The effects of the bovine respiratory syncytial virus on the ciliated epithelium of fetal bovine tracheal organ culture
by Charles Garrett Cantrell

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Veterinary Science
Montana State University
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Abstract:
Histological and ultrastructural studies were undertaken to examine the effects of the bovine respiratory syncytial virus, FS1-1, on the ciliated epithelium of fetal bovine tracheal organ cultures. Data were obtained concerning the functional aspects of replication of the virus and the alterations of cells initiated by the virus infection.

Tracheal sections were removed from culture and fixed at certain time periods up through 22 days in culture for light and transmission electron microscopy. The sections for light microscopy were fixed using each of two fixatives, Bouin's or Telley's and stained with H and E. The sections for electron microscopy were fixed in glutanaldehyde and post-fixed in osmium tetroxide. The thin sections were stained with uranyl acetate and Reynolds lead citrate. Control and viral inoculated cultures were processed simultaneously.

Virus recovered from the tracheal cultures attained a titer approximately equal to the titer of the initial inoculum. The histological changes in the epithelial layer included intracytoplasmic eosinophilic inclusions, multifocal areas of distinct cellular disorgan ization, and ultimately reduction of the epithelium to a single layer of spindle shaped cells. The single cell layer discontinuously covered the basement membrane. Intracytoplasmic inclusions were also observed in the lamina propria adjacent to the basement membrane beneath areas of increased epithelial disruption. Virus-induced changes were also observed in the glandular epithelium within the lamina propria.. The ultrastructural studies demonstrated an increase in number of microvilli, a decrease in number of cilia, chromatin clumping, cytoplasmic clumping and vesiculation, swelling of mitochondria, enlargement and vacuolization of the endoplasmic reticulum, swelling of the Golgi apparatus membranes, and an increase in number of lysosomes. These changes may all be associated with viral infections and are characteristic for paramyxovirus infections. In addition to these changes, spiked virus-like particles, having approximately the dimensions and characteristics of other respiratory syncytial viruses, were observed budding from the cellular membranes.

The results indicate that the bovine RS virus may indeed be one of the more underestimated respiratory pathogens in cattle. Having the tracheal defense functions so reduced the host is left predisposed to further attack by other viral and bacterial respiratory pathogens.

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THE EFFECTS OF THE BOVINE RESPIRATORY SYNCYTIAL VIRUS
ON THE CILIATED EPITHELIUM OF FETAL
BOVINE TRACHEAL ORGAN CULTURE

by

CHARLES GARRETT CANTRELL

A thesis submitted in partial fulfillment
of the requirements for the degree

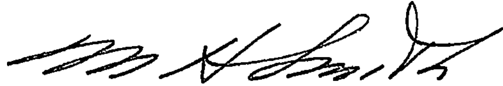
of

MASTER OF SCIENCE

in

Veterinary Science

Approved:



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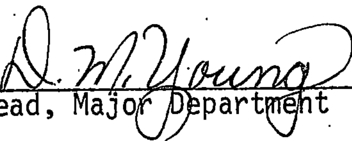

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August, 1979

ACKNOWLEDGMENTS

The author is indebted to Dr. M. H. Smith for his patience and support throughout this study. Dr. Smith has been a teacher, and instilled within the student an appreciation for veterinary medicine and insights far beyond the scope of this project.

Thanks go to Susie Patterson and Jonne Shearman for their technical assistance in the preparation of the slides used in the light microscopy aspects of this study. He would also like to especially thank Drs. Glenwood Epling and William Quinn for their aid in evaluation of the prepared tissues and for their encouragement.

He would like to extend a sincere thanks to his friends and family both near and far for their unwavering support and understanding. Frequent unspoken thanks go to his wife, Laura, for her support throughout this study.

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ABSTRACT

Histological and ultrastructural studies were undertaken to examine the effects of the bovine respiratory syncytial virus, FS1-1, on the ciliated epithelium of fetal bovine tracheal organ cultures. Data were obtained concerning the functional aspects of replication of the virus and the alterations of cells initiated by the virus infection.

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The results indicate that the bovine RS virus may indeed be one of the more underestimated respiratory pathogens in cattle. Having the tracheal defense functions so reduced the host is left predisposed to further attack by other viral and bacterial respiratory pathogens.

CHAPTER 1

INTRODUCTION

Research on respiratory diseases in man as well as in cattle has led to an increased understanding and corresponding interest in the etiology of the clinical diseases. An infection due to a single respiratory virus is rarely fatal, however respiratory viral infections are frequently complicated by multiple viral involvement and secondary bacterial infections. It is therefore rarely possible to determine to what extent the viral or the bacterial infection is responsible in a clinical disease or a given lesion. This is further complicated when trying to evaluate the individual role of a specific virus in the disease complex. It is necessary that each aspect of the complex be understood for that agent's individual role and potential as a contributing part of the multietiological disease in order to most effectively deal with the clinical complex.

Experimentally the respiratory viruses, with some exceptions, invoke only mild clinical responses as compared to the severe clinical manifestations observed in natural infections. Respiratory syncytial virus (RSV) is considered to be the most important respiratory tract pathogen in human infants under the age of 18 months (23). The exact role of bovine respiratory syncytial virus (BRSV) in bovine respiratory disease complex is not so well understood or documented. However, since 1970 bovine respiratory syncytial virus has been

isolated and found to be widespread in Switzerland, Belgium, Japan, England, Australia, and the United States (65, 69, 75, 117, 126, 135, 136, 154).

The difficulty in extrapolating in vitro results to in vivo situations has led to the use of organ cultures. Organ cultures aim at maintaining differentiated cells histologically and functionally intact. In the study of the effect of a given pathogen, organ cultures may reflect the functional state of the cells in the experimental animal more closely than the conventional forms of cell culture.

The purpose of this study was to examine the effects of the bovine respiratory syncytial virus on the ciliated epithelium of fetal bovine tracheal organ cultures. Histological and ultrastructural data were obtained concerning the functional aspects of replication of the virus and the alterations of cells initiated by the virus infection.

CHAPTER 2

LITERATURE REVIEW

Interest and research on bovine respiratory syncytial (RS) viruses began somewhat by chance in 1963 when English investigators studying human RS virus found that the calf serum which they were using in their cell culture medium contained factors that inhibited syncytium production in human RS virus infected cells (41). They determined that these inhibiting factors were specific antibodies, and therefore a very closely related virus must be present in cattle. With this as the beginning of bovine RS virus research it is pertinent to review the work with human RS viruses which laid the foundation for research on the bovine RS virus.

Human RSV

In 1956 Morris, Blunt, and Savage isolated an agent from a colony of chimpanzees experiencing respiratory illness. They named the virus chimpanzee coryza agent (CCA) due to the clinical signs of coryza produced. When one of the laboratory workers developed a moderate respiratory infection, interest and concern about this virus grew. Although the CCA was not recovered from the laboratory worker, there was serological evidence which indicated that it was probably the same virus involved in the chimpanzee and human infections. These findings resulted in a serological survey of different age groups from patients

at the Walter Reed Army Medical Center. There appeared to be a fairly widespread antibody response in the human population. This finding in turn stimulated larger surveys and work directed towards isolating the same or an antigenically similar agent from humans with respiratory disease.

A similar virus was recovered from a child with pneumonia and from an infant with croup in Baltimore in 1956 (27). Serologic studies indicated that infection with the virus occurred commonly in the pediatric population of Baltimore (22, 24). In fact it was shown that seasonal epidemics of human respiratory syncytial virus occur in February and March (22, 86). The isolates produced characteristic syncytia in several types of cell cultures. Because the cytopathogenic effects of the two isolates were indistinguishable from those produced by the CCA and the agents appeared antigenically identical they were grouped together and named respiratory syncytial viruses (27).

As more research was conducted there was sufficient evidence to establish that the RS virus was the greatest single cause of severe respiratory disease, accounting for at least one-fifth of all serious respiratory illnesses of infants (1, 18, 23, 24, 82, 102, 118). Beem and Hamre observed that recovery of RS virus was facilitated when throat-swab specimens were inoculated immediately into tissue culture without prior freezing and thawing; this greatly increased the efficiency in recovering the virus (7).

In adults the virus appears to be associated with a much less severe respiratory illness (77, 78, 106). Several surveys have examined serum samples from all age groups to detect antibodies to the RS visus (1, 51). At the age of five years, and in all older age-groups, over 66 percent of the sera contained both neutralizing and complement-fixing (CF) antibodies. The lowest incidence of antibodies was in the six months to one year age group. A high incidence of infection of RS visus in the population was indicated by the finding of neutralizing antibody in 93 percent of sera from persons over fifteen years of age.

With the recognition of the importance and severity of the RS virus there developed considerable interest and work towards a vaccine against this agent. However, in preparing an effective vaccine there had to be a thorough knowledge of the antigenic composition and extent of antigenic variation of the virus. It also had to be determined if the virus was capable of antigenic drift (30, 32, 46). There were no differences found by using antiserum from infants recovered from RS virus infection (32). Although limited studies utilizing human serum pairs suggested that antigenic variation of RS virus was of little importance in naturally occurring human disease, such variation might assume importance when inactivated vaccines were tested for efficacy in infants (30). In 1966 Coates, et al. conducted a comprehensive study of ten strains of RS virus. Her findings indicated that

antigenic differences do exist but they are all reasonably closely related and re-infection in both children and adults with an antigenically indistinguishable virus occurs commonly (30). There was no indication of antigenic drift in the strains isolated over an eight-year period. During this time they also observed a seasonal relationship to the frequency and severity of the viral infection (31).

In 1969 an inactivated virus vaccine was used in two vaccine trials with children (83, 87). The vaccine against RS virus not only failed to protect the vaccinated children from subsequent natural outbreaks of RS virus infection but produced a more severe clinical illness which resulted in two deaths. Since the serum antibody levels against RS virus were significant at the time of the outbreak, the altered reactivity was completely unexpected. Although there is no apparent explanation, it is suspected that vaccine induced serum antibody, or maternally transmitted serum antibody may in some way contribute to the severity of the illness (18, 23).

