtained by centrifugation at 10000 *g* at 4°C for 10 minutes; the supernatant was aspirated and the pellet was stored in 250 μL 95% EtOH at -70°C until use for RT-PCR[20].

4.12.5 REVERSE TRANSCRIPTION. The conditions for reverse transcription of total cellular RNA from guinea pig lung homogenates into cDNA were similar to the method of template-specific RT-PCR recently described by Shulinder *et al.* (234). Each RNA sample was prepared by aspiration of 95% EtOH from the pellet, washing briefly in 250 μL 70% EtOH containing 0.1% DEPC and drying at room temperature in a vacuum desiccator (Wheaton Dry Seal, Millville, NJ). The dried RNA pellet was dissolved in 25 μL 0.1% DEPC-dH_2O, heated for 10 minutes at 65°C to remove secondary RNA structure and quick chilled on ice.

The reverse transcriptase reaction occurred at 37°C over 75 minutes in a total volume of 20 μL per sample consisting of 5 μL RNA template, 50 mM KCl (Fisher Scientific), 10 mM Tris, pH 8.3 (BDH Chemicals), 8 mM $MgCl_2$ (BDH Chemicals), 200 μM of pooled ultrapure deoxynucleotide triphosphates (dNTPs) (Pharmacia, Montreal, PQ), 2 μM of the oligonucleotide, 5' GCG ATG TCT AGG TTA GGA AGA G 3' (i.e. the primer for reverse transcription in the direction complementary to *genomic* viral RNA), 40 units of RNasin (Boehringer Mannheim, Montreal, PQ) and 16 units of Moloney murine leukemia virus reverse transcriptase (MoMuLV RT) (Pharmacia). The reverse transcriptase was inactivated by heating

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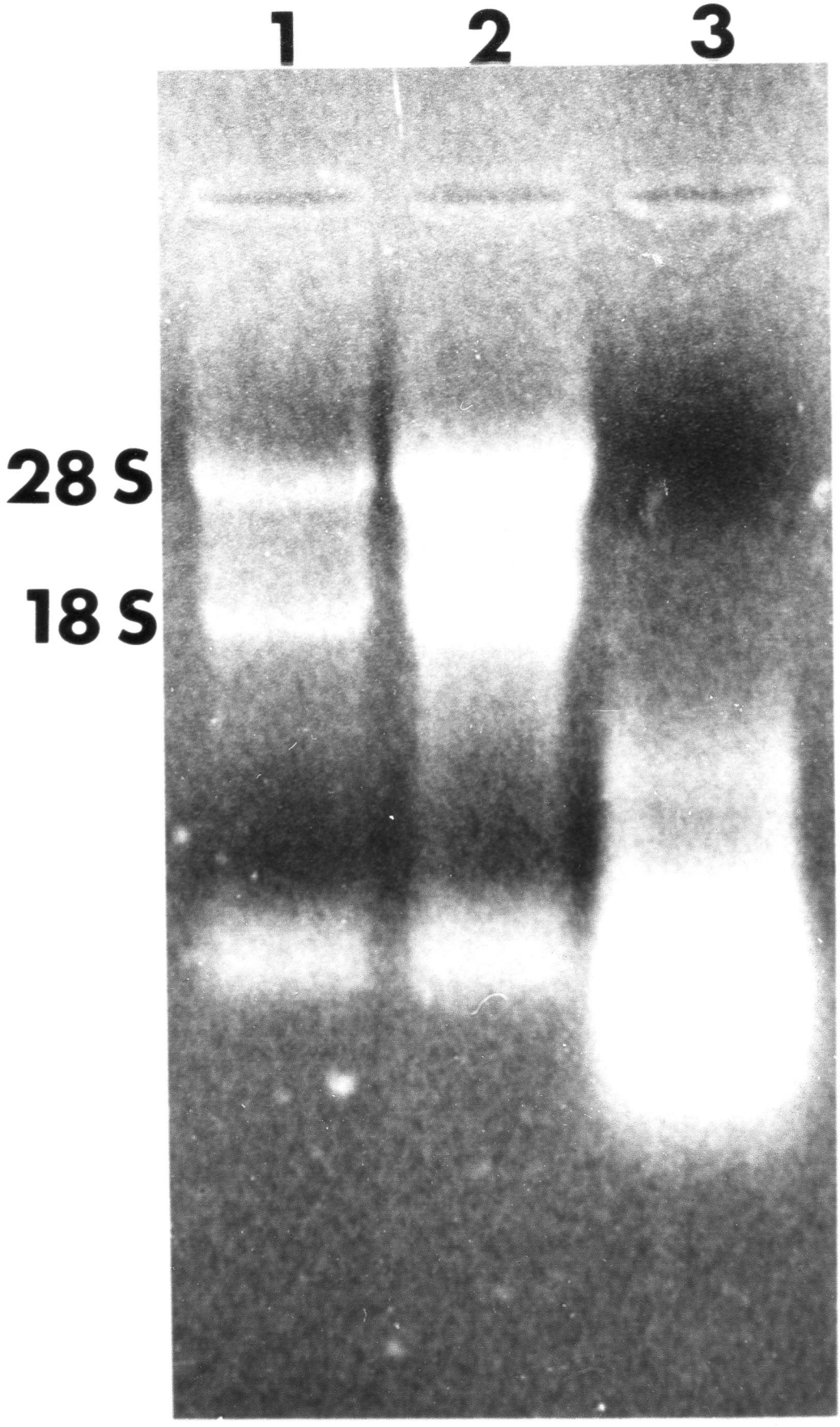
20 One aliquot of each specimen was run on a formaldehyde-containing agarose gel (245) to evaluate the degree of RNA preservation. Only those samples which had well-defined 28S and 18S bands indicative of good RNA preservation (Figure 5) were used for RT-PCR. Formaldehyde gel electrophoresis consisted of preparing a 1% agarose gel in DEPC-dH_2O containing 1X running buffer (5X running buffer is 0.1 M 3-[N-morpholino]-propane sulfonic acid (MOPS), pH 7 (Sigma), 40 mM Na acetate and 5 mM EDTA) and 2.2 M formaldehyde (BDH Chemicals). RNA samples were resuspended in 0.1% DEPC-dH_2O as described in section 4.12.5, of which 4.5 μL was added to 2 μL 5X running buffer, 3.5 μL formaldehyde and 10 μL deionized formamide (Fisher Scientific). After heating each specimen at 65°C for 10 minutes and quick chilling on ice (section 4.12.5), 1 μL of 1 mg/mL ethidium bromide in DEPC-dH_2O and 2 μL formaldehyde gel loading buffer (50% glycerol (BDH Chemicals), 1 mM EDTA, pH 8, 0.25% bromophenol blue (Fisher Scientific), 0.25% xylene cyanol) were added to each tube. In the Fisher Biotech minigel apparatus (section 4.12.7), the gel was immersed in 1X running buffer, pre-run for 5 minutes at 30 volts, and samples were added to the wells. After a 1 hour run at 30 volts, the gel was removed and photographed as described in section 4.12.7.

Figure 5. Agarose-formaldehyde gel electrophoresis of total cellular RNA. The distinct 28S and 18S bands of 30 μg samples of total cellular RNA from the Day 6 RT-PCR study (Lane 1) and the Day 125 study (Lane 2) are indicative of good RNA preservation compared to the markedly denatured RNA from the Day 60 study (Lane 3).


1
2
3
28 S
18 S

the specimen to 99°C in an Isotemp Dry Bath 147 (Fisher Scientific) for 5 minutes. Specimens were kept on ice until PCR amplification.

4.12.6 PCR AMPLIFICATION. Each PCR amplification reaction occurred in a total volume of 50 μL consisting of 5 μL reverse transcriptase cDNA product, 50 mM KCl, 10 mM Tris, pH 8.3, 5 mM $MgCl_2$, 200 μM of pooled ultrapure dNTPs, 5 mM of both flanking primers and, after initially heating the mixture to 100°C for 10 minutes, addition of 2.5 units (0.5 μL) of *Taq* polymerase (GIBCO BRL) to each tube. A GeneAmp PCR System 9600 (Perkin Elmer Cetus) was used for 35 cycles of PCR amplification, each cycle consisting of denaturation (94°C, 1 minute) and annealing/extension (70°C, 2 minutes)[21]. Following completion of the last cycle, tubes were kept at 4°C until required for agarose gel electrophoresis.

4.12.7 AGAROSE GEL ELECTROPHORESIS (246). Eight well, 1.5% agarose (BRL) gels in 1X TBE (10X TBE is 0.9 M Tris, pH 8; 0.9M boric acid (BDH Chemicals); 20 mM EDTA) and 0.01% ethidium bromide (BRL) were cast for electrophoresis using a Fisher Biotech FB 103 minigel apparatus (Fisher Scientific) with 1X TBE as the electrophoresis buffer.

Twenty-five microlitres of each PCR product were added to 5 μL of loading buffer (15% Ficoll 400 (Pharmacia); 0.1 M EDTA, pH 8; 1% SDS (Fisher Scientific); 0.25% xylene cyanol (Eastman Kodak Company, Rochester, NY)). Samples were loaded into designated wells and electrophoresis performed at 100 volts over 45 minutes. Gels illuminated over a Fisher Biotech FBTI 816 UV light source (wavelength: 312 nm) were photographed using a Polaroid MP-3 Land Camera (Polaroid Corporation, Cambridge, MA) (*f* 5.6, shutter speed 0.5 seconds) with Polaroid 667 ISO 3000 Professional Print Film.

4.12.8 SOUTHERN TRANSFER (247). Following photography, gels were immersed in denaturing solution (1.5 M NaCl (BDH Chemicals); 0.5 M NaOH (Fisher Scientific)) for 30

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21 In the first cycle, the denaturation time was 5 minutes and in the last cycle, the annealing/extension time was 10 minutes to ensure completeness of the extension (234).

minutes at room temperature, washed briefly in dH_2O and immersed in neutralizing solution (1.5 M NaCl; 1 mM EDTA; 0.5 M Tris, pH 7.5) for 30 minutes at room temperature.

Each gel was then placed on top of a moist strip of Whatman 3MM Chromatography Paper (Whatman International Ltd., Maldstone, UK) whose ends were immersed in a baking dish containing 20X SSC transfer buffer (20X SSC is 3M NaCl; 0.3 M Na citrate·$2H_2O$, pH 7 (BDH Chemicals)). A Hybond-N membrane (Amersham, Arlington Heights, IL), cut so that its length and width were each 2 mm greater than the gel's, was placed against the upper surface of the gel, covered with a sheet of Whatman 3MM paper (cut to the same size as the Hybond-N) and Saran Wrap was placed around the outer 2 mm edges. Paper towels were placed on top of the Whatman paper and a 500 g weight was placed on top of the paper towels to facilitate capillary transfer of DNA from the gel to the Hybond-N membrane over 18 hours.

Following Southern transfer, DNA was crosslinked to the Hybond-N membrane by exposure to UV light (wavelength: 250 nm) (UVP Inc., San Gabriel, CA) for 4 minutes at room temperature and stored in a sealed plastic bag at -70°C.

4.12.9 PREPARATION OF OLIGONUCLEOTIDE PROBE. The 5' labeling of the third synthetic oligonucleotide (section 4.12.2) with gamma ^{32}P-ATP (Amersham) was accomplished by the polynucleotide kinase reaction followed by DEAE-cellulose chromatography (248). In a 1.5 mL microfuge tube, 1 μL (20 pmoles) of oligonucleotide was mixed with 2 μL 10X kinase buffer (1 M Tris, pH 8; 0.1 M $MgCl_2$; 1 mM EDTA; 1 mM spermidine (Sigma)), 2 μL 0.1 M dithiothreitol (DTT) (Sigma), 5 μL of gamma ^{32}P-ATP (2000 Ci/mmole; 10 μCi/μL) and 10 μL dH_2O. One microlitre (4.5 units) of T4 polynucleotide kinase (Pharmacia) was added to the tube, the contents briefly spun on a benchtop centrifuge and incubated in a Magni-Whirl water bath (Blue M Electric Company) at 37°C for 45 minutes. The polynucleotide kinase reaction was stopped by heating the tube in a New Brunswick Scientific Model G76 Gyrotory Waterbath (Edison, NJ) at 65°C for 10 minutes. Two hundred microlitres of TE (10 mM Tris, pH 8; 1 mM EDTA, pH 8) and 10 μL of 10 mg/mL *E. coli* tRNA were then added to the tube.

A 0.7 cm x 10 cm Bio-Rad column was packed with 0.5 mL of DEAE-cellulose (Sigma), rinsed with 50 mL of 1 M NaCl in TE and equilibrated with 50 mL of TE. The sample was loaded into the column, rinsed with 1 mL of TE and the effluent was collected. The unincorporated nucleotide was eluted with 1 mL 0.2 M NaCl in TE, repeated four times. The labeled oligonucleotide was eluted into two 0.5 mL fractions with 1 M NaCl in TE. The fraction containing the highest amount of radioactivity (measured on a 5 μL aliquot in a Beckman LS 7500 scintillation counter) was used as a probe in filter hybridization.

4.12.10 FILTER HYBRIDIZATION AND AUTORADIOGRAPHY. In a sealed plastic bag, the Hybond-N membrane was incubated with 40 $\mu L/cm^2$ prehybridization solution consisting of 6X SSC, 5X Denhardt's solution (100X Denhardt's stock solution is 10 g Ficoll 400, 10 g polyvinylpyrrolidone (Sigma) and 10 g BSA Fraction V per 500 mL dH_2O), 0.5% SDS, 0.05 M sodium phosphate buffer, pH 6.8 (Fisher Scientific) and 20 μg/mL *E. coli* tRNA (Boehringer Mannheim) in a gyrotory water bath at 65°C for 2 hours. Following prehybridization, the radiolabeled probe was added to the bag for an 18 hour hybridization at 65°C.

Posthybridization washes included three 5 minute washes in 6X SSC at room temperature and, to increase stringency, a 60 minute wash in 6X SSC at 65°C. For autoradiography, the membrane was taken to a darkroom, covered with Saran Wrap, apposed to a sheet of Kodak Ektascan IR Diagnostic Xray Film and kept inside a Kodak X-OMatic Film Cassette with regular intensifying screens. Xray films were exposed from 4 hours (at room temperature) to 72 hours (at -70°C) and developed in a Kodak RP X-OMat Processor.

4.13 PHOTOGRAPHY. Light microscopic photomicrographs (H&E, PAS and immunoperoxidase slides) were shot on an Olympus AH-2 photomicroscope using 100 ASA Kodak Tmax film. This film was also used for photography of Polaroid prints from ethidium bromide-stained agarose gels and autoradiographs. Negatives were developed in Kodak Tmax developer, printed by an Agfa-Gevaert Rapidoprint DD3700 printer (Germany) onto Ilford multigrade paper (Ilford, Cheshire, UK). Electron micrographs were shot on a Philips 400

transmission electron microscope using Kodak 4489 3 1/4" x 4" EM plate film. Negatives were developed in Kodak D19 developer and printed as described above.

4.14 STATISTICAL ANALYSES. Statistical analyses were performed on clinical, gross pathological and histological data (Specific Aim 1) from the day 6, 14 and 60 studies using SYSTAT® Version 5.1 software (Systat, Inc., Evanston, IL). No statistical analysis was performed for the day 125 study because there was only one control animal.

For the purposes of documenting the natural history of intrapulmonary RSV (Specific Aim 2), viral cultures, TEM, immunohistochemistry and RT-PCR were interpreted as either positive (unequivocal signal observed) or negative (no signal observed). An animal was considered to have evidence of RSV lung infection whenever a positive signal was observed by *any* of the four methods, as opposed to reliance on a "gold standard" such as viral culture. This approach was used for three reasons: (a) the possibility of persistent, non-lytic infection producing "false negative" viral cultures on HEp-2 cells (sections 2.5.2 and 3.3); (b) the use of known positive and negative control specimens *for each method* (permitting concomitant evaluation of the method's sensitivity and specificity during each experiment); (c) the protocols were designed to be highly *specific*: the inevitably lowered sensitivities made quantitative or semi-quantitative analyses inappropriate.

4.14.1 CLINICAL AND GROSS LUNG EXAMINATION. The Student's t-test was used to compare mean body weights, lung wet weights and lung to body weight ratios (all continuous variables) between RSV-inoculated and control groups. A p value ≤ 0.05 was considered to be statistically significant.

4.14.2 RSV BRONCHIOLITIS HISTOLOGICAL SCORING SYSTEM. The intraobserver variation and interobserver variation of the RSV bronchiolitis histological scoring system were evaluated by calculating the Pearson coefficient of mean-square contingency (R_2) for each histological feature and expressing R_2 as a fraction of the maximum possible value, R_{2max} (249). The Pearson coefficient of mean-square contingency is an extension of the Pearson chi-square test (the null hypothesis being that the rows and columns of a matrix are independent).

The histological scores from two analyses of the same airways were expressed as a 3 x 3 matrix, with 100% agreement being found along the diagonal (a score of "0" from run 1 also being scored "0" on run 2, etc.). However, in contrast to the null hypothesis of the Pearson chi-square test, the histological scoring system was intended to be highly *reproducible* (i.e., scores ideally being identical between and within observers); therefore, the rows and columns were expected to show marked *dependence*. The Pearson coefficient of mean-square contingency was used to evaluate dependence between rows and columns in the matrix by the calculation of R_2:

$$R_2 = \sqrt{\frac{T}{N+T}},$$

where T = Pearson chi-square statistic and N = number of observations. The interpretation of R_2 is facilitated when R_2 is expressed as a proportion of the maximum possible value, R_{2max}:

$$R_{2\,max} = \sqrt{\frac{q-1}{q}},$$

where q = the number of rows (or columns) in a square matrix. Concerning the histological scoring system, R_{2max} would represent the value of the Pearson chi-square coefficient if there is 100% agreement in the airway scores: for a 3 x 3 matrix, $R_{2max} = \sqrt{2/3} \approx 0.82$.

The intraobserver variation was assessed by rescoring 50 airways from randomly selected slides three months after the initial scoring and the interobserver variation was assessed by two pathologists (Dr. Sergio Gonzalez Bombardier (SGB), Catholic University, Santiago, Chile and the author (RGH)) independently scoring 30 airways on an Olympus BH-2 microscope with a teaching head attachment. For analysis of both intra- and interobserver variation, an R_2/R_{2max} ratio ≥ 0.75 was considered to represent acceptable reproducibility.

4.14.3 LIGHT MICROSCOPIC EVALUATION. For each study, the nonparametric Mann Whitney *U* test (250) was used to compare observed scores (ordinal variables) between RSV-inoculated and control groups for each histological feature. In addition, this test was used to

compare histological scores from 9 control animals at day 6 (given uninfected cell culture supernatant) to 6 "historical" (unmanipulated) controls from the preliminary studies described in section 4.1. The rationale for using the Mann Whitney *U* test was twofold: (a) this test does not require particular assumptions about the guinea pig populations' airway scores (in comparison, the underlying assumption of the Student t-test would be that the airway scores of the guinea pig populations are normally distributed with equal variances); (b) this test permits comparison between *categories* (as represented by the standard photomicrographs). The concomitant scoring of six histological features represented a scenario of multiple comparisons; to account for multiple comparisons, the sequential rejective Bonferroni procedure (251) was used to determine statistical significance at sequential *p* values: ≤ 0.0083 (0.05/6); then ≤ 0.01 (0.05/5) etc. until all six histological features had been analyzed. A sequential rejective Bonferroni procedure was used in preference to unmodified Bonferroni analysis (i.e., setting a significant *p* value at ≤ 0.0083 for *all* histological features) because the latter method is an overly conservative analysis with low statistical power (252).

4.15 GENERAL TECHNICAL NOTE. Distilled H_2O was prepared through a Milli-Q Filtering Apparatus (MilliPore Corporation, Bedford, MA) and autoclaved in an Amsco Vacamatic S (Amsco, Erie, PA) for 20 minutes at 121°C under 20 lb/in^2 steam pressure. For RNA work, DEPC was added to dH_2O at a concentration of 0.1% (by volume), vigorously shaken into solution and autoclaved as above. Solutions containing Tris buffer were prepared using a previously unopened container of fresh crystals added to dH_2O that did not contain DEPC (253). Pyrex® glassware (Corning) and metal utensils were either autoclaved for 30 minutes at 132°C under 30 lb/in^2 steam pressure or, for RNA work, baked overnight at 200°C in a Single-Wall Transite Oven (Blue M Electrical Company, Blue Island, IL). Reagents were weighed on Mettler Model AE-50 or PC 4400 balances (Mettler Instruments AG, Zurich, SW) and pH adjustments of solutions were made using a Beckman Model 4500 digital pH meter (Beckman Corporation, Irvine, CA). Latex gloves (Smith & Nephew Perry, Massillon, OH) were worn

for all experiments involving virus and animal handling (sections 4.1-4.7), viral culture (section 4.9) and RT-PCR (section 4.12).