As a result of these vaccination trials attention shifted towards the development of a stable temperature sensitive mutant of RS virus. This would be a live attenuated vaccine capable of replicating in the relatively cool temperatures in the upper respiratory tract but unable to cause the more severe lower respiratory tract infection (50). Following preliminary results in hamsters, Wright, et al. (159, 160),

demonstrated protection against wild-type RS virus challenge in adults using a temperature-sensitive mutant virus.

Since 1973 the ts-1 temperature sensitive mutant of RS virus has been investigated extensively both in vitro and in vivo in conjunction with its development and subsequent evaluation as a candidate strain designed for use in a live virus vaccine (59, 81, 85, 103, 158). The mutant ts-1 was shown in vitro to be markedly restrictive in growth at 39 C and unable to form plaques at 37 C and above, whereas wild-type virus grew and formed plaques without restriction at 39 C (50). However ts-1 occasionally exhibited genetic instability as indicated by the emergence of genetically altered virus with decreased temperature sensitivity (59, 85, 132).

Bovine Respiratory Syncytial Virus

With the increased understanding of the ubiquitous nature and severity of the human RS virus, research expanded rapidly in the bovine area when the specific antibodies to the human RS virus were found in bovine sera (41).

In 1979 Paccaud and Jacquier (117) isolated a virus antigenically related to the human RS virus from the conjunctivo-nasal specimens of one animal in each of two farms. Evidence of the bovine origin of both strains was demonstrated by the sero-conversions displayed by the cattle under study in neutralization and complement fixation tests and by some biological traits of the virus as well.

The outbreak of bovine respiratory disease started in 1967 involving several farms in Switzerland. The course of the disease was more severe in older animals, although there was no disease in animals older than seven years of age. The disease lasted from three to ten days with no deaths having been reported.

Neutralization tests clearly showed that the two isolates were serologically identical and that both strains were the same agent. Although the viruses were shown to be of bovine origin, cross neutralization tests with a human RS virus proved that the bovine and human viruses were antigenically closely related but not identical. Further serological studies indicated that one-third of all bovine serums tested contained serum neutralizing antibody to the bovine isolates. Horse serum did not neutralize the bovine viruses. One of the bovine isolates was neutralized by up to 80 percent of the human sera, depending on the age-class sampled; thus further supporting the close relationship of the human and bovine viruses. The investigators concluded that the isolates were bovine respiratory syncytial viruses.

Beginning in 1970, reports on isolating and characterizing bovine RS virus came from a number of different countries, suggesting that the bovine RS virus may be as prevalent as the human virus.

In 1970 Inaba, et al. (69, 71, 72) described an apparently new epizootic respiratory disease in cattle which occurred during 1968 and 1969 in Japan. Their report (69) showed that the virus isolated from

this outbreak was clearly a bovine respiratory syncytial virus. They further demonstrated its close antigenic relationship to the human RS virus. This was the first time a bovine RS virus infection has been reported in Japan.

Also in 1970, Wellemans (154) reported isolating a RS virus from cattle in Belgium. He conducted a serological survey of cattle throughout Belgium and concluded that bovine RS virus was the most frequent pathogen in Belgium associated with respiratory symptoms of the cold season in cattle. He indicated that over 40 percent of the animals tested had an antibody titer to bovine RS virus (152). He later experimented with an attenuated bovine RS virus vaccine (153). This experimental vaccine was available only in Belgium.

Jacobs and Edington (75) during 1971 reported the isolation of a bovine RS virus from calves in Britain. The isolation of a RS virus from calves confirmed the implications of their preliminary serological results which suggested that infection with bovine RS virus was widespread in Britain. In 1975 they were able to isolate bovine RS virus from a four month old calf with fatal pneumonia (76).

Since RS virus had been implicated in several countries it seemed reasonable that this virus might be involved in some of the undiagnosed epizootics of bovine respiratory disease in the United States. In 1974 Smith, Frey, and Dierks reported the isolation and characterization of a RS virus from Iowa feedlot cattle with respiratory disease (135, 136).

Although it was not identical, antigenic comparison studies established the close relationship to a human RS virus. They found the RS virus to be widespread in Iowa, as 81 percent of the animals tested were seropositive, and 36 percent of these cattle seroconverted. In experimental infection studies, Smith, et al. noted that the virus produced a more severe illness in those calves with apparent maternal antibody. This was in agreement with findings that human infants who possess maternally derived humoral antibody, yet lack local secretory antibody, experience the most severe type of respiratory disease during RS virus infection (23, 83, 87).

Rosenquist (126) in Missouri described the isolation and identification of a bovine RS virus in the United States from calves affected with acute respiratory disease. The extent of neutralization of these bovine isolates by an antiserum prepared against a human strain of RS virus was comparable to that reported by investigators in other counties. He observed that the bovine RS viruses were more easily recovered during the colder months of the year, indicating a temporal pattern possibly similar to that reported for human epidemics (31).

Mohanty, et al. (107) found that low levels of serum neutralizing antibody to the virus were prevalent in 38 percent of Maryland cattle. Lehmkuhl and Gough (92) reported 55 percent seroconversion in an Iowa cow-calf herd during a respiratory disease outbreak in 1977. Potgieter

and Aldridge (120) demonstrated that 74 percent of mature cows in 160 Oklahoma herds were seropositive for the RS virus.

Smith and Phillips in the Veterinary Research Laboratory, Montana State University (personal communication), have found the bovine RS virus to be widespread in Montana cattle. Examination of serum antibody levels in three herds in the state indicated widespread exposure to bovine RS virus. Precolostral and postcolostral serum samples were also collected. A substantial number of calves had precolostral antibody levels to bovine RS virus. This indicated either transplacental leakage of bovine RS antibodies or transplacental infection by the RS virus.

Clinical and Experimental Infections

The primary site of virus replication in natural bovine RS infection is unknown. Virus was most commonly recovered from nasal secretions and nasal mucosa of infected calves; less frequently from tracheal mucosa, bronchial mucosae, and lung; and rarely from tonsils (76). In bovine respiratory syncytial virus disease a wide range of pathological changes have been reported. In experimental infections some calves with clinical signs may fail to demonstrate pathological changes at necropsy while other calves show signs similar to natural infection (111). In cases of fatal natural infections, pulmonary consolidation, severe pulmonary emphysema, hemorrhage, tracheobronchial epithelial lesions, and bronchopneumonia were observed (72, 115).

Natural infections of bovine RS virus in cattle frequently run their course unrecognized as inapparent infections, mild respiratory disease, or are misdiagnosed due to concurrent infections. However, in many cases where the virus was isolated, investigators describe more severe respiratory disease. Jacobs and Edington (75) isolated bovine RS from a calf showing increased respiratory rate, serous nasal discharge, pyrexia, and coughing. In a second incident they isolated bovine RS virus from a four month old calf with fatal pneumonia (76). In experimentally infected calves clinical signs included nasal discharge, coughing, and pyrexia. They also were able to successfully infect a gnotobiotic calf with the bovine RS virus which they isolated. The gnotobiotic calf showed a marked biphasic pyrexia (75).

In the 1968-1969 outbreak of bovine RS virus disease in Japan Inaba, et al. (72) reported anorexia, depression, decreased lactation, profuse lacrimation, and abortion. Rosenquist (126) reported clinical signs of fever, increased respiratory rate, and mucopurlent nasal discharge. Lehmkuhl and Gough (92) described central nervous system depression, coughing, and serous rhinitis in calves from an outbreak in Iowa.

Mohanty, et al. (107) exposed calves to intranasal and intratracheal inoculation of bovine RS virus and found a transient rise in body temperature lasting for two days, a mild cough, and serous nasal discharge. Histopathologic changes indicative of mild interstitial

pneumonia along with multinucleated giant cells and eosinophilic inclusions were observed in alveolar lumena. The virus was recovered from infected calves although their serologic response was poor.

Smith, et al. (136) conducted experimental infectivity studies in calves with the bovine RS virus they isolated from an Iowa outbreak. On the fourth day post-infection two of the five calves under study developed a temperature spike and clinical signs involving increased respiratory rate, anorexia, serous nasal discharge, dry muzzle, and malaise. A third calf became ill on the fifth day. Virus was recovered from this calf on the sixth day post-infection. More recently Smith (personal communications) has described mild clinical signs including anorexia, fever, nasal discharge, and coughing in experimentally infected calves. Clinical signs observed included severe respiratory distress, fever, and weight loss in twenty-four calves between the ages of one and twelve weeks. Antibiotic treatment did not diminish the clinical signs. At seven days post-infection the temperature was as high as 108 F and the virus could be recovered from nasal secretions. Most of the exposed animals seroconverted, and developed neutralizing antibodies in nasal secretions.

Host Range

Currently little data are available on the host range of bovine RS virus. Chimpanzees, mice, hamsters, guinea pigs, rabbits, and ferrets

have been experimentally infected with either the human strain or the bovine strain of the RS virus (6, 100). Matumoto, et al. (100) conducted a study of the experimental host range of the Japanese isolate of the bovine RS virus. The virus could be propagated serially in the brain of one day old mice, although the virus yield was low and no illness was observed. However, the virus failed to infect adult mice by various routes of inoculation. Young guinea pigs inoculated with virus intracerebrally, intranasally, intratracheally, or intraperitoneally developed no illness but produced low titers of specific neutralizing antibody, indicating the occurance of an inapparent infection.

Complement-fixing antibodies have been demonstrated in the sera of several groups of dogs indicating the presence of a RS virus or some antigenically related agent (96). Several investigators have shown serological evidence indicating that RS virus is prevalent in sheep (25, 136). Berthiaume, et al. (9) found complement-fixing antibodies in 81 percent of sheep sera tested, 6 percent of horses tested, yet no RS virus antibodies were detected in pigs, goats, and ferrets. Smith, et al. (136) tested bovine sera against bovine RS virus and found antibodies to be widespread in sheep in Iowa. Most recently, Smith (personal communication) isolated a syncytium forming agent, antigenically related to bovine RS virus, from African pygmy goats

following a respiratory disease outbreak in goats at Montana State University.

Characteristics of RS Virus in Culture

Respiratory syncytial virus replicates in a wide variety of host cells. Bovine kidney cells including primary fetal kidney, Georgia bovine kidney (GBK), and Madin Darby bovine kidney (MDBK) are perhaps the most frequently used cell types for bovine RS virus growth; however, cell cultures from lung, testicle, turbinate, trachea, thyroid, thymus, spleen, aorta, synovium, duodenum, and rectum are all susceptible (100, 136). Both human and bovine RS viruses are found to replicate in the above cells types to varying extents. Human RS viruses are most commonly grown in green monkey kidney cells (Vero), HeLa, human epithelial no. 2 cells (HEp-2), human embryo fibroblast cells, and HL cells--a more recently developed sensitive line for isolation of RS virus (2, 21, 100, 114). Both human and bovine RS viruses fail to multiply in embryonated eggs (21, 27). The noticeable cytopathic effect in cell culture is the production of syncytia in many cell lines.

Richman and Tauraso (125) studied the adsorption and growth cycle of RS virus in suspension cultures of HEp-2. They found that the adsorption of RS virus to HEp-2 cells was slow but efficient; more than 98 percent of the inoculated virus was adsorbed by twelve hours. Peak virus titers which occured five days after infection compare

favorably with the highest titers achieved in HEp-2 monolayers. They concluded that continuously agitated suspension cultures might provide a better alternative to monolayers for the production of large amounts of high-titered RS virus pool and complement-fixing antigen.

Jordan (80) examined the growth characteristics of the virus in various cell types, different media, and in media with varying defined components. He observed that the ability of the virus to cause syncytia was dependent to some extent not only on the type of cell culture employed, but also on the composition of the nutrient fluid used to maintain the infected cultures. Bennett and Hamre (8) also noted variations in cell sensitivity to the virus. They confirmed earlier reports of the instability of the virus under storage conditions (7). In studying the temperature and storage stability of the virus they found that the maximum virus titer was reached thirty-six hours after infection. No increase in virus titer after thirty-six hours of incubation, even though CPE was not complete, was interpreted as being an equilibrium point between virus production and inactivation since the virus was rapidly inactivated at 37 C.

Hambling (52) conducted a study on the stability of the RS virus during storage in tissue culture medium under various conditions of pH and temperatures. He found that loss of virus infectivity occured at any temperature during storage; in general the lower the temperature the greater the survival of infective virus. Freezing and thawing

caused a marked loss of infectivity. Raised concentrations of serum in the storage fluid did not increase the survival of infective virus at 4 C or -65 C. The virus was susceptible to changes in the pH of the storage medium; maximum infectivity was maintained at pH 7.5.

Law and Hull (91) also studied methods to enhance the stability of the RS virus in storage. They studied the effects of various concentrations of sucrose upon retention of RS virus viability during storage at different temperatures. A concentration of 44.5 percent sucrose with storage at -70 C appeared to be optimal for retention of infectivity.

Immunofluorescence studies by Kisch, Johnson, and Chanock (88) confirmed observations of the replicative cycle observed by Bennett and Hamre (8). However, in their more detailed observations, they noted specific fluorescence only in the cytoplasm throughout the replicative cycle (88). In agreement with earlier findings (27) they found no evidence of hemagglutination. They noted a perinuclear fluorescence at all stages of infection, but intranuclear fluorescence was never observed. Intracytoplasmic inclusions were visible with May-Grunwald-Giemsa and immunofluorescent staining but not with acridine orange. Mitotic nuclei were never observed in syncytia regardless of the method of staining.

Following the immunofluorescence studies by Kisch, Johnson, and Chanock (88) and the nucleic acid inhibitor experiments by Hamparian,

Hilleman, and Ketler (53), Hornsleth (66) examined the effects of proflavine in accelerating the growth of RS virus. Proflavine exerts an action similar to that of the antibiotic actinomycin D. Both compounds inhibit RNA polymerase as well as DNA polymerase, proflavine being most active against DNA polymerase. Inhibition of either of these enzymes leads to a decrease in the synthesis of RNA. The effect of proflavine in accelerating the growth of RS virus suggests that multiplication of the virus is independent of the DNA-dependent synthesis of RNA in the host cell. This effect of proflavine can possibly be explained as a support in the "take over" process of viral infection (66).

Baldridge and Senterfit (4) described the development of a persistent infection of HEp-2 cells with RS virus. The production of cytopathic effect (CPE) on HEp-2 cells has been found to be dependent upon the passage level of the cell line. Cells infected at higher passage levels are covertly infected and continue to produce large amounts of infectious virus which remains cell-associated. The continued presence of infectious virus in the infected cells on serial passage demonstrates, that under the conditions employed, RS virus is capable of infecting HEp-2 cells without the production of CPE and that on continued passage the cell line remains infected with the virus. They reported that the probability of alteration or mutation of the RS virus in these experiments was minimal and that the factors responsible

for the development of the infection were cell-related. This development of an *in vitro* covert infection suggests the possibility that RS virus in nature might produce covertly infected cells in the respiratory tract resulting in the persistence of residual virus or viral genome in such tissue (4).

Physiocochemical Properties

The RS virus is currently classified with the Pneumovirus genus of the Paramyxoviridae family, however, this has been under debate for several years. The enveloped viral particle has an average diameter range of 90 to 300 nm as measured by electron microscopy of negative stained and ultrathin sectioned preparations. The virus consists of a distinctively fringed lipoprotein envelope surrounding an internal helical nucleocapsid component. No hemagglutinin or neuraminidase has been demonstrated (74).

The replication of the bovine RS virus is inhibited by IUDR (5-Iodo-2'-Deoxyuridine). This is in agreement with other reports on the growth of human RS viruses and indicates that the viral nucleic acid is ribonucleic acid (RNA) (53, 69, 70, 74, 117, 136). RS viruses have been found to be rapidly inactivated at pH 3.0 (52, 53, 69, 70, 117, 136). The virus is readily inactivated at 56 C or 55 C (8, 52, 70, 117), however the rate of inactivation varies slightly from study to study. The half-life of bovine RS virus at 56 C, 2.8 min, is in

agreement with the half-life of 2.8 min reported for a human strain of RS virus (26, 126).

Bovine RS virus showed a buoyant density of 1.225 by CsCl equilibrium density gradient centrifugation (70). This agrees with Coates, Forsyth, and Chanock (33) who determined a density of 1.22 to 1.24 for RS virus of human origin by the same method. The RS virus was found to be sensitive to ether and chloroform (8, 27, 69, 70, 136).

Ultrastructure

Attempts to define the structure of the RS virus have met with the problem of preserving the integrity of a fragile virus particle. Artificial distortion of the virion and its components during purification and concentration procedures has probably been responsible for the slightly conflicting results reported in the literature.

The RS virus has been classified among the myxoviruses on the basis of several biological properties, including its syncytium forming effect in tissue cultures (27), its gross structure (13), its RNA content (53), its ether lability (27), and physicochemical studies of the virus and its degradation products (14, 15). It had, however, not been conclusively demonstrated whether the internal component of RS virus had the characteristic myxovirus-like appearance.

It was becoming increasingly obvious that one of the most important pieces of information that could be obtained about the virus

particle was its morphological structure. Since the application of electron microscopic negative staining to the study of virus particles (17) a large number of viruses have been morphologically characterized and their structural features used as a basis for classification. Myxovirus originally referred to viruses capable of adsorption to red cells, and elution from them by means of the enzyme neuraminidase. Myxoviruses fall into two major morphological groups, those with a structure similar to the influenza viruses and those similar to the parainfluenza virus group (149). Zakstelskaya, et al. (161) argues that RS virus cannot be identified with either of the two sub-groups of myxovirus. The surface projections on the RS viral envelope do not resemble those on either the influenza or parainfluenza group. They concluded that on morphological grounds, having a unique compound helical structure, the RS virus should be classified in a sub-group of its own.

It was proposed that the structure of the nucleocapsid of myxoviruses may be a useful parameter for their separation into subgroups (16). Some early reports (14, 43) dealing with the ultrastructure of the internal component of RS virus had not given any conclusive information about its detailed structure. Bloth and Norrby (16) in an electron microscopic analysis showed that the internal component of RS virus had a "herringbone" like structure similar to that of the paramyxoviruses. There was a regular periodicity along its axis of about

7 nm, and a central canal with a diameter estimated to be 4 nm. This was in agreement with other paramyxoviruses, except for the fact that the pitch was somewhat longer than that of other paramyxoviruses (5 nm). They concluded that the grouping of RS virus together with paramyxoviruses was justified by the structure of the internal component, however, they had several other reservations. The characteristics which distinguished RS virus from other paramyxoviruses were: the extreme lability of the internal component; differences of cytopathological effects in infected cells, features of the syncytium forming activity; and some biological characteristics including the fact that neither hemagglutinating activity nor neuraminidase had been demonstrated (16, 161).

Bloth, Espmark, Norrby, and Gard (13) reported on the pleomorphic character of the RS virus. Many particles appeared spherical, others exhibited elongated or distorted forms in negative stained electron microscopy. They observed the internal component to have an average diameter of 12 nm, however in their next paper they concluded, from more measurements, that the nucleoprotein strands had diameters from 16.0 to 18.0 nm with an average of 17.0 nm.