CHAPTER 5: RESULTS[22]

5.1 PRELIMINARY EXPERIMENTS. Inoculation of approximately 4000 pfu of Long strain human RSV into guinea pigs did not produce clinical evidence of acute respiratory disease and no animal died during the course of the study. Histological evaluation of lung parenchyma from the RSV-inoculated group showed a patchy inflammatory process, centered around bronchioles from all lung lobes, evident by day 4 post-inoculation and maximal on day 6. Respiratory epithelial necrosis, mononuclear cell peribronchiolar infiltrates, PMN bronchiolar wall infiltrates and airway wall edema were present (Figure 6) and the extent of inflammation was similar between intranasally inoculated animals and intratracheally inoculated animals. Neither alveolitis nor bronchopneumonia was observed in RSV-inoculated animals. By day 14 the inflammatory process had substantially resolved.

Some bronchioles in control animals had one or more of the histopathological features of acute bronchiolitis. The patchy nature of bronchiolar inflammation in the RSV-inoculated group and the presence of inflammatory background "noise" in the control group necessitated the development of the semi-quantitative, pictorially-based histological scoring system (section 4.8) to discriminate between bronchiolar inflammation attributable to RSV inoculation and non-specific inflammatory background. Figure 7 shows the results and statistical analysis for intraobserver variation and Figure 8 shows the results and statistical analysis for interobserver variation. There was substantial agreement in airway scoring within and between observers for all six histological features examined: in particular, there were no instances of any airway being scored "0" on one occasion and "2" on another. The acceptably low intra- and interobserver variation of this relatively simple scoring system were encouraging for airway scoring in the larger studies of the final protocol.

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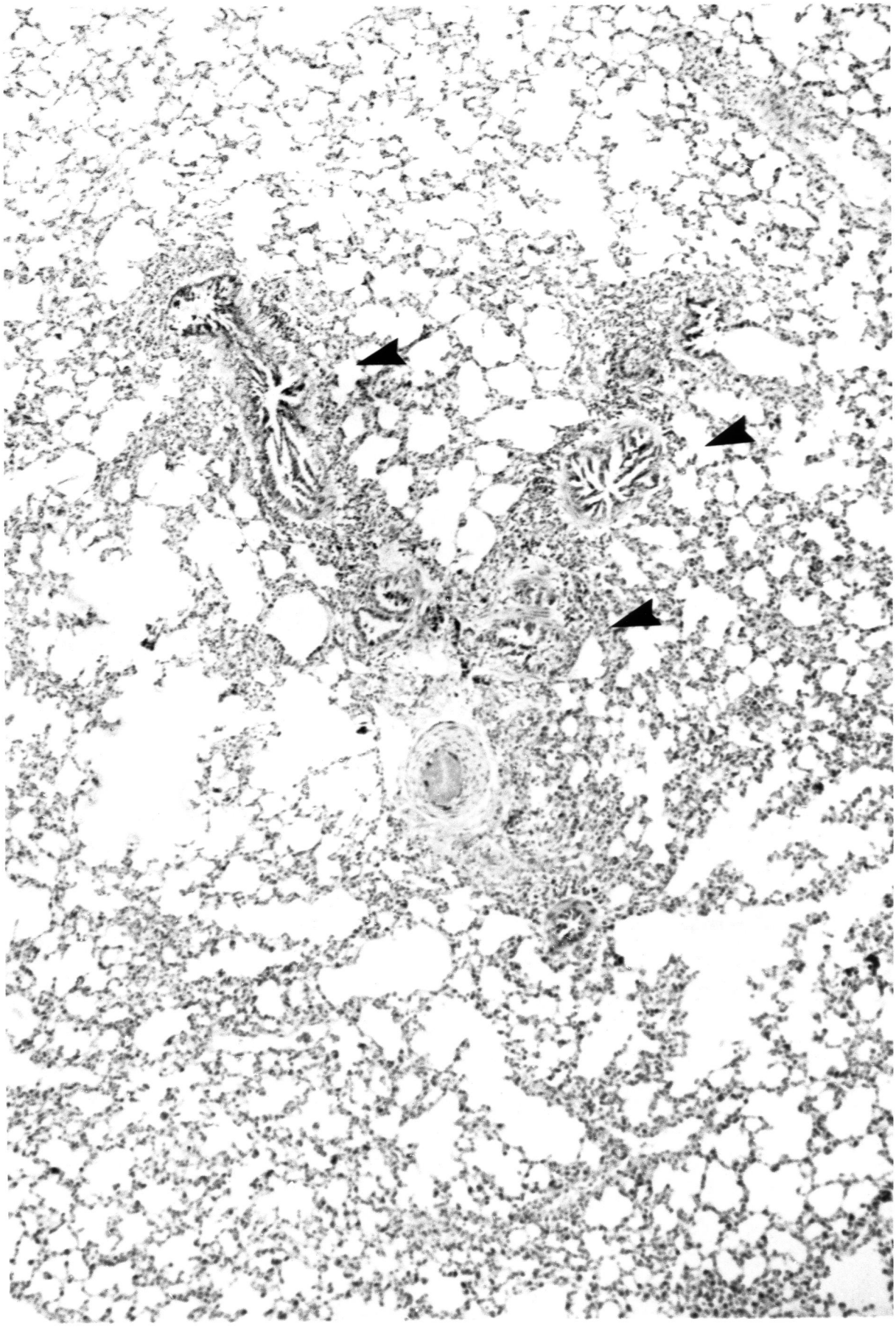
22 Appendix A shows all data collected for each guinea pig studied in this thesis. Appendix B shows the statistical analyses for airway histological scores.

Figure 6. Low power photomicrograph of guinea pig lung, day 6 following intranasal RSV-inoculation (preliminary experiment). There is marked peri-bronchiolar inflammatory cell infiltration and edema (arrowheads) with relative sparing of the alveoli. The bronchiole at top left has epithelial cell necrosis with sloughing into the airway lumen. (H&E stain, final magnification: x52).



EPITHELIAL NECROSIS

run 2 \ run 1	**0**	**1**	**2**
0	24	2	0
1	3	14	0
2	0	1	6

R_2: 0.76

R_2/R_{2max}: 0.93

MONONUCLEAR INFILTRATES

run 2 \ run 1	**0**	**1**	**2**
0	35	2	0
1	1	10	0
2	0	1	1

R_2: 0.73

R_2/R_{2max}: 0.90

PMN INFILTRATES

run 2 \ run 1	**0**	**1**	**2**
0	21	2	0
1	2	21	3
2	0	1	3

R_2: 0.76

R_2/R_{2max}: 0.94

AIRWAY WALL EDEMA

run 2 \ run 1	**0**	**1**	**2**
0	13	3	0
1	2	26	0
2	0	0	6

R_2: 0.78

R_2/R_{2max}: 0.96

BALT HYPERPLASIA

run 2 \ run 1	**0**	**1**	**2**
0	40	0	0
1	1	6	0
2	0	1	2

R_2: 0.77

R_2/R_{2max}: 0.94

GOBLET CELL METAPLASIA

run 2 \ run 1	**0**	**1**	**2**
0	20	1	0
1	0	12	2
2	0	0	15

R_2: 0.79

R_2/R_{2max}: 0.97

Figure 7: Intraobserver variation of the RSV bronchiolitis histological scoring system: n = 50 airways; run 1 = airway score on first run; run 2 = airway score on repeat evaluation. Boldface numbers indicate the score (**0**, **1** or **2**). Each box within the 3 x 3 matrix contains the number of observations (plain type) corresponding to each score from a given run. The Pearson chi-square coefficient of mean square contingency (R_2) is expressed on its own and as a ratio of the maximum possible value (R_2/R_{2max}) as described in section 4.14.2.

EPITHELIAL NECROSIS

obs 2 \ obs 1	**0**	**1**	**2**
0	11	2	0
1	1	14	0
2	0	1	1

R_2: 0.78

R_2/R_{2max}: 0.89

MONONUCLEAR INFILTRATES

obs 2 \ obs 1	**0**	**1**	**2**
0	16	0	0
1	1	12	0
2	0	0	1

R_2: 0.81

R_2/R_{2max}: 0.99

PMN INFILTRATES

obs 2 \ obs 1	**0**	**1**	**2**
0	23	1	0
1	1	5	0
2	0	0	0

R_2: 0.62

R_2/R_{2max}: 0.76

AIRWAY WALL EDEMA

obs 2 \ obs 1	**0**	**1**	**2**
0	16	3	0
1	0	11	0
2	0	0	0

R_2: 0.63

R_2/R_{2max}:0.77

BALT HYPERPLASIA

obs 2 \ obs 1	**0**	**1**	**2**
0	28	1	0
1	1	0	0
2	0	0	1

R_2: 0.71

R_2/R_{2max}: 0.87

GOBLET CELL METAPLASIA

obs 2 \ obs 1	**0**	**1**	**2**
0	12	1	0
1	2	11	0
2	0	0	4

R_2: 0.78

R_2/R_{2max}: 0.96

Figure 8: Interobserver variation of the RSV bronchiolitis histological scoring system: n = 30 airways; obs 1 = score given by RGH; obs 2 = score given by SGB. Boldface numbers indicate the score (**0**, **1** or **2**). Each box within the 3 x 3 matrix contains the number of observations (plain type) corresponding to the scores given by the two observers. The Pearson chi-square coefficient of mean square contingency (R_2) is expressed on its own and as a ratio of the maximum possible value (R_2/R_{2max}) as described in section 4.14.2.

5.2 SPECIFIC AIM 1: CLINICAL-PATHOLOGICAL EVALUATION.

5.2.1 CLINICAL AND GROSS LUNG EXAMINATION. No RSV-inoculated guinea pig developed clinical evidence of coryza, cough, tachypnea, ruffled fur or decreased activity. Table 4 summarizes the results of body weights, lung wet weights and lung to body weight ratios which revealed no statistically significant differences between RSV-inoculated animals and uninfected controls. In summary, RSV-inoculation into guinea pigs did not produce clinical or gross pathological evidence of pulmonary disease.

5.2.2 LUNG HISTOPATHOLOGICAL EVALUATION. Figure 9 shows the results of scoring using the histological bronchiolitis scoring system. On day 6 post-inoculation, juvenile guinea pigs inoculated with low dose (~4000 pfu) RSV had statistically significantly greater respiratory epithelial necrosis ($p \leq 0.002$), bronchiolar PMN infiltrates ($p \leq 0.008$) and mononuclear cell infiltrates ($p \leq 0.01$) compared to controls (Figure 9a). One control animal was omitted from the analysis due to severe aspiration pneumonia. For juvenile guinea pigs inoculated with high dose (~28000 pfu) RSV (Figure 9b), there was also statistically significant epithelial necrosis, mononuclear infiltrates and PMN infiltrates compared to uninfected controls but none of these features was significantly different from the low dose RSV group[23]. In contrast to juvenile guinea pigs on day 6 post-inoculation, adolescent guinea pigs inoculated with RSV had no statistically significant differences in airway histological scores compared to controls (Figure 9c). In the day 6 RT-PCR study (Figure 9d), there was a trend for the RSV-inoculated group to have higher scores for epithelial necrosis, mononuclear infiltrates, PMN infiltrates and goblet cell metaplasia but the small number of animals in each group did not reveal any statistically significant differences.

At days 14 (Figure 9e) and 60 (Figure 9f) there were no statistically significant differences in histological scores between the RSV-inoculated group and the control group.

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[23] Histological scores between the control animals of these groups were also similar (Appendix B).

TABLE 4. GUINEA PIG BODY WEIGHTS, LUNG WET WEIGHTS AND LUNG TO BODY WEIGHT RATIOS

STUDY (EXPT #)	GROUP	n	INITIAL BODY WEIGHT* (g) (mean ± SD)	FINAL BODY WEIGHT (g) (mean ± SD)	LUNG WET WEIGHT (g) (mean ± SD)	LUNG TO BODY WEIGHT RATIO (%) (mean ± SD)
DAY 6 (1755)	RSV	10	-	395 ± 26	1.61 ± 0.24	0.38 ± 0.04
	CONTROL	10	-	412 ± 26	1.62 ± 0.29	0.41 ± 0.05
	p value**			NS	NS	NS
DAY 6 (1791)	RSV	10	303 ± 16	335 ± 18	1.23 ± 0.15	0.37 ± 0.05
	CONTROL	10	308 ± 19	359 ± 18	1.20 ± 0.11	0.36 ± 0.04
	p value		NS	NS	NS	NS
DAY 6 (1830)	RSV	10	-	519 ± 15	2.32 ± 0.13	0.44 ± 0.05
	CONTROL	10	-	515 ± 18	2.38 ± 0.21	0.46 ± 0.03
	p value			NS	NS	NS
DAY 6 (1979) (RT-PCR)	RSV	4	292 ± 5	326 ± 7	1.16 ± 0.04	0.36 ± 0.01
	CONTROL	2	318 ± 13	355 ± 34	1.25 ± 0.21	0.36 ± 0.02
	p value		NS	NS	NS	NS
DAY 14 (1779)	RSV	10	-	469 ± 24	1.70 ± 0.32	0.38 ± 0.08
	CONTROL	9	-	463 ± 41	1.90 ± 0.32	0.39 ± 0.11
	p value			NS	NS	NS
DAY 60 (1889)	RSV	10	364 ± 19	650 ± 53	3.12 ± 1.37	0.48 ± 0.22
	CONTROL	11	360 ± 20	661 ± 63	2.89 ± 1.34	0.43 ± 0.18
	p value		NS	NS	NS	NS
DAY 125 (1950)	RSV	4	388 ± 24	826 ± 48	3.07 ± 0.61	0.37 ± 0.07
	CONTROL	1	340	722	3.26	0.45

* -: not done

** NS: *p* value > 0.05 by Student t-test

Figure 9. Scores from the RSV bronchiolitis histological scoring system. Each point represents the sum of individual scores for 10 airways in a single animal. Legend for all panels: EPITH NECROSIS = respiratory epithelial necrosis; MONO = mononuclear cell infiltrates; PMN = polymorphonuclear cell infiltrates; EDEMA = airway wall edema; BALT = BALT hyperplasia; GOBLET = goblet cell metaplasia. Detailed statistical analyses appear in Appendix B.

Panel **a**: Day 6 study: juvenile guinea pigs, low dose RSV.

Panel **b**: Day 6 study: juvenile guinea pigs, high dose RSV.

Panel **c**: Day 6 study, adolescent guinea pigs, low dose RSV.

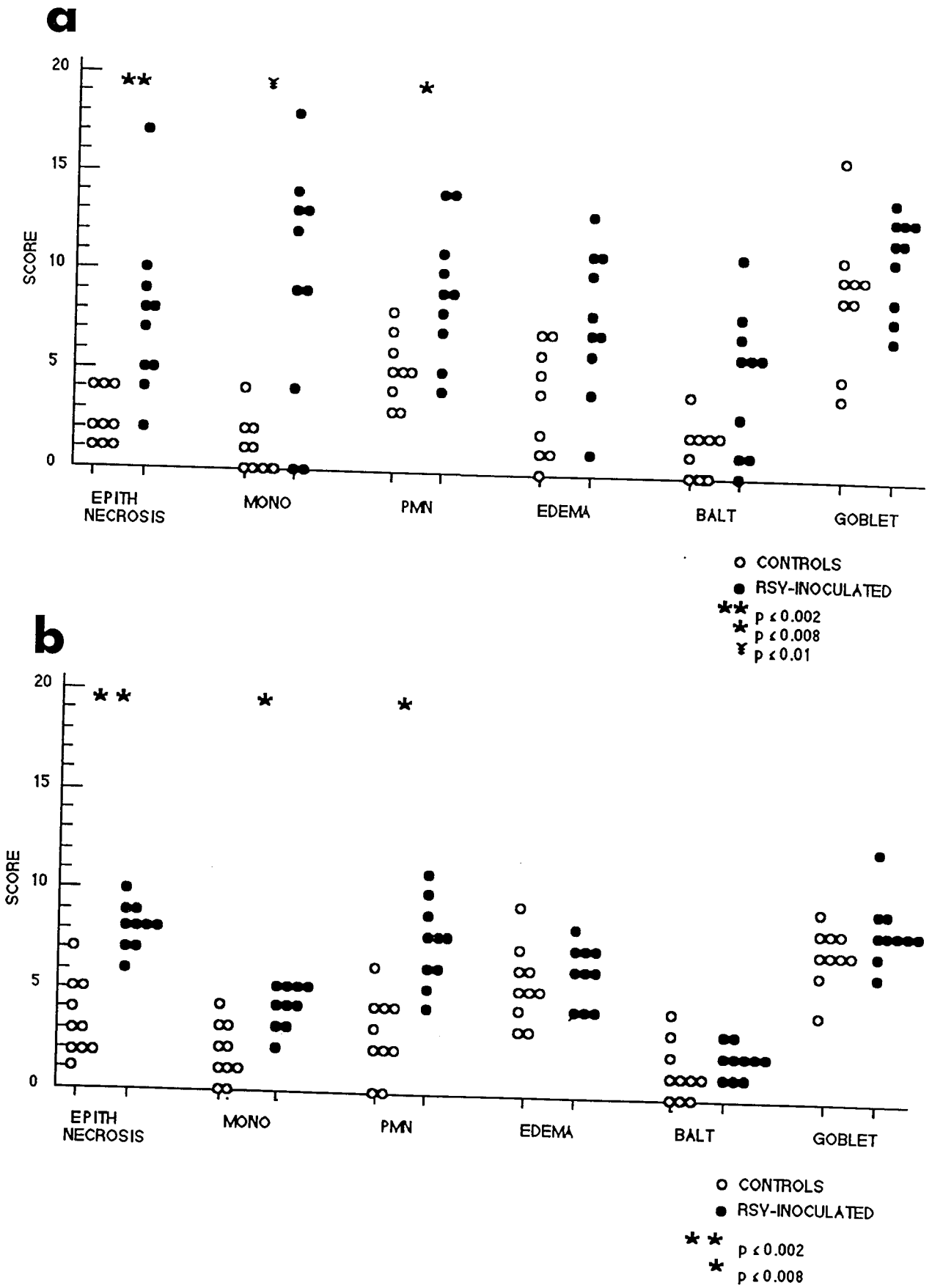
Panel **d**: Day 6 RT-PCR study, juvenile guinea pigs, low dose RSV.

Panel **e**: Day 14 study, juvenile guinea pigs, low dose RSV.

Panel **f**: Day 60 study, juvenile guinea pigs, low dose RSV.

Panel **g**: Day 125 study, juvenile guinea pigs, low dose RSV.

Panel **h**: Day 6 control guinea pigs (same controls as panel **a**) compared to unmanipulated "historical" controls of the preliminary study.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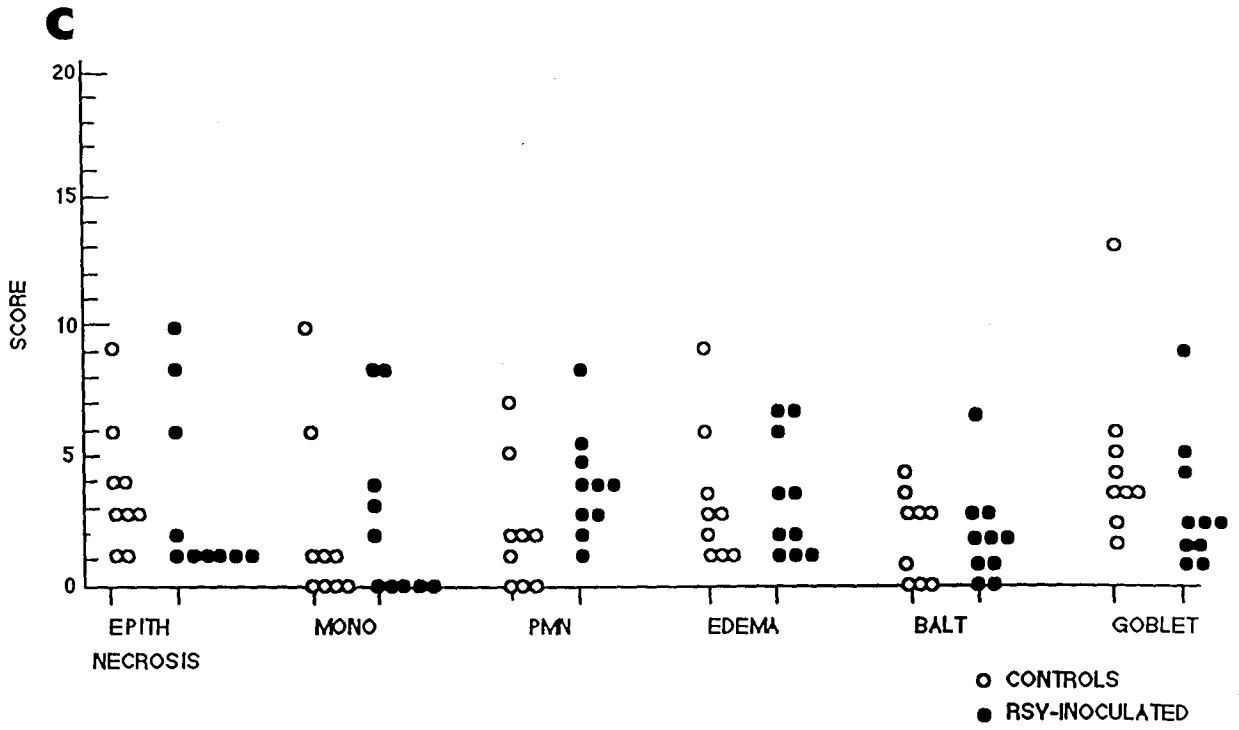
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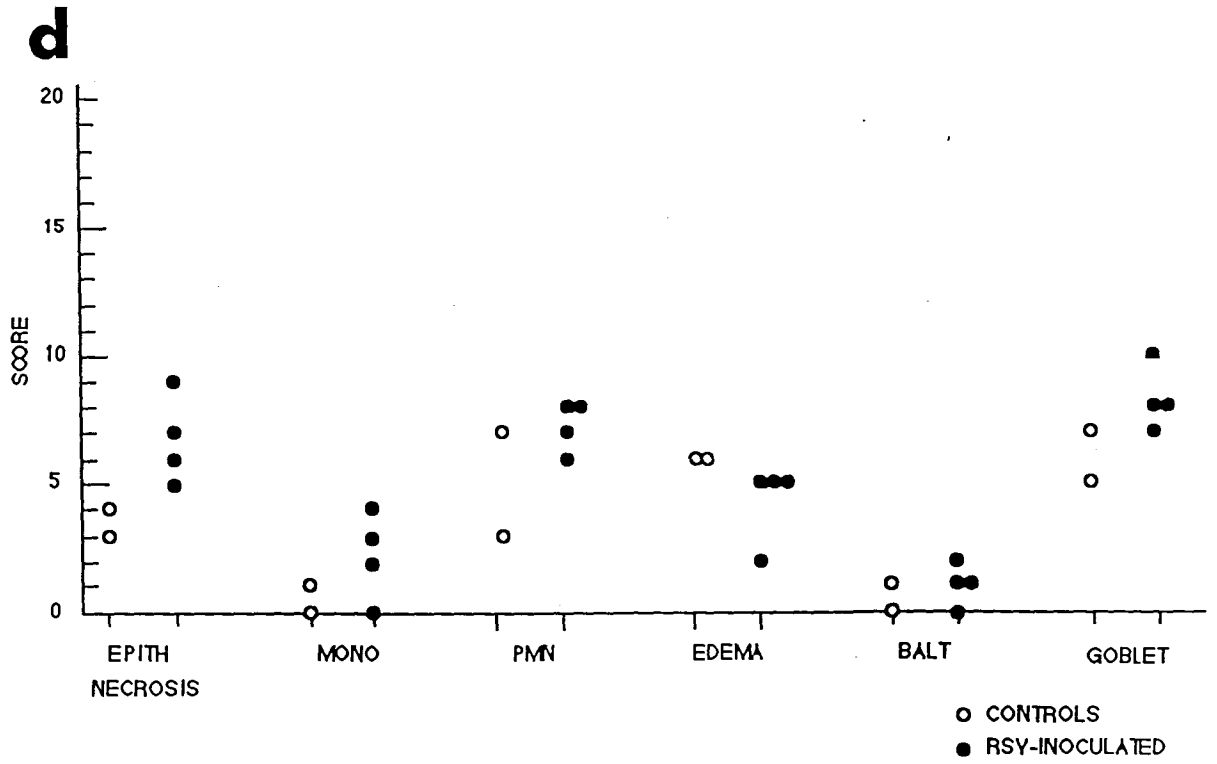
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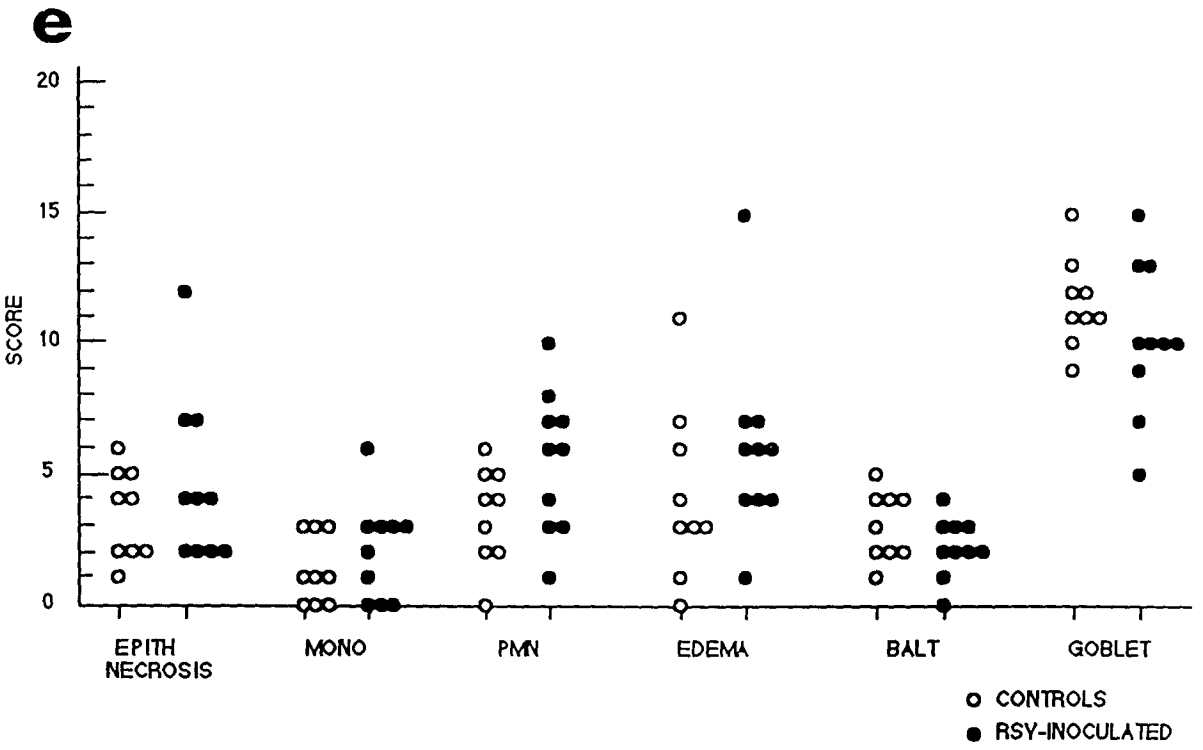
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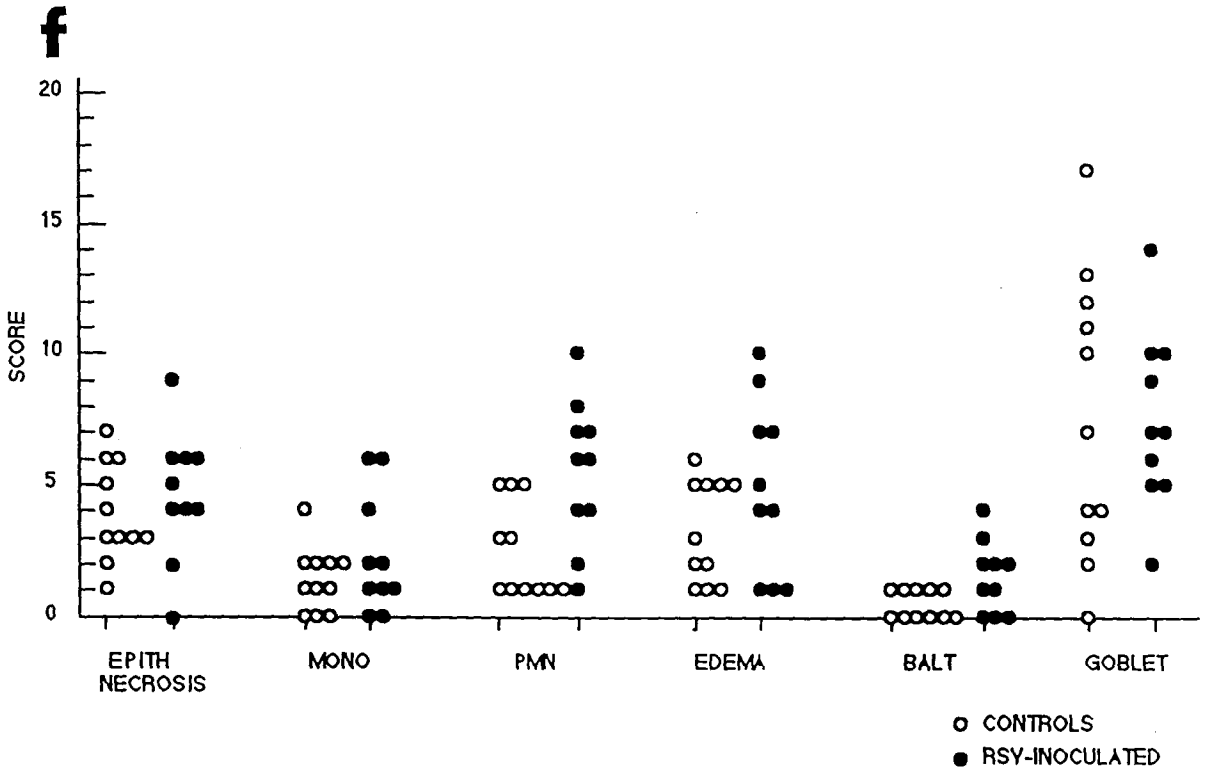
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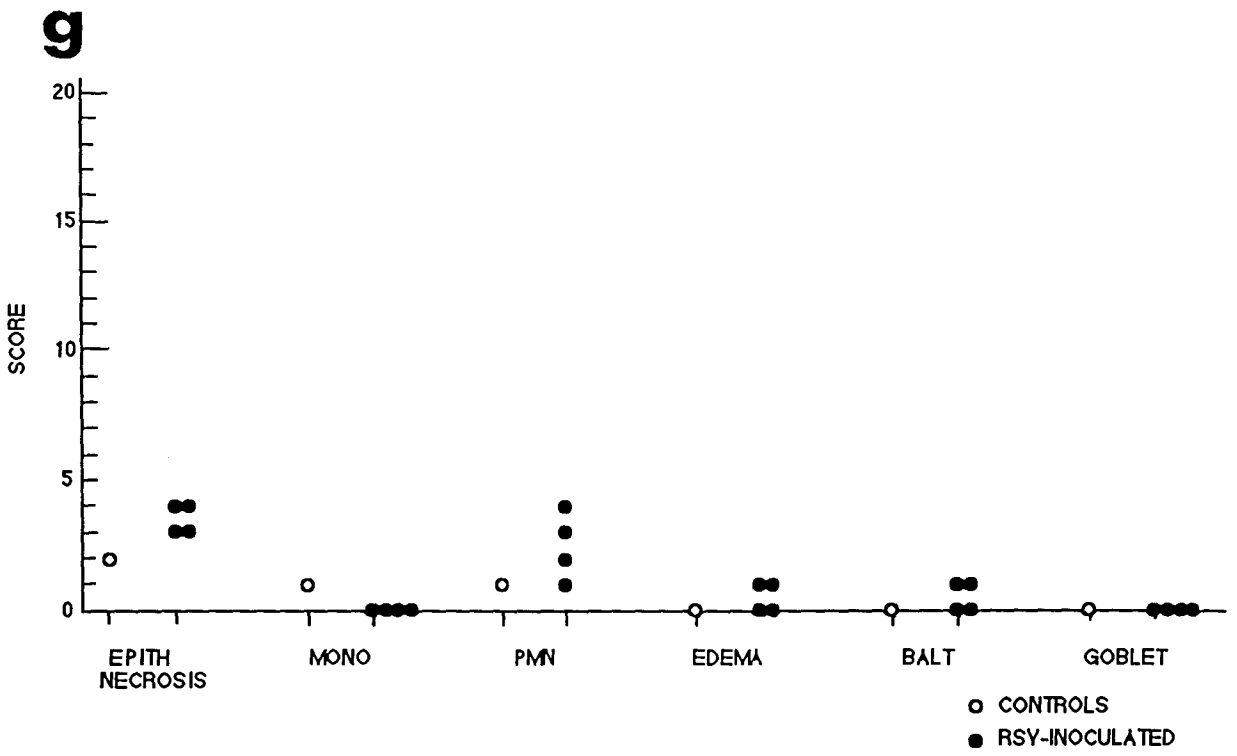
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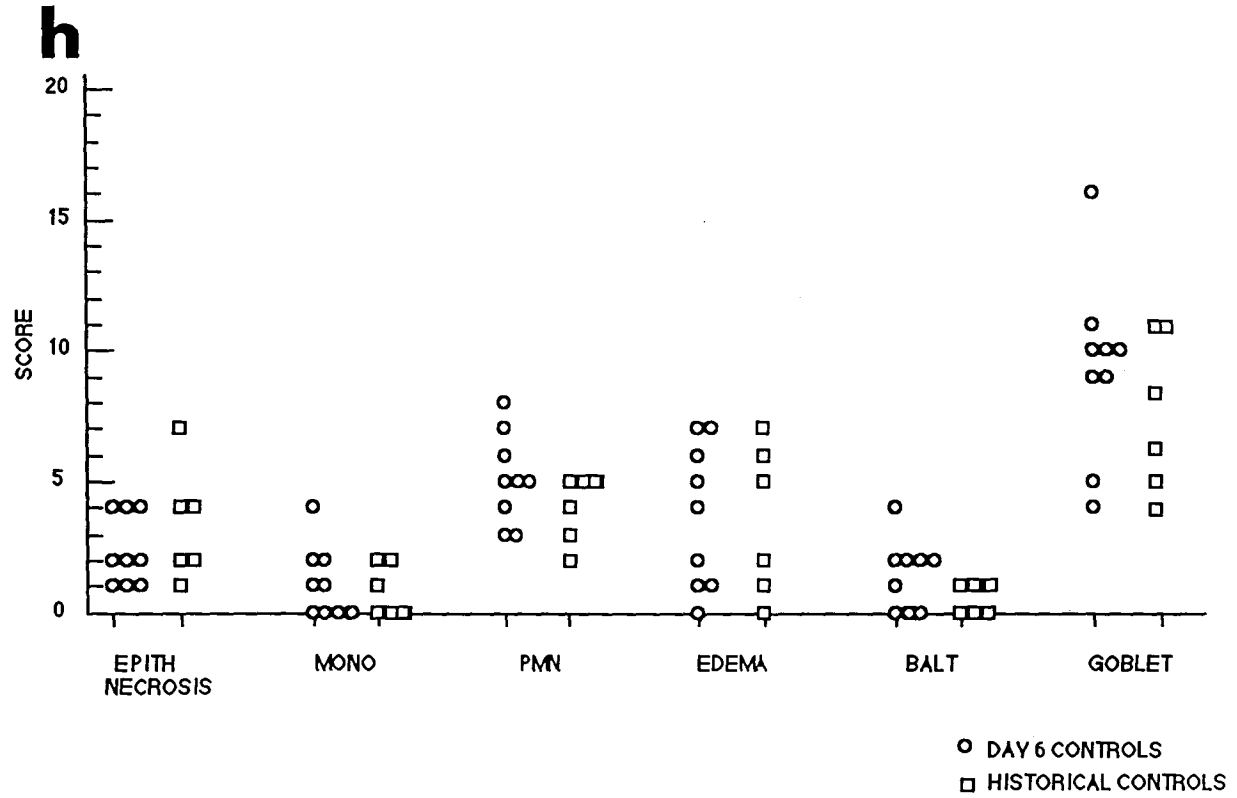
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DAY 6 CONTROLS
HISTORICAL CONTROLS

On day 125, the control animal had a comparable score to the RSV group for each histological feature (Figure 9g). Finally, there were no statistically significant differences for any of the six histological features between the control groups given uninfected cell culture supernatant and the "historical" (unmanipulated) controls from the preliminary study (Figure 9h).

In summary, juvenile guinea pigs inoculated with as few as 4000 pfu of RSV developed histological features of human acute RSV bronchiolitis on day 6 which resolved by day 14. Secondly, on day 6 post-inoculation, no significant bronchiolar inflammation developed in adolescent guinea pigs given a similar amount of RSV. Thirdly, the intranasal delivery of uninfected cell culture supernatant did not elicit significant bronchiolar inflammation in control guinea pigs compared to "historical", unmanipulated controls.

5.3 SPECIFIC AIM 2: NATURAL HISTORY OF INTRAPULMONARY RSV. Table 5 shows the results of studies performed on juvenile guinea pigs given low dose RSV. These results will be presented in further detail below.

5.3.1 VIRAL CULTURE. Whether inoculated with low dose or high dose RSV, 9/10 juvenile guinea pigs on day 6 showed evidence of viral infection by the development of the characteristic RSV cytopathic effect (CPE) in HEp-2 cells (Figure 10). RSV CPE was observed in 2/10 RSV-inoculated juvenile guinea pigs on day 14 but not on days 60 or 125. No HEp-2 cell culture showed CPE characteristic of other viruses. In the day 6 study of adolescent guinea pigs, the cell culture medium was contaminated with yeast which killed the HEp-2 cell monolayers; thus, viral cultures in this group were not completed. There were no sporadic bacterial or fungal infections in HEp-2 cell cultures containing digested guinea pig lung (note that the cell culture medium for the day 60, day 125 and day 6 RT-PCR studies did not contain antibiotics or amphotericin B). In the day 6 RT-PCR study, viral plaque assay revealed a mean ± SD of $1.7 \pm 0.3 \times 10^3$ pfu RSV/g wet weight fresh lung.

TABLE 5: DOCUMENTATION OF INTRAPULMONARY RSV IN VIRUS-INOCULATED GUINEA PIGS *

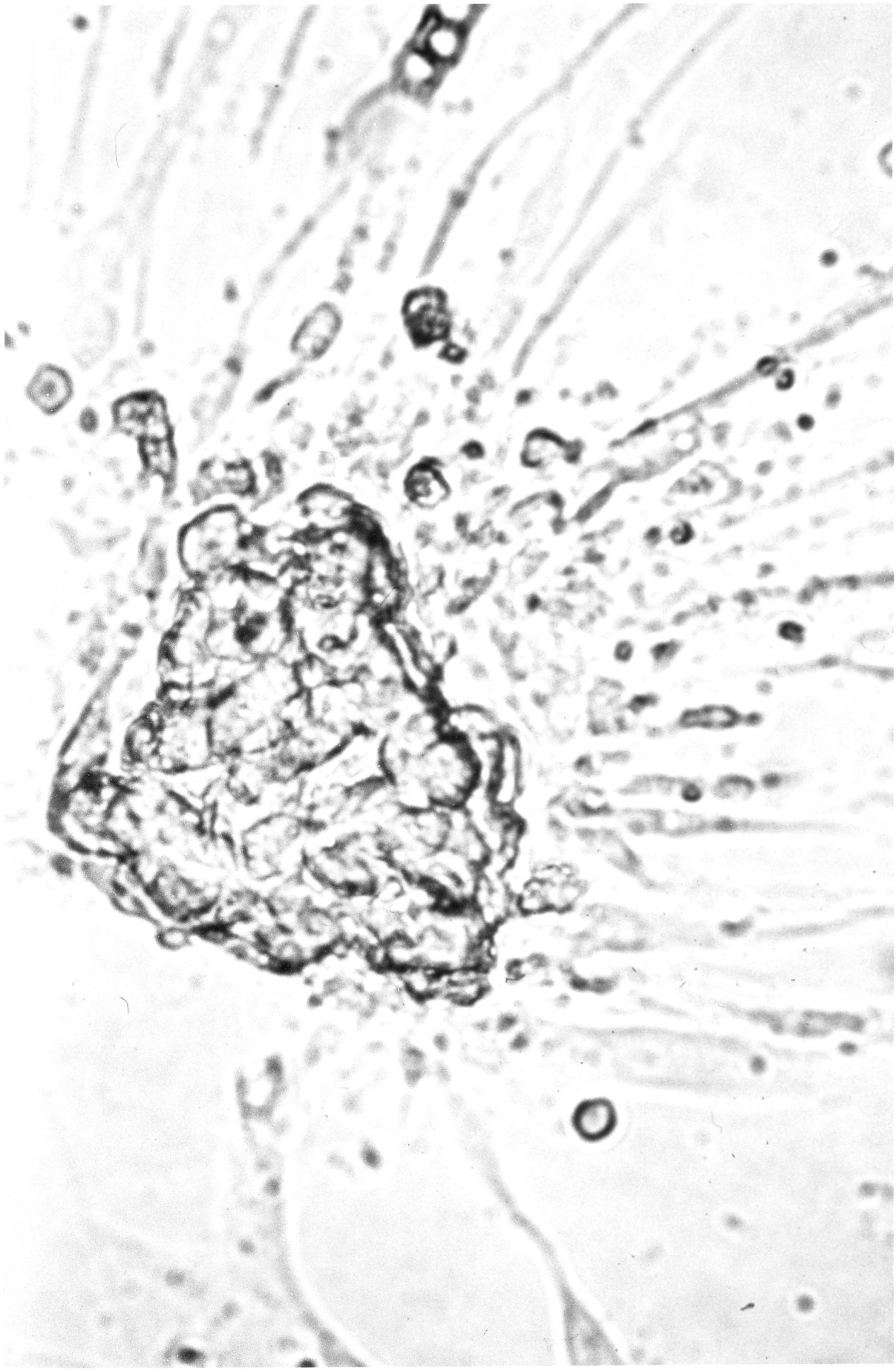
STUDY (EXPT #)	CULTURE	TEM	IMMUNOHISTO-CHEMISTRY	RT-PCR
DAY 6 (1755)	9/10	2/10	7/10	-
DAY 6 (1791)	9/10	-	8/10	-
DAY 6 (1830)	**	-	6/10	-
DAY 6 (RT-PCR) (1979)	4/4 $1.7\pm O.3 \times 10^3$ pfu/g	1/4	2/4	4/4
DAY 14 (1779)	2/10	0/2	6/10	-
DAY 60 (1889)	0/10	-	1/10	-
DAY 125 (1950)	0/4	-	0/4	3/4

* Results are presented for RSV-inoculated guinea pigs only because no control animals studied had a positive result by any method.