The overall viral particle diameters have been reported to vary from 80 to 860 nm (13). Joncas, Berthiume, and Pavilanis (79) reported the particle diameters to vary from 80 to 500 nm. They observed spherical, filamentous, as well as pleomorphic forms. Spherical forms

accounted for 47 percent of the virus particles measured and had diameters ranging from 100 nm to 350 nm. The diameter of the nucleoprotein strand was fairly constant, averaging 13.5 nm. The pitch of the helix averaged 6.5 nm. Projections seen on the surface of the viral particle measured 15 nm. They noted the similarity of RS virus to pneumonia virus of mice (PVM) which, however, is known to hemagglutinate mouse red blood cells.

Ito, et al. (73) also noted the great pleomorphism of the RS virus negative stained preparations. Spherical particles varied in diameter from 80 to 450 nm; on the average enveloped spherical viruses covered with spikes measured 200 nm in diameter. The only structural detail visible in intact virions was the outer 7 to 15 nm thick membrane covered with projections. The projections appeared mainly club-shaped, had a length of 13 to 17 nm and a width of 4 to 7 nm; their center to center distance was 7 to 9 nm (73). In ultrathin sections of RS virus infected BK cells they observed the maturation of virus particles displayed a certain pleomorphism; besides spherical particles measuring 80 to 130 nm in diameter there were filamentous forms of 100 to 130 nm in diameter with varying lengths. In the budding particles, distinct dots with a diameter of 11 to 15 nm were observed. These dots were thought to represent cross sections of filamentous components. They concurred with others (35, 79, 114) that the RS virus and PVM should be

classified in a third subgroup of myxovirus, the name of metamyxovirus was suggested (105).

In another report by Berthiaume, Joncas and Pavilanis (10) by negative staining, the diameter of the RS virus particle was found to be 100 to 350 nm. The diameter of the nucleocapsid averaged 13.5 nm and the pitch of the helix was 6.5 nm. The projections of the RS virus were 12 nm in length and 10 nm apart. Their measurements in ultrathin sections agreed with those from negative staining. In addition, when the filamentous forms were cross sectioned the nucleocapsids appeared as dots 12 nm in diameter. Cytoplasmic inclusions containing similar 12 nm structures were observed occasionally contiguous to the cytoplasmic membrane with budding viral particles.

In freeze-etching experiments Bachi and Howe (3) were able to remove the envelope only and expose the convex structure of the core. This revealed distinct structure consisting of oblique striations with a pitch of 6 nm. The striations seemed to form a helical structure with an angle of 60 degrees around the core exposed by the removal of the envelope.

Morphogenesis

The maturation of virus particles occurred at the cytoplasmic membrane or at the membrane of vesicles present within the cytoplasm as observed in ultrathin sections of Vero cells (114). The latter

structures could represent invaginations of the cytoplasmic membrane. Morphological modifications of membrane structures were seen at the surface of cells. A submembraneous occurrence of distinct dots with diameters of 11 to 15 nm were frequently observed. Four to five dots appeared beneath the membrane of individual particles half-way through their budding-off process, however, a maximum number of 10 to 12 dots were seen in separated particles.

Elongated forms budding from the cytoplasmic membrane were also encountered. In their center, they contained filamentous structures; the tips of the filaments often had the appearance of a budding-off virus particle (114).

Berthiaume, et al. (10) described the cytoplasmic inclusions as pleomorphic, and frequently in the vicinity of the nucleus or the cytoplasmic membrane. At higher magnification they had a granular or thread-like appearance.

Bachi and Howe (3) observed that the virus-associated bulging of the cell surface membrane displayed an electron dense structure measuring 4 nm in thickness opposed to the inner leaflet of the cytoplasmic membrane. On the outside of the plasma membrane, budding areas were distinguished by the presence of a fuzzy coat of variable electron density. Ferritin labled antibody to RS virus reacted specifically with these discrete segments. They observed many spherical as well as filamentous particles which did not contain electron dense dots. These

occurred simultaneously with particles containing electron dense dots in infected cultures of Vero cells.

Organ Culture

Undifferentiated cells in cell culture may support virus growth and may show evidence of a CPE, yet the corresponding tissue cells in the intact host may be completely insusceptible. On the other hand, when dispersed in cell tissue culture, cells from the target areas will not always support growth of the appropriate virus.

Organ culture aims at keeping differentiated cells histologically and functionally intact. In the study of the effect of a given virus, organ culture may reflect the functional state of the cells in the experimental animal better than the conventional forms of cell cultures.

Like other tissue culture methods, the organ culture technique allows control of variables (i.e., pH, temperature, humidity, sterility, and nutrients) under bacteria-free conditions, but it also offers an opportunity to study the effects of the virus on the differentiated target cells of the host.

The infection in an organ culture is more complex than that in a monolayer cell culture. Fully differentiated cells are present which presumably vary widely in their susceptibility to the virus. Cultures of respiratory mucous membrane also secrete and transport mucus. However, the absence in organ cultures of a circulation bearing both

humoral factors (including antibody), and inflammatory and immune cellular responses implies that the complexity of the infection falls far short of that which actually occurs in vivo. Organ cultures are structurally similar to the natural in vivo host target organ and may bear on the mechanisms operating in vivo. It seems probable that persistent infection commonly occurs if organ cultures can be maintained in a healthy state for a sufficiently long time.

With organ culture techniques one should be able to determine mechanisms of viral infection at the cellular level while maintaining differentiated cells in their normal structural relationship. Tissues from one animal can be used both as the principal and the control in the same experiment, thus eliminating some biological variation. Using virus infected organ cultures as the model system, it is obvious that one should be able to apply histopathologic, immunofluorescent, electron microscopic, and other procedures to study certain aspects of the pathogenesis of viral diseases. With this in mind a brief history of organ cultues is of interest.

In 1897 Carl-August Ljunggren (94) reported that human skin could survive a few months if stored in ascitic fluid. The viability of the skin explants was demonstrated by reimplantation. This was the first time a piece of tissue has been maintained in vitro.

In 1914, Thompson (140) observed that isolated parts of chicken embryos could undergo "controlled" growth in contrast to the

"uncontrolled" secondary outgrowth of dedefferentiated cells from cut surfaces of explants. Maximow (101) in 1925 studied the different types of tissue culture further and made a distinction between "unorganized" or cytotypic growth and "organized" or organotypic growth.

Carleton (20) also in 1925, was the first to observe ciliated epithelium in culture. This was in explants of cat and rabbit lung, but he found the ciliated cells degenerated within a day or two. He did not observe any ciliary movement. Strelin (48), in 1929, observed ciliary activity using explants of rabbit bronchi. He was able to observe activity for up to nine days. Strelin, as well as most later investigators aiming at maintaining the differentiated state of ciliated epithelium in tissue culture, used the plasma clot technique developed by Fell in 1926.

The first investigator to use a synthetic medium for explant cultures, was Trowell (143) in 1959. He was able to maintain rat trachea for nine days. Up until 1966 the plasma clot technique with medium containing serum was the accepted procedure for virus studies in organ culture. The plasma clot technique imposed time limitations as the lysis of the clot, which usually occurred after a few days, caused the explants to lose their adhesion.

Bertil Hoorn (61) began an investigation in 1961 to develop a reliable method for preparation, maintenance and study of organ

cultures of ciliated epithelium from the respiratory tract and their ability to support growth of known viruses capable of infecting the respiratory tract. Following this, he and Tyrrell (147) reported cultivating a variety of viruses, including enteroviruses, rhinoviruses, adenoviruses, myxoviruses, and paramyxoviruses in organ cultures of respiratory epithelium. In another study they were able to isolate, in organ culture, a virus from a person with respiratory disease, which they were unable to isolate in any other culture system (63).

Tracheal organ cultures have also been used extensively in bacterial studies, especially studies involving various mycoplasma species (28, 34, 44, 49, 67, 68, 99, 138). Organ cultures have also been demonstrated to be a useful technique for the study of mechanisms of local antibody formation and to attempt to "unmask" latent viruses in animal tissues not demonstrable by more conventional means of cell culture (57). Other uses of organ culture have included the testing of vaccines to determine the pathogenicity of certain viral strains and the evaluation of drugs for the treatment of viral diseases (134).

In an effort to evaluate the organ culture as a system for defining the host range of influenza viruses, Schmidt, Maassab, and Davenport (131) conducted studies to characterize, on a comparative basis, several strains of influenza A virus in tracheal organ cultures from various species. They demonstrated that host-virus interactions in

tracheal organ cultures showed a correlation between the natural history of influenza A viruses and their effect on cultures prepared from natural hosts. For homologous interactions involving virus and tissue obtained from the same host group, infection was established at low titer, high yields were obtained, and CPE usually developed. The majority of heterologous infections required higher input doses and resulted in lower yields and no CPE. In comparative experiments involving ferret host systems, virus infection in the tracheal cultures corresponded well with reaction of live ferrets in inoculation of the same virus strains. They concluded that the tracheal organ cultures exhibited a specificity to influenza A viruses, which was clearly a manifestation *in vitro* of the basic susceptibility of the intact host.

Hara, Beare, and Tyrrell (54) also found a strong association between the ability to reduce ciliary activity of human and ferret tracheal organ cultures and the ability to produce human disease. This association was more evident in the human organ cultures than in the ferret organ cultures. The study used human influenza A viruses and recombinants. All the viruses had previously been given to man and their virulence had been defined by clinical observation. Several other investigators also working with influenza viruses have come to the same conclusion; using ferret, mouse, and hamster tracheal organ cultures, the reproduced infectious patterns paralleled those in the live animal (5, 113, 130).

Westerberg, et al. (156) has also used mouse tracheal organ cultures to study influenza virus infections. They described the effects of influenza A virus infection on ciliary activity and histological structure of mouse trachea in organ culture. In a second study Westerberg, et al. (155) employed mouse tracheal organ cultures to study the role of dual infections in respiratory disease, and to examine the effects of the infectious agents on ciliary activity, one of the nonspecific, host defense mechanisms. The infectious agents studied were Mycoplasma pulmonis and influenza A/PR-8 virus.

It was found that in studying the suitability of organ cultures for propagation of influenza A viruses that adult rabbit tracheal cultures failed to support virus growth (60). Tracheal epithelium from adult rhesus monkeys, likewise, did not support the replication of the virus, whereas nasal respiratory epithelium did support growth (62). Organ cultures from adult and embryonic human respiratory epithelium readily supported the growth of influenza A2 (Asian) virus (61).

Tracheal organ cultures have also been used to study murine cytomegalovirus infections in mouse organ cultures (97), and canine adenoviruses in young adult dog tracheal cultures (141).

Several researchers have examined the consistency of the virus infected tracheal organ culture due to the age of the fetus or the host animal (54, 130, 157). They agree that the age of the fetus has no apparent affect on the susceptibility of the organ culture to the viral

infection. No significant differences in virus yield were noted between cultures from four week-old and twelve week-old or from male or female mice (157). Virus production was accelerated in cultures of organs from three-day old mice. This finding was consistent with _in vivo_ studies indicating that respiratory organs from newborn mice produced higher titers of parainfluenza viruses than did respiratory organs from adult mice (37).

Schiff (130) examined different age groups of hamster trachea in regard to their interferon responses to an influenza virus. He saw no significant difference of interferon production; in all age groups maximum interferon levels were detected in the first five days of infection. Craighead (37) was unable to demonstrate interferon in organ cultures of nasal mucosa from guinea pigs infected with PI-3, in contrast to Willems, et al. (157) who found interferon production in Sendai virus infected mouse nasal mucosa cultures.

Cummiskey, et al. (39), in studying a persistent Newcastle disease virus (NDV) infection in embryonic chicken tracheal organ cultures, were unable to detect interferon activity at any time throughout the course of infection or in culture medium harvested from uninfected explants. They were still unable to detect interferon levels after treatment with either of two interferon inducers, poly (IC) or UV-inactivated NDV. Furthermore, no evidence was found to implicate antibody as a necessary factor in the establishment or maintenance of the

persistent infection. Maternal antibody was not required to establish persistence, and the course of infection was similar in explants derived from immunized or nonimmunized flocks. Immunoglobulin-containing cells were not detected by direct immunofluorescence in frozen sections from either infected or control explants harvested at intervals from time of excision through 93 days in culture. IgM and IgA were not detected in the culture medium from either infected or control explants. However, immunoglobulins of the IgY class were present in the initially harvested culture medium. However, once the medium had been renewed IgY was no longer detected. These findings may reflect an immunological incompetence of the embryonic chick trachea (39).

Heuschele and Easterday (57) used chicken tracheal organ cultures to study antibody formation and persistence of virus in the organ cultures when infected with NDV. They provided evidence that there is local production of specific NDV neutralizing substances. The fact that there was no significant activity against an interferon sensitive virus, VSV, suggests that this substance was not interferon, they called it antiviral factor (AVF). They concluded that following exposure of chickens to NDV, whereby the respiratory tract becomes infected, a persistent infection with NDV may become established. In birds exposed by the aerosol route, local formation and secretion of specific antibody occurs in the trachea and probably elsewhere in the respiratory route. The presence of such antibody in secretions of the respiratory tract is probably the major mechanism of resistance to reinfection of the

respiratory tract. The possibility of interference by persistent virus may also be a factor. It has been suggested that the mechanisms involved in the appearance of such secreted antibody may be similar to those described for man (127, 142).

In a following report Heuschele and Easterday confirmed their organ culture investigations (57) with immunofluorescent and histopathologic studies (58). They concluded that formation and secretion of specific antibody does occur in the chicken trachea; and that NDV may persist in the trachea of chickens as long as 120 days following infection of the respiratory tract with NDV administered as an aerosol.

Finkelstein, McWilliams, and Huizenga (45) reported that chicken tracheal resistance to virus infection mediated at least in part by interferon, was dependent upon the route used to administer the interferon inducer. Interferon was not detected in the trachea or serum of protected chickens at the time studied. Antiviral resistance could also be stimulated in the donor chicken by prior immunization. Tracheal resistance developed only in those chickens immunized by the intratracheal route. Resistance correlated with tracheal, but not with serum antibody titer. It was suggested that some of the antibody responsible for the protection was located in superficial sites.

Chick embryo organ cultures may not be as sensitive as the organ cultures of human embryonic nasal and tracheal epithelium for isolation of human respiratory viruses (63, 104, 146), however, because of its

greater availability, it has a definite advantage over cultures of human origin, and consequently may prove to be very useful for routine diagnostic work (11, 12). However, it should be kept in mind that unlike mammals, the chicken possesses small lymphoid accretions scattered throughout its tracheal submucosa. It is therefore possible that sensitized lymphocytes in the trachea might contribute to an immunologically specific immunity (45).

Organ cultures of respiratory tract tissue have been employed in a wide variety of experimental studies involving viruses and bacteria; in addition to the infectious disease oriented studies long-term maintenance of respiratory tissues in organ culture affords a means of evaluating the morphological effects of oncogenic chemicals and the occurrence of malignant transformation in differentiated epithelial cells. However, many investigators have found it difficult to maintain organ cultures of differentiated respiratory tract tissue in a viable state for extended periods of time. With this in mind several studies were undertaken to assess the nutritional factors and culture conditions that might make long-term maintenance possible (44, 98, 110).

Mossman and Craighead (110) reported on the comparative effects of synthetic media on the long-term growth properties and differentiation of the hamster tracheal epithelium in organ culture. They found that differences in the composition of media and the presence or absence of serum had profound effects on the physiologic state of the

tracheal mucosa maintained in vitro. Unique proliferation of epithelial elements was observed in organ cultures maintained in "complex" media containing serum, whereas use of these media without serum produced disorganized epithelial changes resembling squamous metaplasia. Minimum essential media at low serum concentrations preserved the columnar structure of the normal tracheal epithelium for eight weeks and longer in vitro. Observation of cultures in "complex" media with serum suggests that the replicative capacity of the differentiated respiratory epithelial cell is limited.

Marchok, Cone, and Nettesheim (98) reported on the in vitro conditions which allow long term maintenance of viable rat tracheal explants in culture. They were particularly interested in the effects of different concentrations of vitamin A on the maintenance and secretory activity of the tracheal epithelium; and the levels of cellular proliferation during the changes in functional activity and cell differentiation which occur under different nutritional conditions. Marchok, et al. concluded that the condition of vitamin A deficiency (i.e., squamous metaplasia) can be induced in vitro by growing the tracheal explants in a defined, vitamin A - free medium. However, squamous metaplasia can be prevented by very low levels of vitamin A (0.2 ug per ml) present in medium supplemented with 10 percent horse serum.

Engelhardt and Gabridge (44) designed a system to study the induction of squamous metaplasia in hamster trachea organ cultures. In

addition, attention was devoted to the consequences of vitamin A deficiency-induced metaplasia on the attachment of M. pneumoniae to the tracheal epithelium. They concluded that the optimal medium for maintenance of ciliated epithelium in an organ culture system for up to 30 days was minimal essential medium supplemented with 10 precent horse serum and 0.2 ug of retinol per ml.

Gabridge, Agee, and Cameron (48) further described the trachea in culture by examining the differential distribution of ciliated epithelial cells in the trachea of hamsters. They examined, in detail, the nature of the epithelial surface of the hamster trachea to define its basic morphology. Their results indicate that there is a differential distribution of ciliated cells and that the assumption that all hamster tracheal rings are equivalent is not valid. There are significant morphological and metabolic differences in hamster tracheal epithelium that are related to the point of origin or anatomical location. The greatest ciliation in the hamster trachea was observed over the strip of trachealis muscle between the open ends of the cartilaginous rings. Areas with the heaviest ciliation also had the greatest activity of cellular metabolism.

In a more recent study Henderson, Hu, and Collier (56) examined the pathogenesis of human respiratory syncytial virus infection of ferret and fetal human tracheas in organ culture. Although the patterns of virus growth were similar in these species, the sites and morphologic

consequences of virus replication differed markedly. In the human tracheal culture, synthesis of respiratory syncytial virus occurred in a population of ciliated epithelial cells, whereas other cells in the epithelial layer were spared. However, in the ferret trachea, virus growth occurred in fibroblasts of the lamina propria and serosa. Ciliated epithelial cells did not contain viral antigen and remained histologically normal. Virus replication in the human tracheal culture was associated with cell injury characterized by ballooning degeneration and syncytium formation.

Klein and Collier (90) employed hamster tracheal organ cultures as a model for the study of the pathogenesis of human parainfluenza type 3 virus (PI-3) over a two week period. The infected tracheal explants exhibited specific cytopathologic alterations including nuclear swelling and chromatin margination, multinucleated syncytia and binucleated epithelial cells, and fibroblasts and chondrocytes. Focal destruction and denudation of the respiratory epithelium occurred in later stages of infection. The interesting ability of PI-3 to induce changes such as these has been attributed to the presence of virus-induced lysolecithin which causes fusion of cell membranes (90).

Human PI-3 has been propagated in calf tracheal organ culture, yielding moderate increases in viral titer but demonstrating no apparent effect on cellular morphology (61). This is in contrast to the results of Campbell, et al. (19) who were able to demonstrate specific cytologic

changes in calf tracheal organ cultures infected with bovine strain of PI-3 (discussed in more detail later). Craighead (37) reported the use of guinea pig tracheal explants in experiments with human PI-3. Although he was able to demonstrate infection of his preparations he was unable to show definite cytologic changes associated with infection. Later, Craighead and Brennan (38) reported a study of human adult and fetal tracheal organ culture preparations infected with a human strain of PI-3. In these experiments they noted viral replication and specific cytologic alterations. These alterations consisted of nuclear enlargement, chromatin aggregation, cytoplasmic vacuolization, cytoplasmic inclusions, and occasionally a multinucleated cell. These experiments appear to support the concept of species specificity involving both the source of the PI-3 virus and the host origin of the tracheal organ culture explants.