** Yeast contamination of culture medium precluded analysis for RSV CPE in this group.

-: not done.

Figure 10. HEp-2 cell culture five days after addition of digested lung from an RSV-inoculated guinea pig (day 6 study). Note the syncytial formation characteristic of RSV CPE (inverted microscope, final magnification: x800).



5.3.2 TRANSMISSION ELECTRON MICROSCOPY. In ultrathin sections of RSV-inoculated guinea pig lung, only rare bronchial epithelial cells contained structures consistent with completely assembled RSV (Figure 11a) (191), incompletely assembled "virus-related particles" (Figures 11b and 11c) (220) and viral nucleocapsids (Figure 11d) (216). The virus-related particles did not have the ultrastructural features of cilia, microvilli or phagocytic vacuoles (254). The viral nucleocapsids were discernible from intermediate filaments (10 nm diameter), microtubules (25 nm diameter) associated with cilia, actin microfilaments (6 nm diameter) associated with microvilli, rough endoplasmic reticulum, immunoglobulin aggregates, lamellar bodies of type II pneumocytes or granules from Clara cells, neuroendocrine cells, neutrophils, eosinophils or mast cells (254).

No virus-related particles were observed in bronchiolar epithelial cells, type I or II pneumocytes, Clara cells, neuroendocrine cells or alveolar macrophages. No virus-related particles or nucleocapsids were identified in two RSV-inoculated animals in the day 14 study and consequently TEM was not performed for either the day 60 or day 125 studies.

5.3.3 IMMUNOHISTOCHEMISTRY. For juvenile guinea pigs on day 6, the sensitivity of immunohistochemistry to detect RSV antigens in lung sections ranged from 50 to 80 per cent (Table 5). Most positive staining was observed within the cytoplasm of bronchiolar and bronchial epithelial cells (Figure 12), with substantially fewer type II pneumocytes and alveolar macrophages staining positively. A similar cellular distribution of staining was observed in 6/10 adolescent guinea pigs on day 6. However, on day 14, most staining was observed within alveolar macrophages, with only scattered bronchiolar and bronchial epithelial cells staining positively. Figure 13 shows unequivocal cytoplasmic staining within an alveolar macrophage of an RSV-inoculated animal on day 60. No positive staining was observed in the mid-sagittal lung sections examined from the day 125 study. The control lung sections from two fatal cases of human acute RSV bronchiolitis stained positively during each run and no staining was ever observed in the negative control from human autopsy lung.

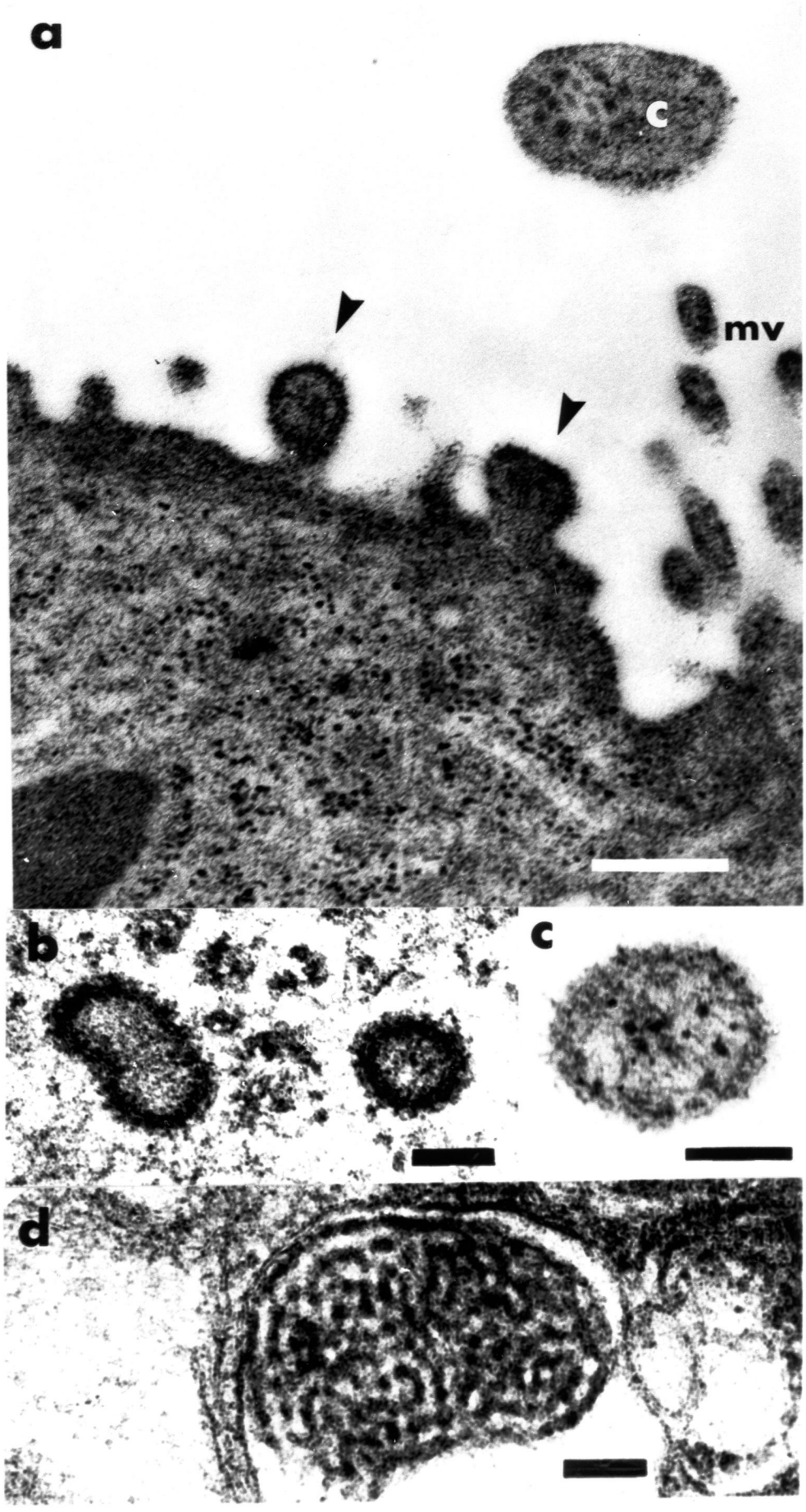
Figure 11. Electron micrographs of RSV-inoculated guinea pig lung (day 6 study).

Panel **a**: Budding viruses (arrowheads) are larger than normal microvilli (mv) of the bronchial epithelial cells but smaller than the cilium (c) seen in oblique section (white bar represents 250 nm).

Panel **b**: Two incompletely assembled "virus-related particles" showing trilaminar membrane with projections (spikes) but paucity of nucleocapsid material (bar represents 100 nm).

Panel **c**: Cross section of a virus-related particle showing central electron dense nucleocapsid material (bar represents 100 nm).

Panel **d**: Intracytoplasmic cluster of filamentous RSV nucleocapsids (bar represents 100 nm).


a
c
mv
b
c
d

5.3.4 RT-PCR. Figure 14 shows the results of agarose gel electrophoresis and corresponding autoradiography for HEp-2 cell samples which underwent RT-PCR. A band of the predicted 410 bp size was observed in samples containing as few as 5 copies of plasmid DNA with the RSV N gene insert (Figure 14a, Lane 8) and was the most prominent band in samples from RSV-infected HEp-2 cells (Figure 14a, Lanes 6 and 7). Of the less prominent, smaller bands present on ethidium-bromide stained agarose gels from RSV-infected HEp-2 cells (Figure 14a, Lanes 6 and 7), one band was faintly positive by autoradiography.

The specificity of RT-PCR for the RSV genomic RNA target sequence was confirmed when no bands were observed in samples that did not contain a nucleic acid template, in samples of uninfected HEp-2 cells and in samples of RSV-infected HEp-2 cells which underwent RNase digestion before reverse transcription. The sensitivity and specificity of RT-PCR was confirmed on multiple runs.

Figure 15 shows the ethidium bromide-stained agarose gels and corresponding autoradiographs of Hybond membranes hybridized with the ^{32}P-labeled oligonucleotide probe from an internal sequence of the predicted PCR product. A specific band of 410 bp was present in 4/4 RSV-inoculated guinea pigs at day 6 and in 3/4 RSV-inoculated guinea pigs at day 125. RT-PCR was not done on RNA extracted from frozen guinea pigs lungs in the original day 6, day 14 and day 60 studies because of extensive RNA degradation in these samples.

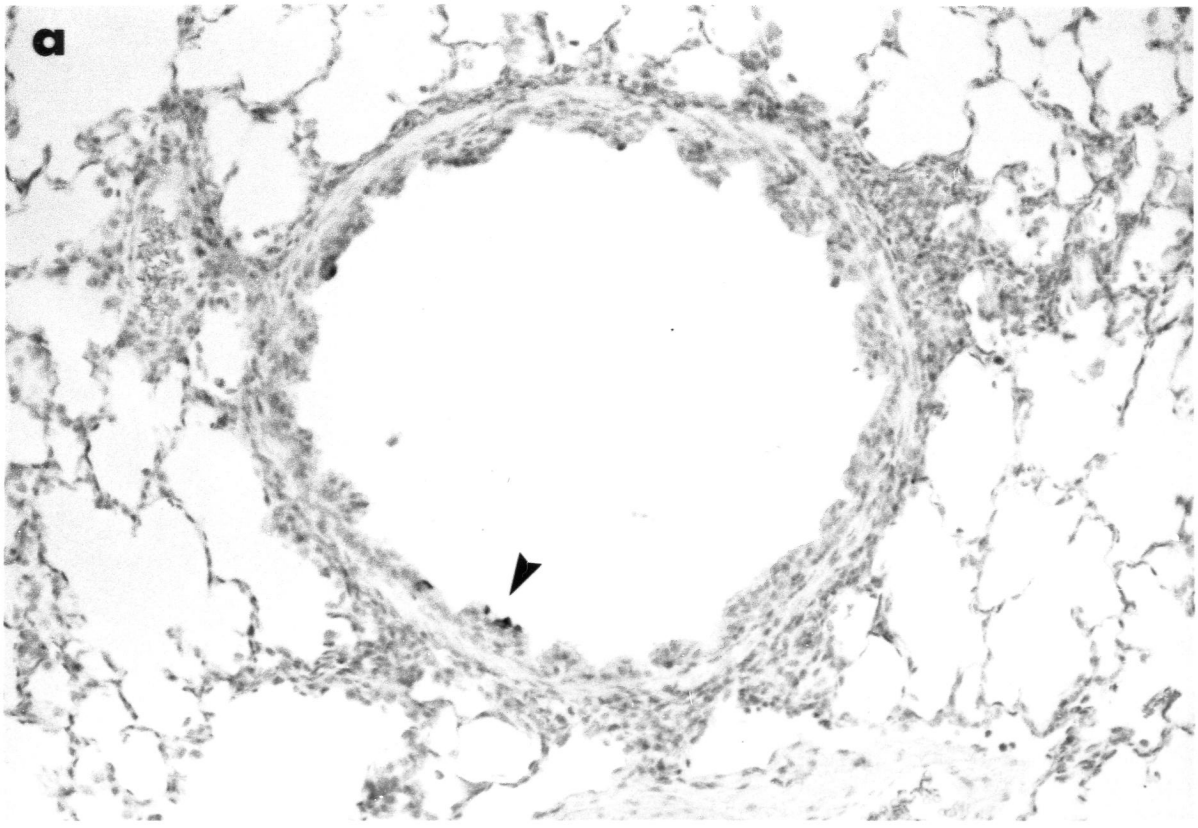
Figure 12. Immunohistochemical staining of RSV-inoculated guinea pig (day 6 study).

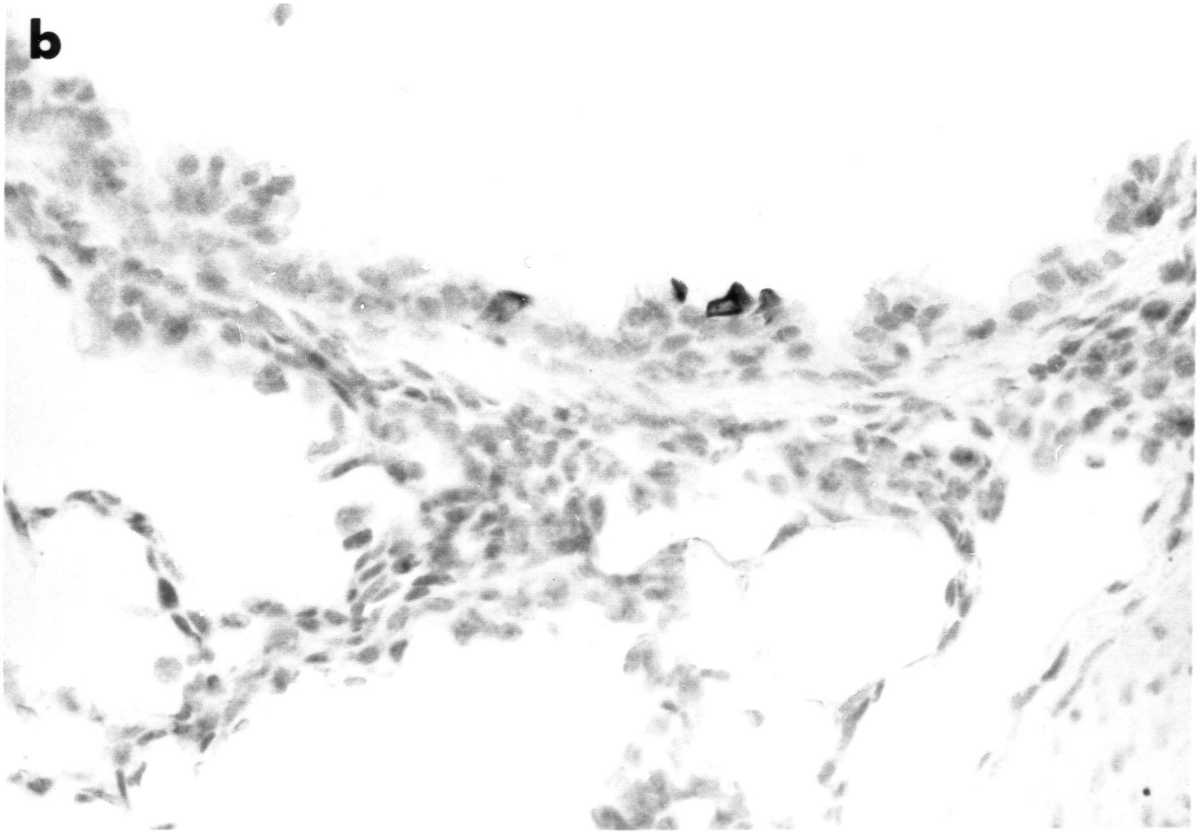
Panel **a**: Low power photomicrograph showing an inflamed membranous bronchiole with a few epithelial cells staining positively with polyclonal anti-RSV antibody (arrowhead). (Hematoxylin counterstain; final magnification: x208).

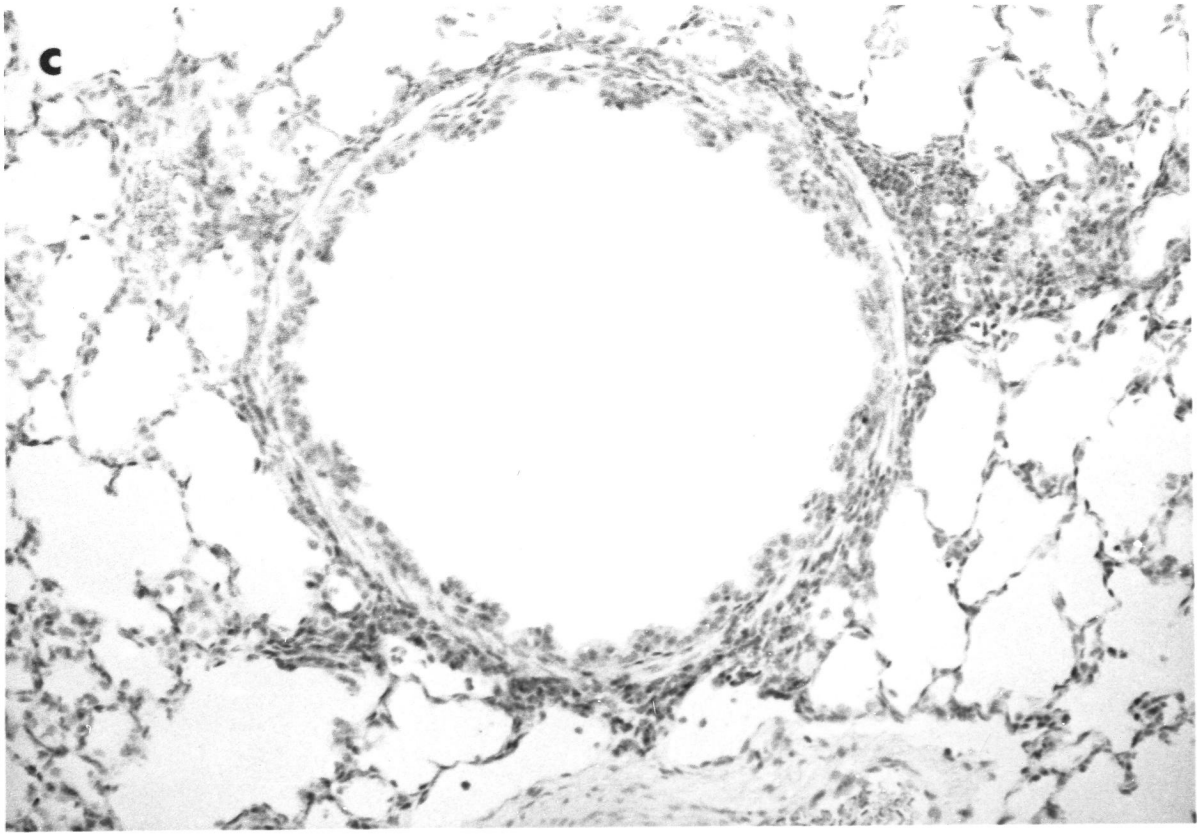
Panel **b**: High power view of RSV-positive cells from the airway of panel **a**, confirming intracytoplasmic staining for RSV antigens. (Hematoxylin counterstain; final magnification: x416).

Panel **c**: Same airway as panel **a** without incubation with anti-RSV antibody. There is absence of non-specific staining in this control section. (Hematoxylin counterstain; final magnification: x208).

Panel **d**: High power view of airway from panel **c**, confirming the absence of non-specific staining (Hematoxylin counterstain; final magnification: x416).


a


b


c

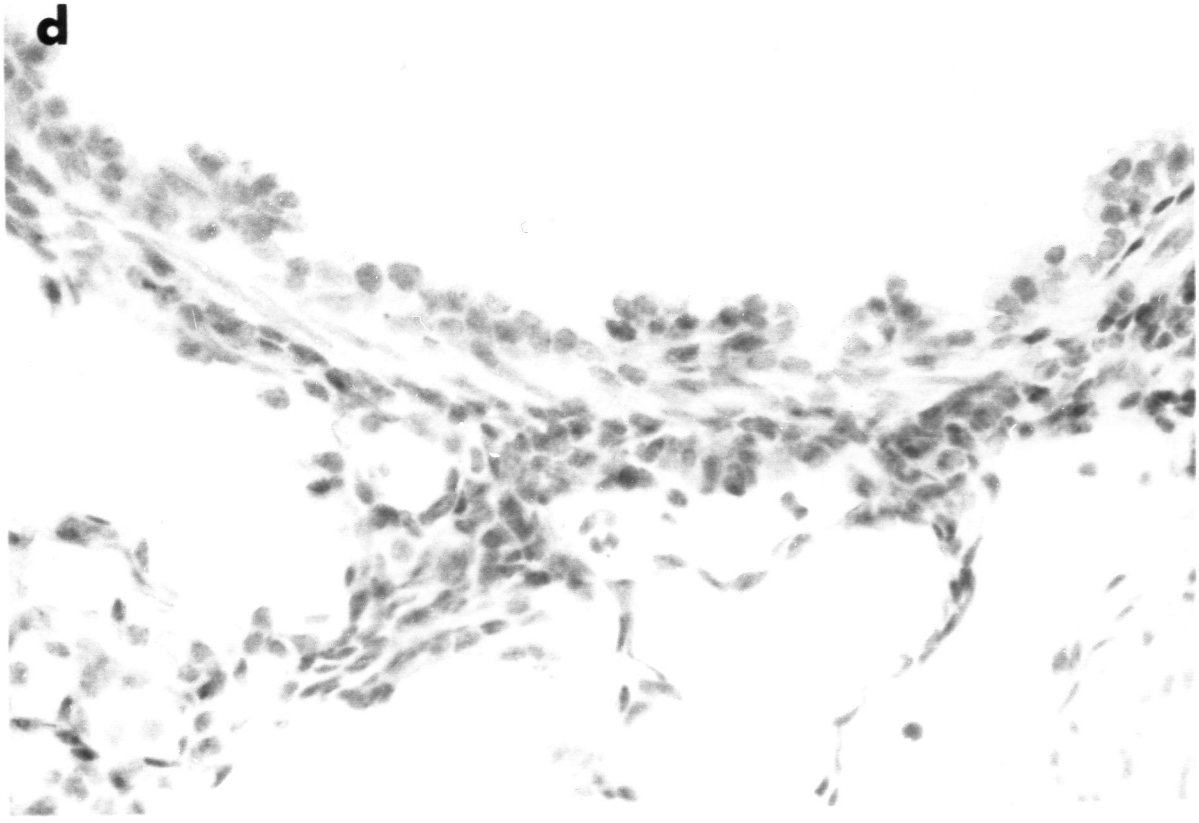

d

Figure 13. Immunohistochemical staining of RSV-inoculated guinea pig lung (day 60 study). There is intracytoplasmic staining in the alveolar macrophage at centre. (Hematoxylin counterstain; final magnification: x800).