In contrast to the common occurrence of PI-3 infection in cattle, documentation of ultrastructural alterations of respiratory tissues induced by PI-3 is limited. Tsai and Thomson (144, 145) described changes of the respiratory tract including the tracheobronchial tree and the alveolar compartments after PI-3 infection in colostrum deprived calves as well as conventionally reared calves.

Tsai and Thomson (144) observed numerous intracytoplasmic inclusions in the ciliated epithelium of the trachea of the two colostrum deprived calves killed at days 5 and 6 postinoculation. The ciliary

arrangement of these infected epithelial cells did not appear to be disturbed by the presence of apical intracytoplasmic inclusions. However in other areas of the same trachea the cilia were highly disorganized, fragmented, or missing. In severely affectly areas, the mucosal surface was completely or partially denuded of epithelium or was lined by a few exfoliated degenerating cells. The most conspicuous feature, at the ultrastructural level, of the infected epithelial cells was the presence of variously sized aggregates of viral nucleocapsids in the cytoplasm. In some affected areas there were dilated granular endoplasmic reticulum, vesicles, osmiophilic granules (lysosomes), and residual bodies. The nuclei were irregular and deeply indented; they contained aggregates of variably condensed chromatin elements. Other cells that contained aggregates of viral nucleocapsids were seen devoid of cilia but possessed irregular projections of microvilli. Virus budding was rarely observed in infected ciliated epithelium, even in cells that appeared to be in the late stage of infection.

Bovine Tracheal Organ Cultures

Nelson (112) examined the microscopic surface features of tissue samples from three bovine tracheas infected with infectious bovine rhinotracheitis (IBR) virus and compared these with tissue samples from three normal bovine tracheas. By using a scanning electron microscope he was able to observe some pathologic effects of IBR viral infection

involving the epithelial surface of the trachea. The effects of viral infection ranged from partial denuding of tracheal cilia to complete sloughing of the columnar epithelium.

Chia and Savan (29) conducted transmission electron microscopic studies on the distribution of IBR virus in bovine fetal tracheal organ cultures. They observed the viral particles to be present in a variety of cell types among the submucosal and the connective tissue as well as the epithelial components of the tracheal organ cultures.

Shroyer and Easterday (134) studied the site of IBR virus replication by histopathologic and fluorescent antibody procedures. They also examined the effect of the virus on the ciliar activity of the tracheal epithelium with an inverted light microscope. The virus lasted in some cultures for as long as 29 days. High levels of virus were detected throughout 7 serial passages in both bovine fetal and calf tracheal explants. Ciliary activity was abolished in infected tracheal epithelium cultures but remained vigorous in control cultures. Immunofluorescent staining and histopathologic changes occurred only in the epithelial cells. The changes seemed to begin focally and then spread. However, there was considerable variation in the amount of epithelial destruction between explants from the same organ culture. These differences were coincident with the variation of ciliar activity from explant to explant in the same culture.

Explants prepared from the trachea of an immune calf were as susceptible as control cultures to *in vitro* infection with IBR virus. Virus could be recovered for long periods (1 to 2 months) from organ cultures prepared from exposed calves killed during the acute stage of IBR (134).

Rossi and Kiesel (128) reported on the susceptibility of bovine fetal tracheal ring cultures to IBR virus as well as PI-3 virus. Both viruses were found to be cytopathogenic for tracheal organ cultures. Cessation of ciliary activity in all cultures of a group was evident on day 7, post inoculation, for IBR virus, and day 11 for PI-3 virus.

In a scanning electron microscopic study of PI-3 infected bovine tracheal organ cultures, Reed and Boyde (123) found that it was necessary to thoroughly wash the cultures prior to fixing. Without vigorous washing the ciliated surface was obscured to a variable extent by mucus. In cultures inoculated with PI-3, epithelial damage was not detected until the sixth day. Histological studies of bovine tracheal organ cultures infected with PI-3 have shown abnormalities in all layers of the epithelium, with partial loss of ciliated cells (150). In the scanning electron microscopic study, the most striking feature of infected cultures was the mass of microvilli covering the exposed surfaces. The appearance contrasted greatly with the finely granular surface of cells in rhinovirus infected cultures. Bovine PI-3, like other paramyxoviruses, buds from the surface of infected tissue culture cells.

Reed and Boyde's findings show that in infected respiratory epithelium the virus buds from cell surfaces which are profusely covered with microvilli. PI-3 eventually largely destroyed the epithelium of the organ cultures by a lytic infection. The sites of viral maturation could be identified among the microvilli at the time of maximal release of infectious virus into the medium. In noting that the formation of microvilli has been shown to be related to the development of polykaryocytes in virus infected cell cultures they postulated that the ability of the epithelial cells to form microvilli may perhaps be a prerequisite for, or a consequence of, lytic infection with PI-3.

In a study on the propagation and cytomorphological effects of bovine PI-3 Kita, Kenney, and Gillespie (89) reported that the virus grew in organ cultures of bovine fetal trachea and lung, although the titers were not so high as those achieved in monolayer cultures. The uninoculated fetal tracheal organ cultures appeared viable after 7, 9, and 11 days. However, only muscle and cartilage were present in sectioned cultures for days 7 and 11. In the epithelial components pyknosis and karyorrhexis were more prominent in the glandular crypts than in the surface epithelium. The cartilage and muscle appeared normal. After 16 days in culture the surface epithelial cells as well as those of the glands were squamous to cuboidal and tended to stratification. The glandular crypts usually contained necrotic cellular debris.

In virus inoculated cultures after 9 days of culture there was more pyknosis and karyorrhexis in the epithelia than was observed in the control cultures. After 14 days there was some degree of necrosis which involved the glandular epithelial cells in particular, although definite inclusions were not seen. After 19 days in culture many of the glands were cystic and lined by low cuboidal to squamous epithelial cells. The surface was covered by the same type of cells, in addition there was distinct margination of the chromatin.

Another investigation to observe the behavior of bovine PI-3 in tracheal organ cultures from neonatal calves with reference to virus growth and the resultant serial evaluation was conducted by Campbell, Thompson, Leighton, and Penny (19). In the uninfected explants good morphological structure persisted in the epithelium for about three weeks; thereafter the pseudostratified columnar architecture changed first to a low columnar and then to a low squamous type. Generally transformation to a squamous stratified layer occurred. Ciliary movement could be observed for periods up to four weeks.

In the infected cultures the ciliary activity declined rapidly after three days and by the seventh day was undetectable. In a second experiment using a lower titered inoculum the effect was similar but slower, taking place between 7 to 14 days. The cytopathic effects consisted of a reduction in height of the pseudostratified epithelium and

some loss of cilia. The columnar structure was lost as the remaining cells became oriented horizontally in low squamous appearance.

It should be noted that in these investigations Campbell, et al. did not subject the explants to any physical interference other than the periodic addition of fresh medium to the dish. This appeared to have no effect on the superficial epithelium. They did not wash the explants in any manner.

In contrast to the destruction of ciliary activity in tracheal ring cultures caused by infection with PI-3, Rossi and Kiesel (128) were unable to demonstrate destruction of ciliary activity by bovine RS virus. However, the bovine RS virus was able to replicate within ciliated epithelial cells of tracheal rings. Three weeks after inoculation, all cultures of bovine RS virus continued to have beating cilia. Their fluorescent antibody studies indicated that the degree of infection with bovine RS virus decreases with time. When cultures inoculated with bovine RS virus showed evidence of waning infection, other cultures from the same lot were inoculated with the same virus. The latter cultures had the same degree of susceptibility as the initial cultures had when they were first examined. As to why bovine RS virus did not produce a more profound effect on the respiratory epithelium of the trachea, Rossi and Kiesel proposed that the affinity of the virus may be for cells deeper in the respiratory tract.

Smith, Frey, and Dierks infected neonatal calf tracheal organ cultures in an attempt to increase the virus titer (136). They failed to produce a virus titer above the titer of virus grown in cell monolayers, however, many of the organ cultures showed a marked decrease in ciliary activity at 5 and 6 days after inoculation. Although the ciliary activity appeared to return to normal within 24 to 48 hours, virus could be recovered from the organ cultures for as long as 40 days after infection.

In a more recent study, Thomas, Stott, Jebbett, and Hamilton (139) examined the growth characteristics of bovine RS virus in bovine fetal tracheal organ cultures under a variety of experimental conditions. In ten separate experiments carried out under standard conditions maximum titers of virus were detected between 11 and 21 days after inoculation. No significant effect on ciliary activity was seen in any of the experiments. Control cultures showed vigorous activity for at least 2 weeks. Histological changes in the RS virus-infected cultures were slight and involved patchy flattening of the epithelium from a columnar to a cuboidal cell layer and loss of confluence in the ciliated border. Large phloxinophilic intracytoplasmic inclusions were seen occasionally in the loose connective tissue on the outer surface of the tracheal ring. The inclusions increased in number with the age of the culture. They were not readily recognized until 15 to 17 days after inoculation with the RS virus.

Staining with fluorescent antibody showed that the RS virus antigen had a similar distribution to the inclusion bodies. Specific fluorescence was located primarily in the peritracheal connective tissue and less frequently and at a reduced intensity in the epithelium. In addition to this they demonstrated that the age of the fetus from which the tracheal cultures were derived had no influence on the virus growth or the ciliary activity.

Table I is a summary of measurements which are reported in the literature and in the text of the literature review.

Table I. Summary of Reported Measurements in nm.

Structure	Average Range
Diameter of RS viral particle	80 - 300
Length of surface spikes	12 - 17
Distance between spikes	7 - 10
Diameter of spikes	4 - 7
Diameter of nucleoprotein strand	12 - 18
Diameter of cilia*	200 - 300

* Reference (124, 129).

CHAPTER 3

MATERIALS AND METHODS

Virus Stock

The Iowa isolate designated FS1-1 (136), a bovine respiratory syncytial virus, was grown on GT cells until 80 percent of the cell monolayer showed signs of cytopathic effect (CPE). Prior to freezing, 10 percent of a solution of sucrose, phosphate and glucose (SPG) was added to the flask medium. The virus was harvested with cells following a single freeze-thaw cycle. This cell-virus suspension was centrifuged for 15 min at 10,000 RPM. The cellular pellet was discarded and aliquots of the virus suspension were stored at -70 C. These were thawed and used as the inoculating virus suspensions when needed.

Cell Cultures

Low passage goat turbinate (GT) cells received from Dr. K. Gilette at NACD, Ames, Iowa were used for virus cultivation. The cells were maintained in Dulbecco's modification of Eagle's minimum essential medium (MEM) (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10 percent goat serum (GS) collected at Montana State University and filter sterilized. No antibiotics were used in cell culture media. The cells were placed in 250 ml plastic tissue culture flasks having a 75 cm^2 surface area for cell attachment (Falcon plastics, Oxnard, California). After incubation at 37 C in a five percent carbon

dioxide atmosphere the cells readily formed a complete monolayer. The medium was removed, and the cell surface was washed with Rinaldini's enzyme solution (R-saline). The cell surface was then flooded with 2 ml of one percent trypsin (Difco Laboratories, Detroit, Michigan) for approximately ten minutes. After this time the cells were readily dispersed by vigorous agitation and repeated pipetting of 5 ml of MEM. The cells were harvested and passed into new flasks using MEM with ten percent GS for continued culture.

When cells were needed for virus growth, they were passed from the 250 ml flask, as described above, into a 650 ml flask having a surface area of 150 cm^2 for cell attachment (Costar, Cambridge, Massachusetts).

Organ Cultures

The tracheal cultures were maintained in MEM or Dulbecco's F-12 medium supplemented with various concentrations (0%, 2%, 5% and 10%) of fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, N.Y.) in an effort to determine the optimal culture medium. The final medium employed for the maintenance of the ciliated epithelium was MEM supplemented with 10% FBS and 0.2 ug/ml of retinol. The fetal bovines, complete with placenta, were purchased from the local abattoir. The fetus was in the laboratory within 1 to 2 hours of the cows death. The age of the fetuses varied but they were generally in the last trimester of development. The trachea of the fetus was aseptically removed and

placed in MEM supplemented with FBS, 5% antibiotics consisting of penicillin G (100 ug/ml), streptomycin (100 ug/ml) (Pen-strep, Grand Island Biological Co.), and 4 percent amphotericin B (10 ug/ml) (Fungizone, E.R. Squibb and Sons, New York, N.Y.). Under a positive pressure hood excessive fat and tissue were trimmed from the exterior of the trachea. Tracheal rings were then separated with a scalpel and further trimmed. The rings were washed and placed, one ring per chamber, in six-chambered plastic tissue culture trays (Linbro Chemical Co., New Haven, Connecticut). Media was added to each chamber to a level which covered the tracheal ring.

The tracheal rings were washed and placed in new trays with fresh medium daily up to the time of inoculation. Three hours following the virus inoculation the rings were placed in new trays with fresh medium, though not washed. They were then washed and placed in fresh trays daily through day 8 post inoculation (PI). After 8 days PI the FBT rings were washed and given new trays with fresh medium on day 10, day 12, day 15 and day 20 PI. The FBT rings were examined using an inverted Olympus light microscope immediately prior to each washing. The tracheal cultures were maintained at 37 C in a 5 percent carbon dioxide incubator. The medium chosen to wash the BFT rings in was MEM plus 10 percent FBS, 5 percent antibiotics, and 4 percent fungizone diluted 1:1 with R-saline. After the second day in culture the tracheal

cultures were maintained in MEM plus FBS and retinol, however, without antibiotics or fungizone.

The tracheal rings were sectioned and fixed for both light microscopy and transmission electron microscopy at the following intervals:

Initial Control	immediately upon excision of the trachea, prior to being placed in culture medium.
Day 0	two days post-excision, immediately prior to virus inoculation

1 hr PI; 3 hr PI; 6 hr PI; 12 hr PI; 18 hr PI.

Day 1	24 hours PI
Day 2	48 hours PI
Day 3	PI
Day 4	PI
Day 5	PI
Day 6	PI
Day 8	PI
Day 10	PI
Day 12	PI
Day 20	PI

Following the light microscopic examination and wash, the FBT ring to be sectioned was placed in a separate petri-dish with fresh medium. A section of the ciliated epithelium covering the trachealis muscle between the ends of the cartilaginous ring was cut from the tracheal ring under aseptic conditions. This section which was to be prepared

for transmission electron microscopy (TEM) was placed in another petri-dish in Sorenson's phosphate buffer and further sectioned into approximately 0.5 mm cubes under a dissecting light microscope. The remainder of the ring was then cut in half for light microscopy fixation.

On the third day in culture, following the wash and transfer to new trays, 0.5 ml of virus suspension was added to the medium in the center of each tracheal ring in each well. Washing was done by means of fairly vigorous agitation in a 100 ml beaker containing approximately 50 ml of the wash medium.

Light Microscopy

Tracheal sections removed from culture according to the previously noted time schedule were fixed for light microscopy using each of two fixatives, Bouin's or Telley's (51). Following fixation all tissues were stained with hematoxylin and eosin (H and E)(51). Slides were examined using a binocular Leitz orthoplan microscope equipped with a standard light source and camera attachment.

Electron Microscopy

The FBT sections were fixed in 4 percent glutaraldehyde in Sorenson's phosphate buffer plus 0.85 percent CaCl for 2 hours at 4 C in the dark. The sections were then washed in cold Sorenson's buffer three times for 15 min each. Following this they were post-fixed in 1 percent osmium tetroxide in Sorenson's buffer for 4 hours at 4 C.

They were again cold washed three times, 15 min each and then dehydrated stepwise in 30 to 100 percent ethanol. The sections were infiltrated with Spurr's embedding medium (95), and allowed to polymerize at 70 C overnight in beem capsules filled with pure Spurr's.

The ultrathin sections were cut with glass knives on a Reichert OM-U2 ultramicrotome between 60 to 100 nm. Thick sections were cut at approximately 600 to 800 nm, stained with Toluidine blue and previewed on a Zeiss microscope with transmitted bright-field illumination. Thin sections were stained with uranyl acetate and Reynolds lead citrate (55). The grids were examined with a Zeiss EM 9S-2 transmission electron microscope at 60 KV.

Controls

The tracheal rings used as controls were always from the same trachea as the virus infected sections. One set of the control cultures were inoculated with a virus-free suspension of GT cells, 10% SPG and maintenance medium following centrifugation for 15 min at 10,000 RPM. Another set of the control cultures were not inoculated at all. They were maintained and washed in the same manner and processed at the same time as the infected tracheal rings for both light and electron microscopy.

As an intermediate point of reference the medium from several fetal bovine tracheal rings, both infected and non-infected, was removed at 12 days post-inoculation and placed on separate flasks of GT cells.

Virus Titrations

The bovine respiratory syncytial virus was titrated using 24 well plastic tissue culture plates (Linbro Chemical Co., New Haven, Connecticut); each well provided a monolayer of GT cells. All titrations were performed in quadruplicate. Each well was inoculated with 0.1 ml of half log. dilutions of virus suspension and overlayed with 1 percent agar. The plates were allowed to incubate for six days, or until plaques were discernible, at 37 C in a 5 percent carbon dioxide atmosphere. Titers were determined as plaque forming units per milliliter (PFU/ml).

To assay the viral growth in the organ cultures, medium was harvested from the FBT rings at the time intervals when the cultures were washed. The medium was frozen at -70 C and titrated as described above.

All cell culture, organ culture, and viral procedures were conducted in a positive air flow hood.

CHAPTER 4

RESULTS

Culture Characteristics

The FS1-1 isolate of the bovine RSV grew readily on the GT cells attaining a titer of 9.4 x 10^3 PFU/ml. CPE was first noted after 4 days with 80 percent cell involvement by 6-7 days.

Various concentrations (0%, 2%, 5%, and 10%) of fetal bovine serum (FBS) were used in maintenance media of both Dulbecco's F-12 medium and MEM in an attempt to prolong the ciliary activity of the fetal bovine tracheal (FBT) cultures. The FBT cultures showed slightly prolonged ciliary activity when grown in MEM plus 10 percent FBS. One such plate of cultures was maintained, showing strong ciliary activity for nine months, however this was an exception. Most cultures could easily be maintained for up to four weeks. In the serum-free MEM the FBT rings did not generate as much debris on the epithelial surface or in the medium as compared to when they were maintained in media with FBS. However, the ciliary activity was not as strong for as long a period of time as when FBS was added to the medium.

There was a temporary lapse in ciliary activity when the trachea was first sectioned and placed in culture. After the first day the cultures generally showed a strong, fairly uniform ciliary activity as observed under the light microscope. The most consistent ciliary activity was observed over the strip of trachealis muscle between the

ends of the cartilaginous rings. After approximately ten days in culture the cilia werenoted to be shortened and, although still active, beating with reduced vigor.

There was marked variability from section to section of the same trachea. There was also distinct variability from trachea to trachea, although there was no correclation between size or age of the fetus and degree or strength of ciliation.

Light Microscopy:

The FBT rings were each cut in half with one half being fixed in Bouin's solution while the opposite half was fixed in Telley's solution. Both fixatives preserved the delicate ciliated epithelium, however Telley's fixative consistently gave a better preservation of the architecture of the mocosa and submucosa layers. Previously 10 percent buffered formalin had been used with a very limited success in preserving the ciliated epithelium.