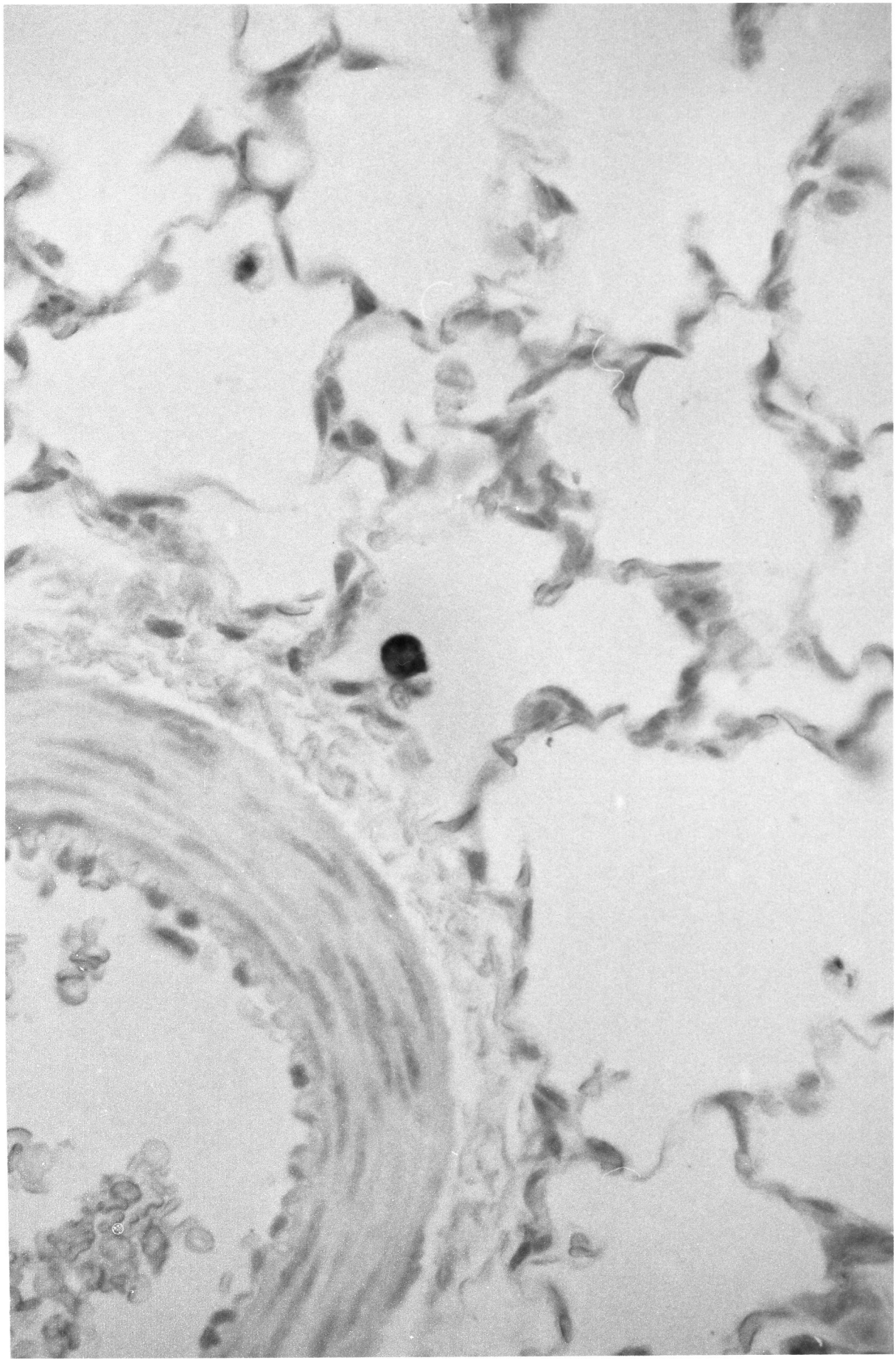


Figure 14. Agarose gel electrophoresis and autoradiography of HEp-2 cell cultures undergoing RT-PCR. Top: agarose gel stained with ethidium bromide; Bottom: autoradiograph after 24 hour exposure.

Panel **a**: Lane 1: PUC 18-*Hinf* I digest (size markers).

Lane 2: blank

Lane 3: no template, RT and PCR steps

Lane 4: no template, PCR step only

Lane 5: uninfected HEp-2 cells

Lane 6: RSV-infected HEp-2 cells

Lane 7: RSV-infected HEp-2 cells (duplicate of sample in lane 6)

Lane 8: RSV N gene cDNA in pGEM3, 5 copies

The agarose gel shows a number of bands in Lanes 6, 7, and 8, with the predicted PCR product of 410 bp being most prominent. The autoradiograph shows a specific band of the predicted 410 bp PCR product in Lanes 6, 7 and 8 corresponding to positive samples. There is also a specific band from a sample containing 5 copies of target cDNA (Lane 8).

Panel **b**: Lane 1: PUC 18-*Hinf* I digest (size markers).

Lane 2: uninfected HEp-2 cells

Lane 3: RSV-infected HEp-2 cells, PCR step only (no RT step)

Lane 4: duplicate of sample in lane 3

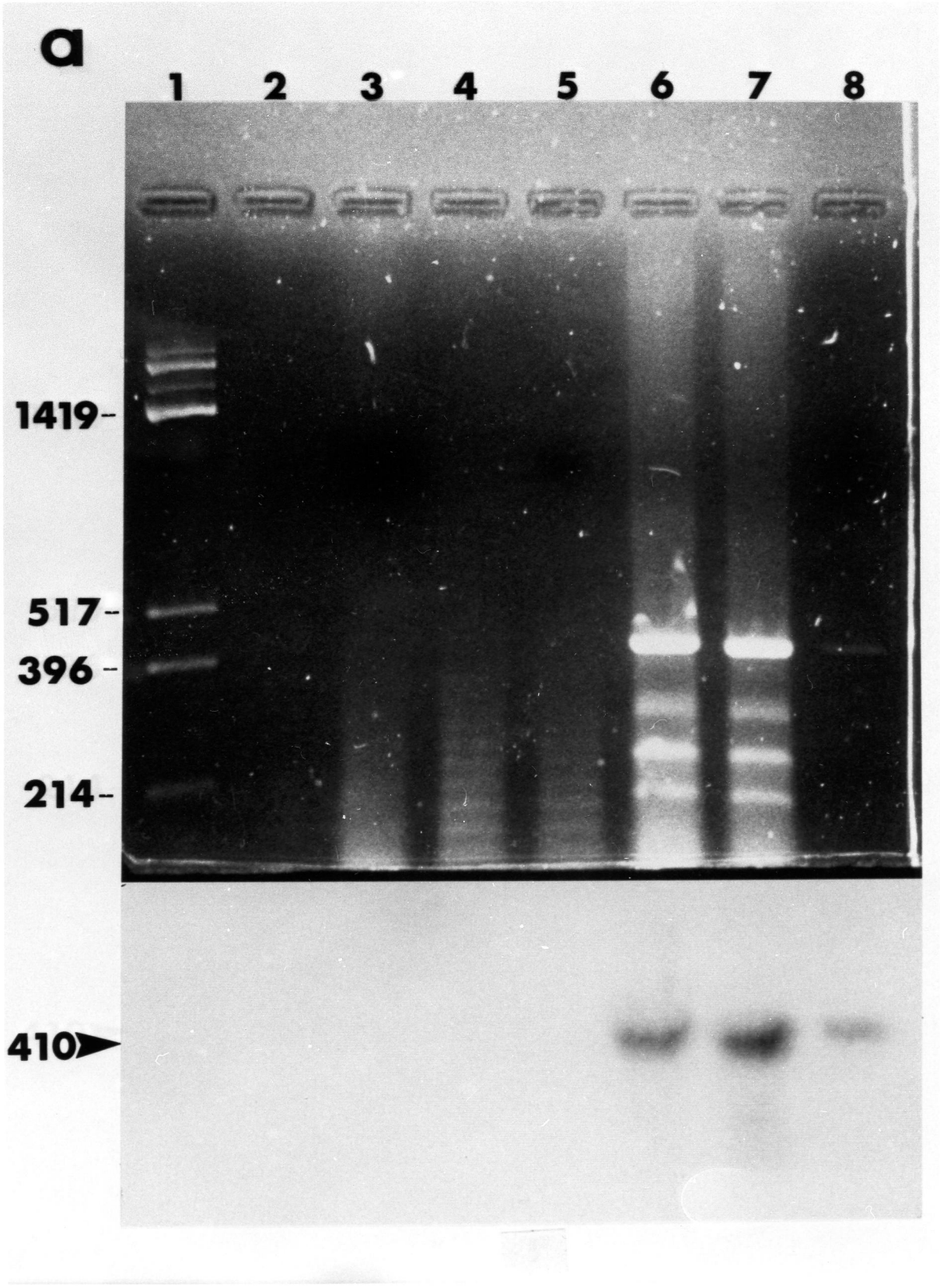
Lane 5: blank

Lane 6: RSV N gene cDNA in pGEM3, 500 copies

Lane 7: RSV-infected HEp-2 cells with RNase pretreatment prior to the RT step

Lane 8: RSV-infected HEp-2 cells

The agarose gel shows that performing PCR without prior reverse transcription did *not* result in amplification of specific DNA from RSV-infected HEp-2 cells (Lanes 3 and 4) and that RNase pretreatment did not result in amplification of PCR product (Lane 7).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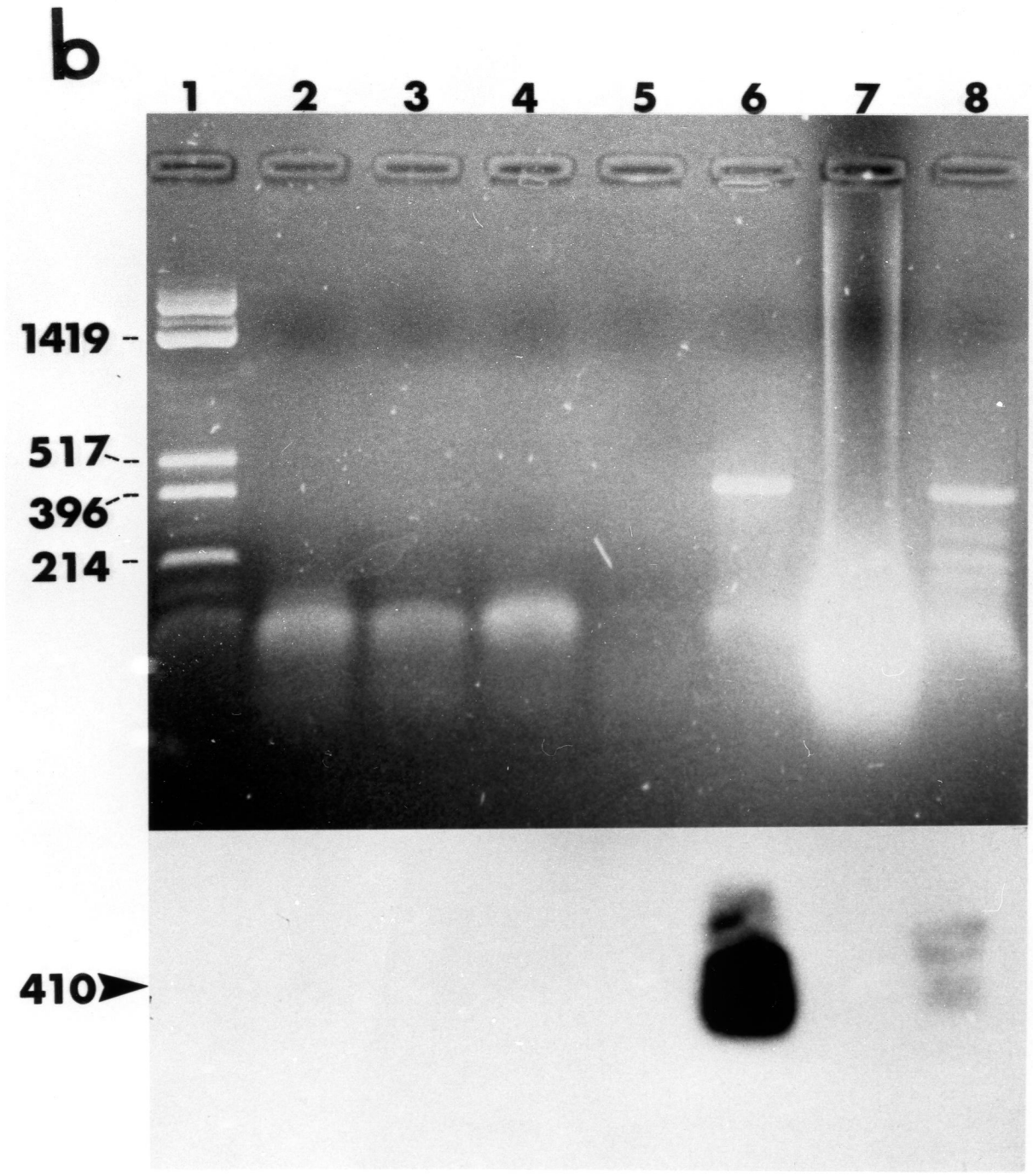
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Figure 15. Agarose gel electrophoresis and autoradiography for day 6 and day 125 RT-PCR studies. Top: agarose gel stained with ethidium bromide; Bottom: autoradiograph after 72 hour exposure.

Panel **a**: Day 6 RT-PCR study.

Lane 1: PUC 18-*Hinf* I digest (size markers).

Lane 2: no template (negative control).

Lane 3: 1979-3 (RSV-inoculated)

Lane 4: 1979-5 (RSV-inoculated)

Lane 5: 1979-6 (RSV-inoculated)

Lane 6: 1979-7 (RSV-inoculated)

Lane 7: 1979-19 (control)

Lane 8: 1979-20 (control)

The agarose gel shows a number of non-specific bands in each of the lanes; the autoradiograph shows a specific band of the predicted 410 bp PCR product in Lanes 3, 4, 5 and 6 corresponding to 4/4 guinea pigs inoculated 6 days previously with RSV.

Panel **b**: Day 125 study.

Lane 1: PUC 18-*Hinf* I digest (size markers).

Lane 2: 500 copies of RSV N gene cDNA in pGEM 3 (positive control of PCR step).

Lane 3: no template (negative control).

Lane 4: 1950-2 (RSV-inoculated)

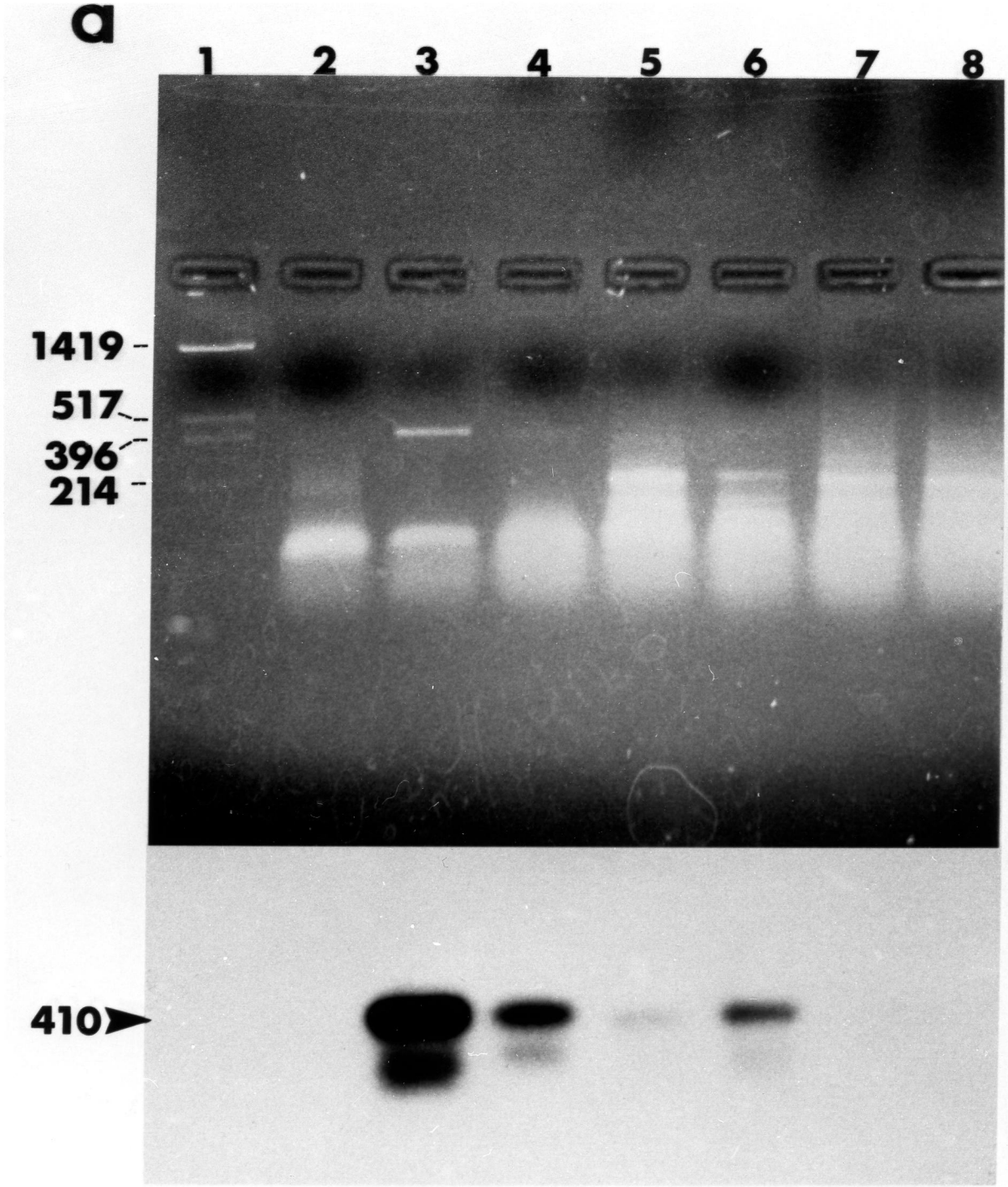
Lane 5: 1950-3 (RSV-inoculated)

Lane 6: 1950-4 (RSV-inoculated)

Lane 7: 1950-5 (RSV-inoculated)

Lane 8: 1950-1 (control)

The agarose gel shows a number of non-specific bands in each of the samples; the autoradiograph shows a specific band of the predicted 410 bp PCR product in Lanes 4, 5 and 6 corresponding to 3/4 guinea pigs inoculated 125 days with RSV.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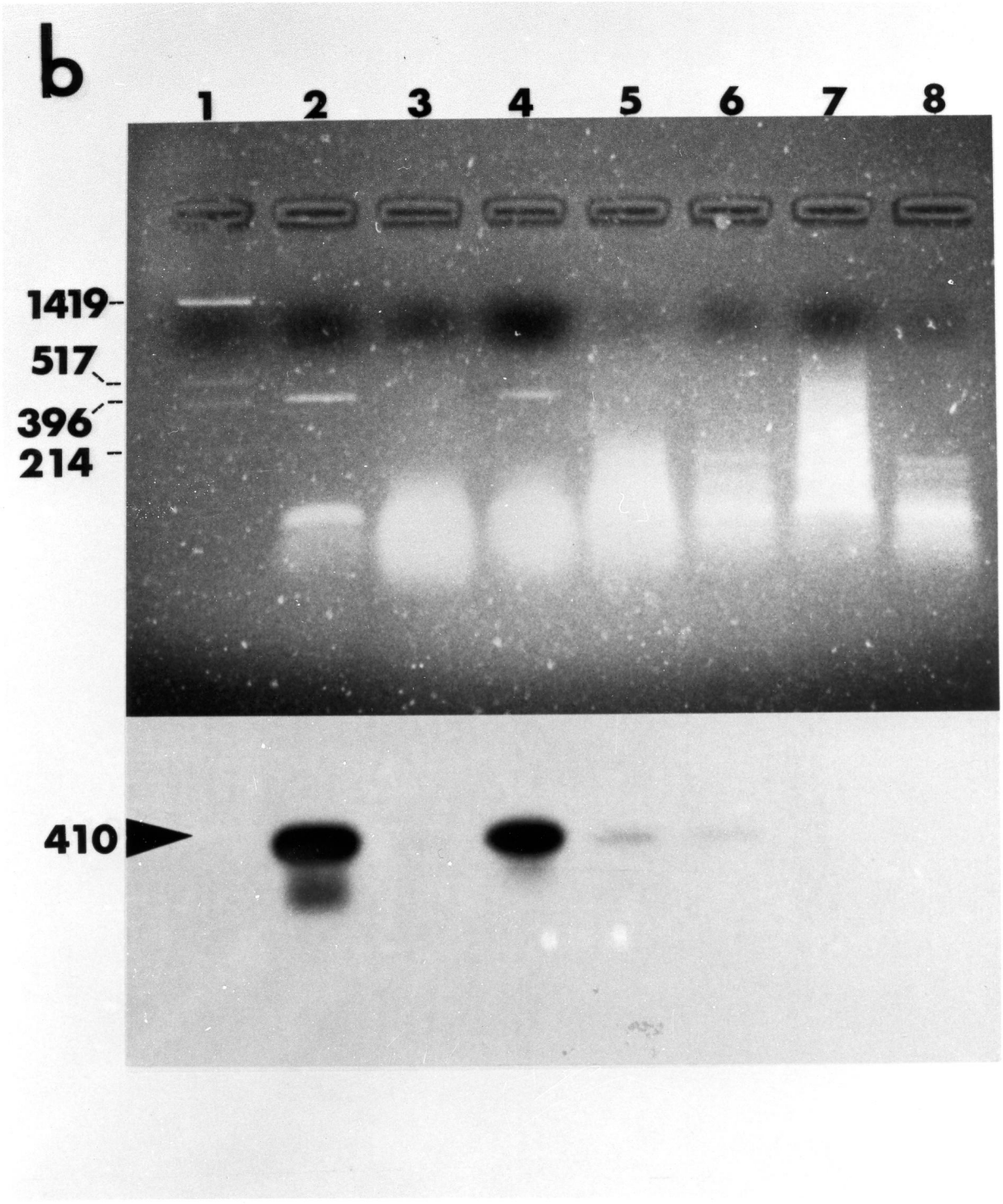
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5.4 SUMMARY. Inoculation of juvenile guinea pigs with human RSV resulted in significant bronchiolar epithelial necrosis, mononuclear cell bronchiolar infiltrates and PMN bronchiolar infiltrates on day 6 post-inoculation in the absence of detectable clinical disease or gross pulmonary lesions. The extent of bronchiolar inflammation was similar whether juvenile guinea pigs were given ~4000 pfu or ~28000 pfu of RSV. By day 14, the bronchiolar inflammation had substantially resolved and there was no chronic bronchiolar inflammation on either day 60 or day 125. In contrast to the juvenile group on day 6, no significant bronchiolar inflammation was observed in a group of adolescent guinea pigs inoculated with ~4000 pfu of RSV.

RSV was cultured from the lung parenchyma in 9/10 juvenile guinea pigs inoculated with ~4000 pfu of RSV on day 6, from 2/10 animals on day 14 and none of the RSV-inoculated animals on days 60 or 125. TEM revealed completely assembled virus, virus-related particles and viral nucleocapsids in rare airway epithelial cells on day 6. On day 6, RSV immunohistochemistry was positive in 7/10 juvenile and 6/10 adolescent guinea pigs inoculated with low dose RSV, in 8/10 juvenile animals given high dose RSV and in 2/4 RSV-inoculated animals in the RT-PCR study. RSV antigens were identified in lung sections from 6/10 juvenile animals on day 14 and 1/10 juvenile animals on day 60. On day 6, positive immunostaining was present predominantly within bronchiolar epithelial cells; on day 14, alveolar macrophages were the predominant RSV-positive cell type and on day 60, positive RSV staining was present only within alveolar macrophages. RSV genomic RNA was documented in the lung of 4/4 infected guinea pigs at day 6 and in 3/4 infected guinea pigs at day 125.

In summary, while human RSV did not produce clinical evidence of acute lower respiratory tract disease in guinea pigs, younger animals had statistically significant bronchiolar inflammation on day 6 which resolved by day 14. Despite resolution of the inflammatory process, evidence of intrapulmonary RSV was documented up to 125 days after primary inoculation.

CHAPTER 6: DISCUSSION

6.1 SPECIFIC AIM 1: PRODUCTION OF ACUTE RSV BRONCHIOLITIS IN THE GUINEA PIG.

The results of these experiments showed that intranasal inoculation of ~4000 pfu of human RSV into anesthetized, one month old juvenile guinea pigs produced maximal histological evidence of acute bronchiolitis on day 6, without concomitant clinical signs of acute respiratory disease or gross pulmonary lesions. Bronchiolar inflammation resolved by day 14 and no chronic inflammatory sequelæ were observed on days 60 or 125. Increasing the inoculated dose of virus to ~28000 pfu did not increase the severity of bronchiolar inflammation on day 6 and inoculation of two month old adolescent guinea pigs with ~4000 pfu of RSV produced no evidence of acute bronchiolitis.

These experiments established that this protocol of human RSV infection produced a self-limited histological bronchiolitis in juvenile guinea pigs which had features of human disease. Similarly to the previously described cotton rat (199) and mouse (198) models, RSV inoculation of guinea pigs did not produce clinical evidence of acute respiratory disease or gross pulmonary lesions. However, the guinea pig model better resembled human disease than either the cotton rat or mouse models in terms of the age susceptibility of animals to develop bronchiolar inflammation. The details of these results are discussed below.

6.1.1 CLINICAL-PATHOLOGICAL EVALUATION.

Bronchiolar inflammation in the absence of clinical disease has been observed in cotton rats inoculated intranasally with 10^4 pfu human RSV (199) and in young mice given 10^7 pfu (198) (in contrast to humans, clinical signs of respiratory disease "paradoxically" developed in *older* mice given human RSV and no explanation was offered for this observation)[24]. The extent of bronchiolar inflammation appeared similar in the guinea pig, cotton rat and mouse, based on review of published photomicrographs and comparison to results from another histological scoring system

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[24] In human infants, the amount of RSV necessary to produce clinical signs of acute bronchiolitis is unknown because it is unethical to perform the experiment. However, a previous study of adult human volunteers showed that intranasal inoculation with $10^{2.7}$ pfu produced upper respiratory infection in 16/16 subjects (255).

developed for cotton rats (156). In this thesis, one month old guinea pigs were used as the juvenile group because the laboratory did not have breeding facilities. Consequently, the effects of intranasal inoculation of human RSV into very young guinea pigs (analogous to infection of human infants) remain unknown. In summary, human RSV produces similar degrees of bronchiolar inflammation in the guinea pig, cotton rat and mouse, but the apparent predilection of younger guinea pigs to develop significant bronchiolar inflammation more closely approximates human infections.

Concerning adolescent guinea pigs, the absence of statistically significant bronchiolar inflammation on day 6 post-RSV inoculation may have been due to a "dilutional" effect from the instillation of a similar viral inoculum into larger lungs (in comparison to juvenile animals). However, the extent of RSV-induced airway inflammation was probably *not* dependent on the amount of virus instilled because a similar degree of bronchiolar inflammation was observed in juvenile guinea pigs given either ~4000 pfu or ~28000 pfu of human RSV (Appendix B). To definitively address this issue would require a separate study of adolescent guinea pigs given the larger amount of human RSV.

Other possibilities to account for the apparent predilection of younger RSV-inoculated guinea pigs to develop significant bronchiolar inflammation include structural characteristics of airways conducive to viral-spread to the bronchioles, a higher intrinsic susceptibility of airway epithelial cells to RSV infection or differences in immune response to RSV (section 2.6.1). Although examination for differences in airway geometry between juvenile and adolescent animals would be difficult experimentally (149), the other two possibilities are amenable to study by the experimental approaches previously used for humans: comparison of susceptibility to RSV infection in different respiratory cell types *in vitro* (136), RSV serology (section 2.7.1) and assays of RSV-specific cytotoxic T-lymphocytes (section 2.7.2). However, the differences between the human and guinea pig immune systems (section 2.9) may limit the interpretation of experiments examining virus-specific immune responses.

Collaborative studies (256) using acetylcholine challenge have shown that the RSV-

inoculated juvenile guinea pigs develop airway hyperresponsiveness by day 6 that resolves by day 14, in parallel with the histological lesions[25]. These observations contrast with those of a recent study (99) of parainfluenza type I (Sendai) virus-infected rats challenged with methacholine: in this study, the authors documented both acute and persistent airway hyperresponsiveness in otherwise asymptomatic animals (unfortunately, histological evaluation to document the duration of airway inflammation was not performed). These differences in the duration of airway hyperresponsiveness may be attributable to intrinsic differences between RSV and Sendai virus, differences in host susceptibilities to viral infection (the rat is a natural host to Sendai virus) or immune responses to these viruses.

The ability of RSV to produce concomitant histological and physiological abnormalities in the setting of subclinical infection may be relevant to the majority of acute RSV lung infections in humans since hospitalization is rarely required (257). Should this be the case, the epidemiological implications are significant because RSV-induced airway inflammation and airway hyperresponsiveness (two hallmarks of asthma) may affect far more individuals than previously suspected, as studies of acute RSV bronchiolitis tend to be limited to hospitalized patients (90, 91). An hypothesis generated from the experiments of this thesis is that, during acute RSV lung infection, asymptomatic or mildly symptomatic children develop airway inflammation and airway hyperresponsiveness. Furthermore, the study of Sendai virus-infected rats suggests that airway hyperresponsiveness might persist (99). While the identification and recruitment of asymptomatic or mildly symptomatic RSV-infected children would be difficult in practice, serial tissue sampling (e.g., nasopharyngeal aspirates to test for presence of RSV) and lung function testing of these children could yield valuable information

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[25] Increased airway inflammation and increased airway hyperresponsiveness were observed for the RSV-inoculated group as a whole. For a given animal, no histological features from the RSV bronchiolitis scoring system independently predicted the presence of coexistent airway hyperresponsiveness, in part due to the number of animals studied (data not shown). There was no augmentation of airway hyperresponsiveness to acetylcholine by using high dose RSV in juvenile guinea pigs or after low dose RSV given to adolescent animals. Additional morphometric analyses revealed no significant airway submucosal thickening or smooth muscle thickening in RSV-inoculated guinea pigs on either days 6 or 14.

about the possible role of persistent RSV infection in the pathogenesis of persistent airway hyperresponsiveness.

6.1.2 HISTOLOGICAL SCORING SYSTEM. The histological scoring system for acute bronchiolitis was simple, reproducible and permitted distinction between RSV-specific bronchiolar inflammation from non-specific inflammation induced by uninfected cell culture supernatant. The results from the scoring system also showed that the uninfected cell culture supernatant did not induce significant airway inflammation because airway histological scores in this group were similar to those of unmanipulated, historical control animals[26]. On day 6 post-inoculation, the statistically significant epithelial necrosis and bronchiolar mononuclear cell infiltrates in juvenile RSV-inoculated guinea pigs confirmed the presence of acute lytic infection resembling human disease (103). Curiously, despite extensive study of the host immune response to RSV in many species (section 2.7), there are no published reports describing the characteristics of the lymphocytes comprising the bronchiolar wall infiltrates. Immunocytochemical characterization of these cells may provide information concerning the host's local immune response to intrapulmonary RSV. Preliminary experiments using antibodies which cross-react with guinea pig B-lymphocyte (anti-CDw75) and T-lymphocyte (anti-CD3) epitopes suggest that T-cells constitute that vast majority of infiltrating mononuclear cells on day 6 (data not shown). Further experiments are planned to further subclassify these cells into CD4+ (helper-inducer phenotype) and CD8+ (cytotoxic-suppressor phenotype) with antibodies directed against guinea pig T-cell antigens. A predominance of CD8+ cells would be consistent with a local cytotoxic T-cell response to RSV while a predominance of CD4+ cells would be consistent with either humoral (i.e., T_{H2} cells) or cell-mediated (i.e., T_{H1} cells) immunity (258).

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26 In general, the unmanipulated historical control group had "near-normal" airway histology. Some focally striking bronchiolar inflammation was observed in a few of the animals inoculated with uninfected cell culture supernatant; however, these findings were not statistically significant for the group as a whole.

A second unresolved issue concerning the mononuclear cell bronchiolar wall infiltrates is the *specificity* of lymphocytes toward RSV. One method to screen for RSV-specific lymphocyte memory is the *in vitro* ^{3}H-thymidine incorporation assay that documents lymphocyte proliferation induced by microbial crude antigen preparations (259). This utility of this assay has been previously shown in several rheumatological disorders, in which T-lymphocytes aspirated from inflamed joints proliferate (i.e., incorporate ^{3}H-thymidine) in a more specific fashion (following exposure to a panel of crude microbial antigen preparations) than the circulating lymphocytes from the same patients (260). If lymphocytes isolated from the guinea pig lung are exposed to a panel of crude microbial antigen preparations in a similar manner, one may screen for RSV-specific lymphocyte proliferation from the inflamed lung. Limitations of this technique include the loss of morphological information regarding the location(s) of the responding lymphocytes within the lung (e.g., bronchiolar wall vs. BALT) and the majority of proliferating lymphocytes having a CD4+ phenotype consistent with memory T-cells rather than cytotoxic T-cells (261). The presence of RSV-specific cytotoxic T-lymphocytes within the lungs of RSV-inoculated guinea pigs may be assessed by *in vitro* chromium release assays (174-178).

The presence of statistically significant bronchiolar wall PMN infiltrates reproduced recent observations of cow lungs infected with bovine RSV (224) and extended previous observations of increased neutrophil adherence to RSV-infected HEp-2 cells induced by RSV-specific antibody *in vitro* (242). Circulating anti-RSV antibodies may play a role in the recruitment of neutrophils to bronchioles *in vivo* because the guinea pig is known to produce RSV-specific antibodies following intranasal RSV inoculation (164, 182). Testing this possibility would require serology on day 6 post-inoculation, but the interpretation of results would be limited by species differences in humoral immunity (section 2.9).

Airway wall edema, BALT hyperplasia and goblet cell metaplasia were not statistically significant between RSV-inoculated guinea pigs and controls. While the lack of significant airway wall edema might be related to species differences in the host inflammatory response to

RSV, another possibility is that extensive airway wall edema may be a feature of *fatal* cases of human acute RSV bronchiolitis (since histological descriptions of human airway lesions have been primarily derived from postmortem specimens) (101-104). Although the experiments of this thesis confirmed the existence of BALT in guinea pigs (similar to rabbits, rats and humans (262)), the lack of BALT hyperplasia following RSV may have been due to several factors. For example, BALT may not be an important constituent of the pulmonary immune system, as recently suggested for human BALT by Pabst (263). Secondly, if cell-mediated immunity is of greater importance than humoral immunity in the host response to acute RSV lung infection, then BALT hyperplasia might not occur since (in humans) BALT consists mostly of B-lymphocytes associated with secretory IgA immunity (264). A third possibility is that lymphocyte traffic through BALT (264) to the bronchioles achieves an equilibrium during acute RSV infection that does not result in detectable BALT hyperplasia. Immunocytochemically characterizing the lymphocytes (see above discussion for bronchiolar wall lymphocytic infiltrates) within guinea pig BALT is a first step toward clarifying any role for BALT in the guinea pig's immune response to RSV.

The lack of significant goblet cell metaplasia of the bronchiolar epithelium may have been due to RSV inducing insufficient epithelial cell lysis to stimulate metaplastic repair. Alternatively, if normal guinea pig bronchioles contain a greater proportion of goblet cells than human airways, subtle goblet cell metaplasia might have been obscured. Another possibility is that, in contrast to humans (243), goblet cell metaplasia is not an important epithelial repair mechanism in guinea pig airways. These possibilities could be addressed by counting goblet cells in guinea pig airways which have undergone extensive experimental epithelial cell injury (e.g., acid injury), comparing the number of goblet cells in normal guinea pig vs. human airways, and possibly re-scoring lung sections for alternate mechanisms of epithelial repair such as squamous cell metaplasia[27].

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27 Squamous cell metaplasia as mechanism of airway epithelial repair following acute lytic RSV infection is *not* favored because inspection of histological sections from RSV-inoculated

6.2 NATURAL HISTORY OF INTRAPULMONARY RSV. The combination of viral culture, TEM, immunohistochemistry and RT-PCR provided new insights into the natural history of intrapulmonary virus (within both lungs) up to 125 days from the time of acute infection—approximately 25% of the expected life span of a guinea pig (209). The details of these studies are summarized below.

6.2.1 VIRAL CULTURE. The ability to culture replicating RSV from the lung in the majority of animals during acute infection has also been reported in the cotton rat (199) and the mouse (198) models. In particular, on day 6 post-inoculation, a mean ± SD of $1.7 \pm 0.3 \times 10^3$ pfu RSV/g wet weight fresh lung in guinea pigs was similar to levels previously reported for cotton rats and mice. An apparent discrepancy between the guinea pig and the other two species was the maximum period of ability to isolate virus post-inoculation: in the guinea pig, RSV CPE was observed in 2/10 RSV-inoculated animals at day 14, while virus was only isolated up to 7 days post-infection in the cotton rat and 8 days in the mouse. However, there was a distinct possibility of false negative cultures in both the cotton rat and mouse models because in both models, excised lungs were frozen and stored for days to weeks prior to processing for culture on HEp-2 cells. In contrast, fresh lungs were used in this thesis because freezing and thawing may inactivate RSV (265)[28]. Another important difference in the viral culture protocol of this thesis was the weekly passaging of samples for up to one month before being called negative[29]: HEp-2 cell monolayers were examined only at 5 days after plating of lung digests in the cotton rat model and after 4 days in the mouse model. In summary, the ability to culture intrapulmonary RSV from the guinea pig substantially longer than the cotton rat or the mouse

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animals did not reveal evidence of squamous cell metaplasia. This observation was the rationale for not including squamous cell metaplasia as part of the histological scoring system.

28 If freezing and thawing caused viral degradation in these studies, then both cotton rats and mice may have actually had *greater* amounts of intrapulmonary RSV than observed in guinea pigs on day 6.

29 One of the two positive RSV cultures in the day 14 study occurred during the second passage of HEp-2 cells.

may have reflected differences in viral culture methods rather than intrinsic species differences in the susceptibility to longer term human RSV infection.

The inability to culture virus in the day 60 and day 125 studies was probably a consequence of insufficient release of RSV from persistently infected alveolar macrophages to infect HEp-2 cells *in vitro*. This explanation is favored because infected alveolar macrophages have a propensity to retain RSV (136) and the immunohistochemistry studies of this thesis have shown evidence of RSV only within alveolar macrophages on day 60. A potential mechanism for the persistent, non-lytic infection of alveolar macrophages is through the production of so-called "defective interfering particles" (237) of RSV following acute infection. Defective interfering particles, although missing portions of the viral genome required for replication, remain capable of producing viral proteins that may be deleterious (without necessarily being lethal) to the infected cell.

The endpoint of viral culture was the documentation of syncytial formation in HEp-2 cell monolayers, the characteristic CPE of human RSV. A potential confounding factor was that "pathogen-free" guinea pigs (214) were not used in the experiments of this thesis such that the observed CPE may have been due to other viruses that form syncytia in HEp-2 cells, e.g., parainfluenza virus or mumps virus (135). However, pulmonary infection by either parainfluenza or mumps viruses was not likely because the guinea pig is not a natural host to either virus (213) and no syncytia were observed in any HEp-2 cell cultures from control animals. In addition, precautions were taken to decrease the likelihood of undesired infections in guinea pigs (section 2.9). The room housing RSV-inoculated guinea pigs was reserved exclusively for the experiments of this thesis and was cleaned prior to the delivery of animals. Furthermore, isolation procedures (gowns, hats, masks, gloves and shoe covers) were employed to protect both guinea pigs and investigators against undesired infections.

6.2.2 TRANSMISSION ELECTRON MICROSCOPY. As discussed in section 3.3, the low sensitivity of TEM in the diagnosis of viral infections resulted in TEM being used as an

ancillary technique in this thesis[30]. Particular effort was made to exclude the possibility of other structures that may have been confused with RSV nucleocapsid or assembled virus. The main utility of TEM was the confirmation that viral replication and assembly took place within infected bronchial epithelial cells, as shown by the presence of free intracytoplasmic nucleocapsids and assembled virus particles budding from the cell membrane. The TEM results indicate that positive viral cultures, immunohistochemical staining and RT-PCR were not solely attributable to free virus left over from the inoculation procedure. The inability to find unequivocal virus-related particles by TEM within bronchiolar epithelial cells, type I and type II pneumocytes or alveolar macrophages has also been reported in cows infected with bovine RSV and in that instance was attributed to sampling error (222).

Immunogold electron microscopy (267, 268) has advanced the capability of TEM to identify the presence of specific antigens in ultrathin sections; however, no protocol using immunogold electron microscopy for the identification of RSV antigens has been published to date. The development of a suitable protocol would permit the unequivocal identification of virus-related structures that might otherwise have been missed or misinterpreted.

6.2.3 IMMUNOHISTOCHEMISTRY. During the acute phase of RSV lung infection on day 6, the polyclonal anti-RSV antibody detected RSV antigens in paraformaldehyde-fixed, paraffin-embedded midsagittal sections of guinea pig lung with 50-70% sensitivity and 100% specificity compared to viral culture. Both the sensitivity and specificity of this antibody were comparable to a previous report that documented RSV antigens in formalin-fixed, paraffin embedded sections of human autopsy lungs (104).

In adolescent guinea pigs, the immunohistochemical demonstration of RSV antigens in 5/10 infected animals confirmed that RSV reached the peripheral airways following intranasal

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30 Neither the cotton rat nor mouse models of RSV lung infection used TEM to document intrapulmonary viral particles. The difficulty in finding virus-related particles in the guinea pig lung prompted a review of *Index Medicus*: since 1973, there have been only 6 reports of TEM documenting intrapulmonary pulmonary RSV in ultrathin sections of lung (104, 191, 222-224, 266). These reports have primarily dealt with the ultrastructural identification of bovine RSV in cows or sheep.

inoculation. Therefore, the lack of statistically significant airway inflammation in adolescent guinea pigs was not due to absence of virus. As discussed in section 4.14, no attempts were made to quantify the number of positive cells with airway inflammation, etc. because the immunohistochemistry protocol was designed be to highly specific rather than highly sensitive[31]. Concerning adolescent guinea pigs, if the lack of significant airway inflammation during acute RSV infection resulted from a lower "burden" of intrapulmonary replicating virus, then quantitative viral plaque assays may have revealed differences. Unfortunately, no viral cultures were completed on the lung digests of adolescent guinea pigs because of inadvertent contamination of the cell culture medium by yeast.

The major new findings by immunohistochemistry related to the changing distribution of RSV antigens in different lung cell types over time. On day 6, RSV antigens were identified primarily in airway epithelial cells (consistent with the cotton rat and mouse models) but by day 14 were present mostly within alveolar macrophages. On day 60, RSV antigens were identified exclusively within alveolar macrophages. These results suggest that persistent non-lytic RSV infection of alveolar macrophages may occur *in vivo* and extend recent observations of RSV-infected human alveolar macrophages *in vitro* remaining viable, with little shedding of RSV into culture supernatants (136). However, these observations do not entirely exclude the possibility of RSV infection of other lung cell types (undetectable by immunohistochemistry), with scavenging of free virus by alveolar macrophages. Alternatively, repeated episodes of RSV aspiration from chronic or recurrent upper respiratory tract infections may have been responsible for the presence of RSV antigens within alveolar macrophages. This mechanism is not favored because repeated aspiration of RSV from the upper respiratory tract would probably have also resulted in infection of other susceptible lung cell types such as airway

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31 Western blotting was attempted to quantify RSV antigens in protein extracts of frozen guinea pig lung. However, multiple runs were negative (data not shown), probably because the amount of virus was small compared to the amount total lung protein, thus rendering detection of RSV beyond the sensitivity of the technique (269). A positive signal by Western blotting requires at least 20 pg of target protein—equivalent to over 10^7 nucleocapsid molecules.

epithelial cells (136). Presumably anti-RSV antibody would have detected RSV antigens in cell types in addition to alveolar macrophages.

The lack of statistically significant bronchiolar inflammation on days 14 and 60, despite documentation of intrapulmonary RSV antigens, suggests that the virus itself is insufficient to stimulate chronic airway inflammation. However, if persistent RSV infection occurs in human alveolar macrophages *in vivo*, one may speculate that virus-induced alterations of macrophage function (section 1.3.5) could play a role in the pathogenesis of chronic airway inflammation to non-specific inhaled environmental agents (115): for example, the lack of "down regulation" of T_{H2} cells by alveolar macrophages in atopic asthma. The possibility of virus-infected macrophages permitting the production of allergen-induced airway inflammation was not examined in this thesis because extensive efforts were made to prevent guinea pigs from being exposed to non-specific environmental agents.

Finally, the documentation of RSV antigens within alveolar macrophages on day 60 provides new information regarding the fate of these cells. Previous studies have suggested that alveolar macrophages are cleared from the lung at the rate of 3.5%/day—the implication being that a population of alveolar macrophages would be completely cleared from the lung within one month (270). In contrast, Brain *et al.* have speculated that alveolar macrophages undergo several possible fates, from clearance up the tracheobronchial tree to migration into the pulmonary interstitium and even migration into regional pulmonary lymph nodes (271). Since alveolar macrophages are not believed to undergo appreciable mitotic activity *in vivo*, the experiments of this thesis suggest that the clearance of RSV-infected alveolar macrophages is delayed from the peripheral lung. The possibility of RSV infection of pulmonary lymph nodes was not examined; regarding other pulmonary lymphoid tissue, there was no evidence of RSV antigens within BALT.

6.2.4 RT-PCR. The RT-PCR methodology developed for this thesis introduced three new aspects to the investigation of pulmonary viral disease. First, this protocol represented the first known attempt to document pulmonary RSV infection using lung specimens rather than

exfoliated cells from the upper respiratory tract. Secondly, the sensitivity and specificity of this RT-PCR protocol were evaluated such that one could be confident that a positive signal would result if a sample contained at least 5 copies of target cDNA after reverse transcription. Thirdly, RT-PCR not only revealed the presence of RSV genomic RNA in the guinea pig lung during acute RSV infection but was also positive in 3/4 guinea pigs studied 125 days post-inoculation—twice as long as previously documented in immunodeficient humans and four times as long as previously documented in immunocompetent humans. The long-term persistence of RSV within the lung is a novel finding which supports the working hypothesis of this thesis that RSV (or at least a portion of its genome) may persist within the lung following resolution of acute bronchiolitis.

In samples of RSV-infected HEp-2 cells, the presence of bands in addition to the main 410 bp PCR product may have represented amplified non-specific human DNA or single-stranded DNA from the viral PCR amplification. Two of the three bands were negative on autoradiography and a viral origin for these bands is essentially excluded. One band was faintly positive by autoradiography and likely represented single-stranded DNA from the viral PCR product (272). Importantly, the absence of extra bands from PCR products in samples from RSV-infected guinea pig lungs confirmed the specificity of the RT-PCR method in this situation.

The RT-PCR technique did not quantify the amount of RSV genomic RNA within a given sample: to do this rigorously would have required concomitant reverse transcription and amplification of a guinea pig *m*RNA target sequence with known cellular levels. This was not pursued in this thesis because the additional variables inherent in quantitative RNA PCR (e.g., the efficiency of the reverse transcription step; factors influencing expression of the *m*RNA sequence chosen to normalize the data) are yet to be resolved in other, more established systems (273-275)[32].

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32 A relatively easy approach for quantitative RT-PCR for RSV genomic RNA is to use specimens of RSV-infected HEp-2 cells containing known amounts of plaque forming units.

Finally, the RT-PCR protocol developed for this thesis did not localize RSV genomic RNA within the guinea pig lung. Although *in situ* hybridization is a technique that would theoretically permit cellular localization RSV RNA, a sensitivity of only 50% has been reported for conventional *in situ* hybridization in the detection of RSV nucleic acid in culture-positive nasopharyngeal secretions (219, 276). The low sensitivity of *in situ* hybridization is related to the requirement for a minimum of 10-20 copies of target RNA per infected cell for a positive signal and the propensity of RNA to be digested by ubiquitous intracellular and exogenous RNases (277). Repeated attempts to demonstrate RSV by *in situ* hybridization (based on the method of Murphy *et al.* (278) for the identification of RSV RNA in cotton rat lung sections) were uniformly negative for sections of RSV-infected guinea pig lung despite consistent positivity in sections of RSV-infected HEp-2 cells (data not shown). The negative results may have been related to low copy numbers of RSV within infected cells and/or the apparent propensity for RNA within the guinea pig lung to degrade easily[33].

Recently, protocols combining *in situ* hybridization with PCR have successfully been used to detect human papilloma viral DNA in formalin-fixed, paraffin embedded tissue sections (279) and human immunodeficiency virus proviral RNA in peripheral blood monocytes (280). These results indicate that morphological correlation to PCR products is now possible. However, the combined use of *in situ* hybridization and RT-PCR *in tissue sections* has not been reported and a further technical advance would therefore be required to apply this combined approach to the guinea pig model of RSV lung infection.

However, the relationship between plaque forming units and the corresponding copy numbers of RSV genomic RNA would still have to be established.

[33] Recall that there was extensive degradation of total cellular RNA extracted in the first three day 6 studies, the day 14 study and in the day 60 study. Two principal investigators in the U.B.C. Pulmonary Research Laboratory (Drs. T. Bai and G. Bondy) have independently experienced problems in extracting intact RNA from the guinea pig lung, despite the use of protocols intended for RNA extraction in organs rich in endogenous RNase such as pancreas (244). A literature review yielded no information concerning this apparent tendency for RNA in the guinea pig lung to degrade easily.

6.3 CONCLUDING REMARKS. The experimental results presented in this thesis support the working hypothesis that human RSV may chronically persist within the lung following resolution of acute bronchiolitis, although the presence of RSV *per se* was insufficient to produce chronic airway inflammation. In the future the guinea pig model could be extended to include studies of: (a) re-infections with RSV; (b) genetic factors influencing the phenomena observed in primary RSV lung infection; (c) the specificity of the host immune response to RSV during acute lung infection; (d) the effects of non-specific inhalational irritants on alveolar macrophage function, airway inflammation and airway hyperresponsiveness in guinea pigs with coexistent RSV lung infection. The guinea pig model is now sufficiently well established to examine whether second or third RSV infections produce progressively milder disease (as is apparently the case for humans). Concerning the role of genetic factors of guinea pigs in primary RSV lung infection, the study of airway histology and lung mechanics using inbred animals such as the BE strain (this strain mounts a poor immune response to most antigens) or the PCA strain (a model of cutaneous anaphylaxis) (206) may yield new information concerning the relative roles of RSV itself vs. the host immune response to RSV in the pathogenesis of acute bronchiolitis. Unfortunately, inbred strains of guinea pigs tend to breed poorly and thus may not be readily available.

Concerning whether the host immune response to RSV is virus-specific in guinea pigs, a threefold approach testing for RSV-specific cytotoxic T-lymphocytes, RSV-specific antibodies and RSV-specific T-cell memory (the ^{3}H-thymidine incorporation assay) may be used. In contrast to previous reports, the evaluation of guinea pig specimens on day 6 post-inoculation will provide a new perspective about the nature and extent of virus-specific immunity during the acute phase of primary RSV lung infection, when bronchiolar inflammation is maximal.

The possible deleterious effects of RSV on alveolar macrophage function may be investigated as previously described for parainfluenza virus (114). Of potentially greater interest is testing the extent of airway inflammation and airway hyperresponsiveness in RSV-infected guinea pigs following challenge with such non-specific environmental agents as

cigarette smoke or inhaled allergens because the combination of RSV infection with non-specific environmental agents may be synergistic (113). Since it is not feasible to perform such studies on humans, the guinea pig model is appropriate for such investigations.

Perhaps the most exciting application of the methods and results of this thesis is a rationale to investigate human asthmatic patients for evidence of persistent RSV lung infection. In particular, the immunohistochemistry and RT-PCR techniques could be used to study human alveolar macrophages obtained by bronchoalveolar lavage (BAL) (281, 282). A less invasive approach would involve testing cells obtained from nasopharyngeal aspirates for evidence of persistent RSV upper respiratory tract infection in children with asthma. In contrast to the guinea pig, it is plausible that repeated episodes of aspiration of RSV from the upper respiratory tract (either as recurrent or relapsing infections) may play a role in the pathogenesis of chronic airway inflammation in childhood asthma because humans are natural hosts to RSV. Although sampling of nasopharyngeal aspirates is less direct than sampling lung cells by BAL, the technical ease and high patient tolerance of nasopharyngeal sampling would facilitate the rapid acquisition of a sufficient number of specimens to permit epidemiologically relevant interpretations, e.g., the prevalence of RSV in the upper respiratory tract in asthmatic patients vs. normal subjects.

In conclusion, investigators have long postulated a role of viral lung infections in the pathogenesis of asthma; however, it is only with the advent of improved, highly sensitive and specific techniques such as RT-PCR that this possibility can realistically begin to be examined. This thesis used the guinea pig to show how the combination of "classical" and contemporary experimental approaches may yield intriguing new information about the natural history of intrapulmonary virus from the time of known primary infection. The application of these techniques to the study of human asthma is now feasible and may culminate in an improved understanding of the role of pulmonary viral infections in the pathogenesis of asthma.

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APPENDIX A: EXPERIMENTAL DATA FOR ALL GUINEA PIGS STUDIED

LEGEND: Note: Blank spaces indicate that no data are available.

ANIMAL Unique guinea pig identifier. The first four digits refer to the Experiment number of the UBC Pulmonary Research Laboratory. The next two digits refer to the animal number within the experiment. For example,

"174203.000" designates Experimental number 1742, Animal 3.

Experiment numbers are as follows:

1742	"historical" controls	juevnile guinea pigs	unmanipulated
1755	day 6	juvenile guinea pigs	low dose RSV
1791	day 6	juvenile guinea pigs	high dose RSV
1830	day 6	adolescent guinea pigs	low dose RSV
1779	day 14	juvenile guinea pigs	low dose RSV
1889	day 60	juvenile guinea pigs	low dose RSV
1950	day 125	juvenile guinea pigs	low dose RSV
1979	day 6 (RT-PCR)	juvenile guinea pigs	low dose RSV

GROUP Designates unique groups of guinea pigs (i.e., handled in the same manner for the same experiment).

RSV RSV inoculation status: (1=uninfected controls; 2= RSV-inoculated animals)

BODYWTI Body weight (g) on the day of the inoculation procedure.

BODYWTF Body weight (g) on the study day.

LUNGWT Lung wet weight (g) on the study day.

LBRATIO Lung to body weight ratio on the study day (= LUNGWT/BODYWTF)

EPITH Histological score for epithelial necrosis (n=10 airways)
MONO Histological score for mononuclear cell infiltrates (n=10 airways)
PMN Histological score for polymorphonuclear cell infiltrates (n=10 airways)
EDEMA Histological score for airway wall edema (n=10 airways)
BALT Histological score for hyperplasia of bronchus-associated lymphoid tissue (n=10 airways)
GOBLET Histological score for goblet cell metaplasia (n=10 airways)

CULTURE Results of one month viral culture (1=no CPE; 2.xxx=positive CPE, xxx = number of days post-plating onto HEp-2 cells until CPE was observed).

For Expt # 1979, values express the number of pfu/g wet weight fresh lung.

TEM Results of transmission electron microscopy (1=negative; 2=positive)

IMMUNO Results of immunohistochemistry (1=negative; 2=positive)

RT-PCR Results of RT-PCR for RSV genomic RNA (1=negative; 2=positive)

ANIMAL	GROUP	RSV	BODYWTI	BODYWTF
174203.000	1.000	1.000	.	.
174206.000	1.000	1.000	.	.
174211.000	1.000	1.000	.	.
174212.000	1.000	1.000	.	.
174218.000	1.000	1.000	.	.
174219.000	1.000	1.000	.	.
175501.000	2.000	1.000	.	433.000
175502.000	2.000	1.000	.	.
175503.000	2.000	1.000	.	438.000
175504.000	3.000	2.000	.	441.000
175505.000	2.000	1.000	.	383.000
175506.000	2.000	1.000	.	380.000
175507.000	3.000	2.000	.	422.000
175508.000	3.000	2.000	.	428.000
175509.000	3.000	2.000	.	387.000
175510.000	2.000	1.000	.	.
175511.000	2.000	1.000	.	427.000
175512.000	3.000	2.000	.	382.000
175513.000	3.000	2.000	.	370.000
175514.000	3.000	2.000	.	382.000
175515.000	2.000	1.000	.	399.000
175516.000	2.000	1.000	.	429.000
175517.000	2.000	1.000	.	407.000
175518.000	3.000	2.000	.	378.000
175519.000	3.000	2.000	.	367.000
175520.000	3.000	2.000	.	394.000
179101.000	4.000	1.000	281.000	321.000
179104.000	5.000	2.000	302.000	324.000
179107.000	4.000	1.000	289.000	322.000
179110.000	5.000	2.000	277.000	307.000
179113.000	4.000	1.000	294.000	315.000
179114.000	4.000	1.000	288.000	327.000
179115.000	5.000	2.000	292.000	331.000
179116.000	5.000	2.000	289.000	316.000
179117.000	4.000	1.000	323.000	337.000
179118.000	4.000	1.000	324.000	355.000
179119.000	5.000	2.000	316.000	351.000
179120.000	5.000	2.000	296.000	328.000
179128.000	4.000	1.000	316.000	350.000
179129.000	4.000	1.000	326.000	352.000
179132.000	5.000	2.000	312.000	345.000
179133.000	5.000	2.000	324.000	362.000
179136.000	4.000	1.000	331.000	374.000
179137.000	4.000	1.000	312.000	341.000
179138.000	5.000	2.000	326.000	359.000
179139.000	5.000	2.000	294.000	333.000
183001.000	6.000	1.000	.	518.000
183002.000	6.000	1.000	.	527.000
183003.000	6.000	1.000	.	497.000
183004.000	6.000	1.000	.	503.000
183005.000	6.000	1.000	.	533.000

ANIMAL	GROUP	RSV	BODYWTI	BODYWTF
183006.000	6.000	1.000	.	526.000
183007.000	6.000	1.000	.	501.000
183008.000	6.000	1.000	.	488.000
183009.000	6.000	1.000	.	539.000
183019.000	7.000	2.000	.	527.000
183020.000	7.000	2.000	.	506.000
183021.000	7.000	2.000	.	541.000
183022.000	7.000	2.000	.	521.000
183023.000	7.000	2.000	.	522.000
183032.000	7.000	2.000	.	545.000
183033.000	7.000	2.000	.	514.000
183034.000	7.000	2.000	.	505.000
183035.000	7.000	2.000	.	513.000
183036.000	7.000	2.000	.	499.000
177901.000	8.000	1.000	.	.
177902.000	8.000	1.000	.	.
177903.000	8.000	1.000	.	495.000
177904.000	9.000	2.000	.	473.000
177905.000	9.000	2.000	.	427.000
177906.000	8.000	1.000	.	475.000
177907.000	8.000	1.000	.	.
177908.000	9.000	2.000	.	532.000
177909.000	9.000	2.000	.	469.000
177910.000	8.000	1.000	.	491.000
177911.000	8.000	1.000	.	449.000
177912.000	9.000	2.000	.	438.000
177913.000	9.000	2.000	.	434.000
177914.000	9.000	2.000	.	487.000
177915.000	8.000	1.000	.	470.000
177916.000	8.000	1.000	.	434.000
177917.000	9.000	2.000	.	518.000
177918.000	9.000	2.000	.	407.000
177919.000	9.000	2.000	.	451.000
188901.000	10.000	1.000	383.000	563.000
188902.000	10.000	1.000	336.000	643.000
188903.000	10.000	1.000	352.000	643.000
188904.000	10.000	1.000	340.000	611.000
188905.000	10.000	1.000	380.000	584.000
188906.000	10.000	1.000	366.000	655.000
188907.000	11.000	2.000	342.000	576.000
188908.000	11.000	2.000	358.000	703.000
188909.000	11.000	2.000	365.000	660.000
188910.000	11.000	2.000	357.000	635.000
188911.000	11.000	2.000	369.000	648.000
188912.000	10.000	1.000	.	746.000
188913.000	10.000	1.000	.	675.000
188914.000	10.000	1.000	.	768.000
188915.000	10.000	1.000	.	704.000
188916.000	10.000	1.000	.	681.000
188917.000	11.000	2.000	.	627.000
188918.000	11.000	2.000	366.000	699.000
188919.000	11.000	2.000	343.000	746.000

ANIMAL	GROUP	RSV	BODYWTI	BODYWTF
188920.000	11.000	2.000	366.000	609.000
188921.000	11.000	2.000	409.000	600.000
195001.000	12.000	1.000	340.000	722.000
195002.000	13.000	2.000	383.000	845.000
195003.000	13.000	2.000	360.000	757.000
195004.000	13.000	2.000	390.000	869.000
195005.000	13.000	2.000	419.000	835.000
197903.000	15.000	2.000	299.000	331.000
197905.000	15.000	2.000	292.000	317.000
197906.000	15.000	2.000	286.000	324.000
197907.000	15.000	2.000	292.000	331.000
197919.000	14.000	1.000	328.000	379.000
197920.000	14.000	1.000	309.000	331.000

ANIMAL	LUNGWT	LBRATIO	EPITH	MONO
174203.000	.	.	2.000	0.000
174206.000	.	.	4.000	2.000
174211.000	.	.	1.000	0.000
174212.000	.	.	2.000	0.000
174218.000	.	.	7.000	1.000
174219.000	.	.	4.000	2.000
175501.000	1.610	0.372	2.000	0.000
175502.000	1.250	.	.	.
175503.000	1.620	0.370	4.000	0.000
175504.000	1.730	0.392	7.000	14.000
175505.000	1.600	0.418	1.000	1.000
175506.000	1.630	0.429	4.000	2.000
175507.000	2.180	0.512	4.000	0.000
175508.000	1.600	0.374	8.000	18.000
175509.000	1.510	0.390	9.000	12.000
175510.000	2.300	.	1.000	0.000
175511.000	1.820	0.426	2.000	4.000
175512.000	1.640	0.429	17.000	13.000
175513.000	1.500	0.405	10.000	9.000
175514.000	1.640	0.429	8.000	13.000
175515.000	1.470	0.368	4.000	1.000
175516.000	1.440	0.336	1.000	2.000
175517.000	1.420	0.349	2.000	0.000
175518.000	1.290	0.341	5.000	0.000
175519.000	1.450	0.395	5.000	9.000
175520.000	1.520	0.386	2.000	2.000
179101.000	1.270	0.396	3.000	1.000
179104.000	1.200	0.370	9.000	4.000
179107.000	1.110	0.345	5.000	2.000
179110.000	1.130	0.368	7.000	4.000
179113.000	1.070	0.340	5.000	4.000
179114.000	1.330	0.407	4.000	3.000
179115.000	1.110	0.335	9.000	4.000
179116.000	1.190	0.376	6.000	2.000
179117.000	1.160	0.344	2.000	1.000
179118.000	1.120	0.315	2.000	1.000
179119.000	1.370	0.390	8.000	3.000
179120.000	1.120	0.341	8.000	5.000
179128.000	1.050	0.300	1.000	0.000
179129.000	1.220	0.347	7.000	3.000
179132.000	1.200	0.348	8.000	3.000
179133.000	1.160	0.320	8.000	5.000
179136.000	1.320	0.353	2.000	0.000
179137.000	1.360	0.399	3.000	2.000
179138.000	1.240	0.364	7.000	5.000
179139.000	1.610	0.483	10.000	5.000
183001.000	2.000	0.386	1.000	1.000
183002.000	2.260	0.429	3.000	1.000
183003.000	2.720	0.547	3.000	0.000
183004.000	2.560	0.509	4.000	0.000
183005.000	2.300	0.431	1.000	0.000
183006.000	2.410	0.458	6.000	10.000

ANIMAL	LUNGWT	LBRATIO	EPITH	MONO
183007.000	2.350	0.469	1.000	0.000
183008.000	2.510	0.514	9.000	6.000
183009.000	2.290	0.425	4.000	1.000
183019.000	2.180	0.414	8.000	8.000
183020.000	2.190	0.405	10.000	8.000
183021.000	2.130	0.409	2.000	0.000
183022.000	2.540	0.488	1.000	0.000
183023.000	2.480	0.475	1.000	4.000
183032.000	2.370	0.435	1.000	0.000
183033.000	2.310	0.449	1.000	0.000
183034.000	2.420	0.479	1.000	2.000
183035.000	2.300	0.448	1.000	0.000
183036.000	2.290	0.459	6.000	3.000
177901.000	.	.	5.000	1.000
177902.000	.	.	2.000	1.000
177903.000	2.490	0.503	5.000	3.000
177904.000	2.140	0.452	2.000	3.000
177905.000	2.360	0.553	4.000	3.000
177906.000	2.670	0.562	2.000	0.000
177907.000	2.360	.	2.000	3.000
177908.000	1.640	0.308	2.000	1.000
177909.000	1.460	0.311	4.000	3.000
177910.000	1.310	0.267	4.000	1.000
177911.000	1.550	0.345	4.000	2.000
177912.000	1.400	0.320	7.000	3.000
177913.000	1.760	0.406	2.000	0.000
177914.000	1.710	0.351	7.000	0.000
177915.000	1.420	0.302	1.000	0.000
177916.000	1.470	0.339	6.000	0.000
177917.000	1.590	0.307	2.000	0.000
177918.000	1.510	0.371	12.000	6.000
177919.000	1.430	0.317	4.000	2.000
188901.000	2.310	0.410	2.000	1.000
188902.000	1.700	0.264	5.000	0.000
188903.000	1.570	0.244	1.000	0.000
188904.000	1.500	0.245	3.000	2.000
188905.000	1.930	0.330	7.000	2.000
188906.000	1.500	0.229	3.000	0.000
188907.000	1.600	0.278	4.000	1.000
188908.000	1.700	0.242	6.000	2.000
188909.000	1.700	0.256	6.000	0.000
188910.000	3.670	0.578	6.000	1.000
188911.000	3.730	0.576	4.000	3.000
188912.000	3.900	0.523	6.000	4.000
188913.000	3.960	0.587	3.000	1.000
188914.000	4.200	0.547	6.000	2.000
188915.000	4.700	0.668	3.000	1.000
188916.000	4.500	0.662	4.000	2.000
188917.000	5.700	0.909	0.000	1.000
188918.000	4.000	0.572	9.000	2.000
188919.000	3.100	0.416	2.000	0.000
188920.000	1.900	0.312	5.000	6.000

ANIMAL	LUNGWT	LBRATIO	EPITH	MONO
188921.000	4.100	0.683	4.000	6.000
195001.000	2.870	0.397	2.000	1.000
195002.000	3.310	0.392	3.000	0.000
195003.000	2.640	0.349	3.000	0.000
195004.000	2.520	0.290	2.000	0.000
195005.000	3.800	0.455	4.000	0.000
197903.000	1.190	0.360	9.000	4.000
197905.000	1.180	0.372	6.000	0.000
197906.000	1.110	0.343	5.000	2.000
197907.000	1.170	0.353	7.000	3.000
197919.000	1.410	0.372	3.000	1.000
197920.000	1.120	0.338	2.000	0.000

ANIMAL	PMN	EDEMA	BALT	GOBLET
174203.000	4.000	1.000	0.000	10.000
174206.000	3.000	6.000	0.000	5.000
174211.000	2.000	2.000	0.000	8.000
174212.000	5.000	0.000	1.000	10.000
174218.000	5.000	7.000	1.000	4.000
174219.000	5.000	5.000	1.000	6.000
175501.000	3.000	0.000	0.000	11.000
175502.000	.	.	.	.
175503.000	6.000	2.000	2.000	5.000
175504.000	9.000	7.000	6.000	14.000
175505.000	3.000	1.000	5.000	5.000
175506.000	8.000	6.000	2.000	10.000
175507.000	5.000	4.000	0.000	13.000
175508.000	14.000	10.000	7.000	13.000
175509.000	7.000	7.000	11.000	12.000
175510.000	4.000	7.000	0.000	10.000
175511.000	7.000	7.000	2.000	16.000
175512.000	14.000	13.000	6.000	8.000
175513.000	11.000	6.000	6.000	13.000
175514.000	10.000	11.000	8.000	9.000
175515.000	5.000	4.000	2.000	9.000
175516.000	5.000	5.000	1.000	10.000
175517.000	5.000	1.000	0.000	9.000
175518.000	8.000	8.000	1.000	12.000
175519.000	9.000	11.000	3.000	11.000
175520.000	4.000	1.000	1.000	7.000
179101.000	3.000	5.000	0.000	7.000
179104.000	9.000	7.000	1.000	8.000
179107.000	4.000	5.000	1.000	9.000
179110.000	4.000	4.000	2.000	9.000
179113.000	6.000	7.000	4.000	4.000
179114.000	2.000	6.000	3.000	6.000
179115.000	5.000	6.000	2.000	7.000
179116.000	6.000	7.000	2.000	8.000
179117.000	2.000	3.000	0.000	7.000
179118.000	0.000	3.000	1.000	8.000
179119.000	6.000	6.000	1.000	8.000
179120.000	10.000	7.000	3.000	8.000
179128.000	0.000	4.000	1.000	7.000
179129.000	4.000	9.000	2.000	8.000
179132.000	8.000	4.000	1.000	6.000
179133.000	8.000	6.000	2.000	8.000
179136.000	2.000	5.000	0.000	7.000
179137.000	4.000	6.000	1.000	8.000
179138.000	8.000	4.000	2.000	9.000
179139.000	11.000	8.000	3.000	12.000
183001.000	1.000	2.000	3.000	7.000
183002.000	2.000	3.000	0.000	4.000
183003.000	0.000	3.000	0.000	3.000
183004.000	2.000	1.000	3.000	5.000
183005.000	0.000	1.000	0.000	2.000

ANIMAL	PMN	EDEMA	BALT	GOBLET
183006.000	7.000	6.000	5.000	13.000
183007.000	0.000	1.000	1.000	4.000
183008.000	5.000	9.000	3.000	6.000
183009.000	2.000	4.000	4.000	4.000
183019.000	8.000	6.000	7.000	5.000
183020.000	6.000	7.000	2.000	9.000
183021.000	4.000	1.000	2.000	3.000
183022.000	2.000	2.000	1.000	3.000
183023.000	4.000	4.000	3.000	2.000
183032.000	1.000	2.000	0.000	1.000
183033.000	3.000	1.000	2.000	1.000
183034.000	4.000	4.000	3.000	2.000
183035.000	3.000	1.000	1.000	3.000
183036.000	5.000	7.000	0.000	6.000
177901.000	5.000	6.000	2.000	11.000
177902.000	0.000	1.000	2.000	10.000
177903.000	4.000	11.000	4.000	13.000
177904.000	7.000	7.000	3.000	15.000
177905.000	6.000	6.000	3.000	5.000
177906.000	2.000	3.000	2.000	12.000
177907.000	3.000	3.000	4.000	11.000
177908.000	1.000	4.000	2.000	7.000
177909.000	7.000	6.000	1.000	9.000
177910.000	6.000	4.000	3.000	11.000
177911.000	5.000	7.000	5.000	9.000
177912.000	10.000	7.000	3.000	10.000
177913.000	3.000	1.000	0.000	10.000
177914.000	6.000	4.000	2.000	10.000
177915.000	2.000	0.000	1.000	12.000
177916.000	4.000	3.000	4.000	15.000
177917.000	4.000	4.000	2.000	13.000
177918.000	8.000	15.000	4.000	13.000
177919.000	4.000	6.000	2.000	10.000
188901.000	1.000	1.000	1.000	13.000
188902.000	1.000	2.000	0.000	17.000
188903.000	1.000	1.000	0.000	11.000
188904.000	1.000	1.000	0.000	10.000
188905.000	5.000	5.000	1.000	7.000
188906.000	1.000	2.000	1.000	12.000
188907.000	6.000	1.000	1.000	5.000
188908.000	7.000	5.000	2.000	9.000
188909.000	2.000	1.000	2.000	14.000
188910.000	6.000	4.000	0.000	2.000
188911.000	4.000	9.000	1.000	6.000
188912.000	5.000	5.000	1.000	3.000
188913.000	3.000	6.000	0.000	4.000
188914.000	5.000	5.000	0.000	2.000
188915.000	1.000	5.000	0.000	0.000
188916.000	3.000	3.000	1.000	4.000
188917.000	4.000	4.000	0.000	7.000
188918.000	10.000	7.000	4.000	10.000

ANIMAL	PMN	EDEMA	BALT	GOBLET
188919.000	1.000	1.000	0.000	7.000
188920.000	7.000	7.000	2.000	10.000
188921.000	8.000	10.000	3.000	5.000
195001.000	1.000	0.000	0.000	0.000
195002.000	2.000	1.000	0.000	0.000
195003.000	1.000	1.000	0.000	0.000
195004.000	2.000	1.000	1.000	0.000
195005.000	3.000	0.000	1.000	0.000
197903.000	8.000	5.000	1.000	10.000
197905.000	8.000	2.000	0.000	7.000
197906.000	7.000	5.000	1.000	8.000
197907.000	6.000	5.000	2.000	8.000
197919.000	3.000	2.000	0.000	6.000
197920.000	2.000	1.000	0.000	5.000

ANIMAL	CULTURE	TEM	IMMUNO	RT-PCR
174203.000	1.000	.	.	.
174206.000	1.000	.	.	.
174211.000	1.000	.	.	.
174212.000	1.000	.	.	.
174218.000	1.000	.	.	.
174219.000	1.000	.	.	.
175501.000	1.000	.	1.000	.
175502.000	1.000	.	1.000	.
175503.000	1.000	.	1.000	.
175504.000	2.007	1.000	2.000	.
175505.000	1.000	.	1.000	.
175506.000	1.000	.	1.000	.
175507.000	2.006	1.000	1.000	.
175508.000	2.006	1.000	1.000	.
175509.000	2.004	1.000	2.000	.
175510.000	1.000	.	1.000	.
175511.000	1.000	.	1.000	.
175512.000	2.005	2.000	1.000	.
175513.000	2.005	1.000	2.000	.
175514.000	2.005	1.000	2.000	.
175515.000	1.000	.	1.000	.
175516.000	1.000	.	1.000	.
175517.000	1.000	.	1.000	.
175518.000	2.007	1.000	2.000	.
175519.000	1.000	2.000	2.000	.
175520.000	2.007	1.000	2.000	.
179101.000	1.000	.	1.000	.
179104.000	2.004	.	2.000	.
179107.000	1.000	.	1.000	.
179110.000	2.006	.	2.000	.
179113.000	1.000	.	1.000	.
179114.000	1.000	.	1.000	.
179115.000	2.005	.	1.000	.
179116.000	2.007	.	2.000	.
179117.000	1.000	.	1.000	.
179118.000	1.000	.	1.000	.
179119.000	1.000	.	2.000	.
179120.000	2.006	.	1.000	.
179128.000	1.000	.	1.000	.
179129.000	1.000	.	1.000	.
179132.000	2.006	.	2.000	.
179133.000	2.006	.	2.000	.
179136.000	1.000	.	1.000	.
179137.000	1.000	.	1.000	.
179138.000	2.005	.	2.000	.
179139.000	2.005	.	2.000	.
183001.000	.	.	1.000	.
183002.000	.	.	1.000	.
183003.000	.	.	1.000	.
183004.000	.	.	1.000	.
183005.000	.	.	1.000	.
183006.000	.	.	1.000	.

ANIMAL	CULTURE	TEM	IMMUNO	RT-PCR
183007.000	.	.	1.000	.
183008.000	.	.	1.000	.
183009.000	.	.	1.000	.
183019.000	.	.	2.000	.
183020.000	.	.	2.000	.
183021.000	.	.	2.000	.
183022.000	.	.	2.000	.
183023.000	.	.	1.000	.
183032.000	.	.	1.000	.
183033.000	.	.	1.000	.
183034.000	.	.	2.000	.
183035.000	.	.	1.000	.
183036.000	.	.	1.000	.
177901.000	1.000	.	1.000	.
177902.000	1.000	.	1.000	.
177903.000	1.000	.	1.000	.
177904.000	2.006	1.000	1.000	.
177905.000	1.000	.	2.000	.
177906.000	1.000	.	1.000	.
177907.000	1.000	.	1.000	.
177908.000	1.000	.	2.000	.
177909.000	2.011	1.000	2.000	.
177910.000	1.000	.	1.000	.
177911.000	1.000	.	1.000	.
177912.000	1.000	.	2.000	.
177913.000	1.000	.	1.000	.
177914.000	1.000	.	2.000	.
177915.000	1.000	.	1.000	.
177916.000	1.000	.	1.000	.
177917.000	1.000	.	1.000	.
177918.000	1.000	.	1.000	.
177919.000	1.000	.	2.000	.
188901.000	1.000	.	1.000	.
188902.000	1.000	.	1.000	.
188903.000	1.000	.	1.000	.
188904.000	1.000	.	1.000	.
188905.000	1.000	.	1.000	.
188906.000	1.000	.	1.000	.
188907.000	1.000	.	1.000	.
188908.000	1.000	.	1.000	.
188909.000	1.000	.	1.000	.
188910.000	1.000	.	2.000	.
188911.000	1.000	.	1.000	.
188912.000	1.000	.	1.000	.
188913.000	1.000	.	1.000	.
188914.000	1.000	.	1.000	.
188915.000	1.000	.	1.000	.
188916.000	1.000	.	1.000	.
188917.000	1.000	.	1.000	.
188918.000	1.000	.	1.000	.
188919.000	1.000	.	1.000	.
188920.000	1.000	.	1.000	.

ANIMAL	CULTURE	TEM	IMMUNO	RT-PCR
188921.000	1.000	.	1.000	.
195001.000	1.000	.	1.000	1.000
195002.000	1.000	.	1.000	2.000
195003.000	1.000	.	1.000	2.000
195004.000	1.000	.	1.000	2.000
195005.000	1.000	.	1.000	1.000
197903.000	2023.000	1.000	2.000	2.000
197905.000	1298.000	1.000	1.000	2.000
197906.000	1554.000	1.000	2.000	2.000
197907.000	1813.000	2.000	1.000	2.000
197919.000	0.000	.	1.000	1.000
197920.000	0.000	.	1.000	1.000

APPENDIX B: STATISTICAL ANALYSES OF AIRWAY HISTOLOGICAL SCORES

DAY 6 STUDY: JUVENILE GUINEA PIGS, LOW DOSE RSV (EXPT # 1755)							
RANK SUM	n	EPITH	MONO	PMN	EDEMA	BALT	GOBLET
RSV	10	139	129	132	126	125.5	117.5
CONTROL	9	51	61	58	64	64.5	72.5
Mann-Whitney *U* statistic		6	14	13	16	19.5	27.5
p value		0.001	0.01	0.008	0.018	0.035	0.15

DAY 6 STUDY: JUVENILE GUINEA PIGS, HIGH DOSE RSV (EXPT # 1791)							
RANK SUM	n	EPITH	MONO	PMN	EDEMA	BALT	GOBLET
RSV	10	153	145.5	150.5	118	124.5	127
CONTROL	10	57	64.5	59.5	92	85.5	83
Mann-Whitney *U* statistic		2	9.5	4.5	37	30.5	28
p value		0.0001	0.002	0.001	0.32	0.13	0.09

DAY 6 STUDY: ADOLESCENT GUINEA PIGS, LOW DOSE RSV (EXPT # 1830)							
RANK SUM	n	EPITH	MONO	PMN	EDEMA	BALT	GOBLET
RSV	10	91.5	102	123.5	103	97	80.5
CONTROL	9	98.5	88	66.5	87	93	109.5
Mann-Whitney *U* statistic		53.5	43	21.5	42	48	64.5
p value		0.46	0.86	0.053	0.80	0.80	0.11

DAY 14 STUDY: JUVENILE GUINEA PIGS, LOW DOSE RSV (EXPT # 1779)							
RANK SUM	n	EPITH	MONO	PMN	EDEMA	BALT	GOBLET
RSV	10	107	111.5	122.5	116.5	104.5	85
CONTROL	9	83	78.5	67.5	73.5	85.5	105
Mann-Whitney *U* statistic		38	33.5	22.5	28.5	59.5	60
p value		0.55	0.33	0.056	0.17	0.22	0.21

DAY 60 STUDY: JUVENILE GUINEA PIGS, LOW DOSE RSV (EXPT # 1889)							
RANK SUM	n	EPITH	MONO	PMN	EDEMA	BALT	GOBLET
RSV	10	121.5	119.5	146	123.5	136	111.5
CONTROL	11	109.5	111.5	85	107.5	95	119.5
Mann-Whitney *U* statistic		43.5	45.5	19	41.5	29	53.5
p value		0.41	0.49	0.015	0.33	0.051	0.92

DAY 6 CONTROL JUVENILE GUINEA PIGS (EXPT # 1755) VS. "HISTORICAL" CONTROLS (EXPT # 1742)							
RANK SUM	n	EPITH	MONO	PMN	EDEMA	BALT	GOBLET
DAY 6	10	77.5	89	94	86.5	96	89.5
HISTORIC	6	58.5	47	42	49.5	40	46.5
Mann-Whitney *U* statistic		37.5	26	21	28.5	19	25.5
p value		0.40	0.64	0.32	0.87	0.21	0.62

DAY 6 JUVENILE GUINEA PIGS: HIGH DOSE RSV VS. LOW DOSE RSV (RSV-INOCULATED ANIMALS FROM EXPT #s 1791 AND 1755)							
RANK SUM	n	EPITH	MONO	PMN	EDEMA	BALT	GOBLET
HIGH DOSE	10	115.5	84.5	90.5	84.5	83	73
LOW DOSE	10	94.5	125.5	119.5	125.5	127	137
Mann-Whitney *U* statistic		39.5	70.5	64.5	70.5	72	82
p value		0.42	0.12	0.27	0.12	0.09	0.015

DAY 6 JUVENILE GUINEA PIGS: CONTROLS FROM HIGH DOSE VS. LOW DOSE RSV STUDIES (CONTROL ANIMALS FROM EXPT #s 1791 and 1755)							
RANK SUM	n	EPITH	MONO	PMN	EDEMA	BALT	GOBLET
HIGH DOSE	10	126.5	122.5	80	124	105.5	84
LOW DOSE	10	83.5	87.5	130	86	104.5	126
Mann-Whitney *U* statistic		28.5	32.5	75	31	49.5	71
p value		0.10	0.18	0.06	0.15	0.97	0.11