Uninoculated Cultures Prior to culturing, the mucosa had a continuous pseudo-stratified columnar epithelium with a full even covering of cilia. The lamina propria contained numerous tubuloacinous glandular structures. After two days of being in culture widely scattered necrosis of individual cells was noted in the mucosa layer adjacent to the basement membrane. These changes were characterized by mild cytoplasmic vacuolization and nuclear pyknosis. The glandular structures

in the lamina propria appeared to be undergoing mild yet widespread degeneration characterized by nuclear pyknosis, cytoplasmic eosinophilia and sloughing of cells into the lumen.

After three days in culture, 24 hrs post RSV inoculation (PI), some cell shrinkage was noted in the deeper layers of the epithelium adjacent to the basement membrane. Perinuclear cytoplasmic vacuolization was mild yet widely disseminated involving many cells of the epithelium. The cilia were observed to be shortened. The lamina propria just beneath the basement membrane was noted to be hypocellular and pale staining. The remainder of the lamina propria was pale and the fibrous stroma was slightly separated. The glandular elements were in varying degrees of continued degeneration, from complete loss of normal architecture to mild invididual cell necrosis.

By the fifth day in culture (3 days PI) there was a smooth transition from the normal ciliated pseudo-stratified columnar cells to areas of less organized cuboidal cells. This formed a thinner epithelial layer covered with cilia which were reduced in number and in length. The glandular structures of the lamina propria were especially reduced in number beneath the areas of cuboidal epithelium. Remaining glandular ducts contained necrotic cellular debris in their lumena.

After seven days in culture (5 days PI) the majority of the epithelium was covered with ciliated tall columnar cells. This blended smoothly with irregular areas of low columnar and cuboidal cells. The

epithelium was several cell layers thick with the exception of a few focal areas of one or two cells in thickness. The cilia were shortened and were markedly reduced in number.

The section taken after eight days in culture (6 days PI) was noted to have an epithelial layer which was mainly covered with ciliated pseudo-stratified columnar cells. The glandular structures of the lamina propria were reduced in number. The remaining structures were moderately necrotic with lumena containing cellular debris.

By the tenth day in culture approximately 50 percent of the epithelium was composed of ciliated pseudo-stratified columnar cells. Some separation of the epithelium from the basement membrane was noted. The section fixed following fourteen days in culture (12 days PI) had an epithelial layer composed of approximately 50 percent pseudo-stratified columnar cells covered with shortened, though numerous cilia. This merged with non-ciliated low columnar cells which joined areas of cuboidal cells.

After twenty-two days in culture (20 days PI) the epithelium was noted to be composed of ciliated, tall pseudo-stratified columnar cells. A single layer of epithelial cells which lay along the basement membrane were noted to be degenerate as characterized by nuclear pyknosis and cytoplasmic eosinophilia. This degeneration was multi-focal involving approximately one third of the epithelium. The lamina propria was hypocellular with a few remaining glandular structure remnants.

Mitotic cells were frequently noted in many of the uninoculated sections throughout the culture period.

RSV Inoculated Cultures The first noteworthy RSV inoculated section was fixed 18 hours PI, after two days and 18 hours in culture. There was no corresponding uninoculated section fixed at this time. Approximately seventy-five percent of the epithelium was covered with ciliated pseudo-stratified columnar epithelium. The ciliated border was discontinuous. There were several focal areas within the ciliated epithelium where the cells were rounded and had lost the pseudo-stratified organization. Some of these rounded areas still had cilia on the border while others did not. The pseudo-stratified columnar cells merged with low columnar and then with cuboidal cells. The cuboidal cell layer had frequent focal areas of rounding and disoranization. Beneath the rounded areas there were increased number of degenerate cells along the basement membrane.

The lamina propria appeared consistent with the control cultures; the glandular structures contained necrotic debris within their lumena.

The 24 hour post RSV inoculated culture had approximately half of the epithelium covered with disorganized columnar cells. These cells had a discontinuous cilial covering. Interspersed among the columnar cells were areas of cuboidal cells varying in thickness from a single cell to several cells. Multifocally throughout the disorganized

columnar and cuboidal areas were areas of cell rounding with increased disorganization.

In the lamina propria several of the glandular structures were noted to be lined by an epithelium which was piled up two or more cells in thickness with large nuclei and multiple nucleoli. The cytoplasmic margins of these cells were indistinct.

The 48 hour post RSV inoculated culture had an epithelium composed of a bilayer of nonciliated cuboidal cells. Interspersed throughout this layer were multifocal nodular aggregates of rounded cells with an occasional cell protruding from the epithelial surface. The cuboidal cells, in one area, blended smoothly with low columnar discontinuously ciliated cells. In another area they blended into tall columnar cells. The tall columnar cells were disorganized, having multifocal areas of rounded aggregates of cells. Contained within the aggregates were noted pocket aggregates of necrotic cells characterized by pyknotic nuclei and shrunken cytoplasm. Several intracytoplasmic, eosinophilic inclusions were noted.

By the third day post RSV inoculation the epithelium was mainly composed of a single layer of cuboidal cells; these merged multifocally with spindle shaped cells. Multifocal nodular aggregates of rounded cells gave the epithelium a somewhat rough appearance. The epithelium was irregularly covered with cilia; these were virtually absent over the flattened spindle shaped cells, yet occurring in clumps in other areas.

Four days following RSV inoculation the epithelium was largely composed of nonciliated cuboidal cells which varied in thickness from one to two cells. There were areas where the cuboidal cells merged with spindle shaped cells which covered the basement membrane as well as areas of pseudo-stratified columnar epithelium. There was a shortened ciliated layer covering the columnar epithelium. Mitotic cells were noted in the cuboidal cell layer and in the columnar epithelium along the basement membrane.

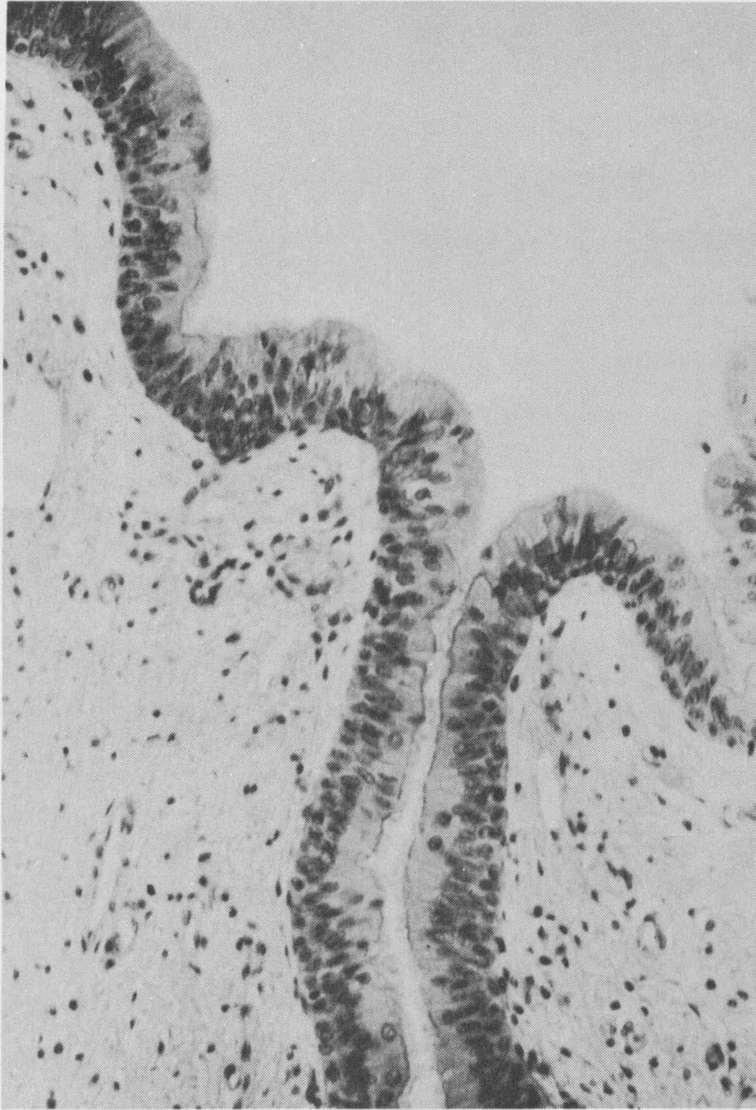
The fifth day post RSV inoculated section had an epithelium which was almost entirely composed of spindle shaped cells stretched over the basement membrane in a single cell layer. The lamina propria was hypocellular; the glandular structures were degenerate and filled with cellular debris. Many of the cells lining the lumen appeared to have undergone nuclear fusion.

By eight days following RSV inoculation the epithelium was reduced to multiple aggregates of cells. The aggregates varied in size from involving several cells to approximately 15 cells. The cells were rounded in shape and many appeared lysed. Between many of the aggregates the lamina propria was covered with the basement membrane alone. Eosinophilic inclusions were observed within the lamina propria, adjacent to the basement membrane beneath epithelial areas showing increased disorganization.

The sections taken 10 days PI had a single layer of spindle shaped cells covering the basement membrane. In multifocal areas the spindle shaped cells were stretched covering the basement membrane yet pulled away from the membrane. On one end of the section the spindle shaped cells merged smoothly with columnar cells. The columnar cells had a shortened ragged covering of cilia. The lamina propria was hypocellular and poor staining. Beneath the spindle cell covering the glandular structures were hypocellular with complete loss of architecture. The glandular structures beneath the columnar cells had remnent cellular structure.

After twelve days PI the epithelium consisted of a single layer of spindle shaped cells, these blended smoothly with a rough layer of cuboidal cells which merged with more organized columnar cells. The columnar cells had a shortened rough covering of cilia. The cells had an increased granularity and multiple nucleoli. Karyolysis was noted in several of the spindle and cuboidal cells. The lamina propria was hypocellular and poor staining with acellular remnants of the glandular structures.

The twenty day PI sections were not very different from the 10 or 12 day PI sections. The epithelium consisted mainly of a single layer of spindle cells. The spindle cells merged with an area of ragged spindle and cuboidal cells interspersed together. This area was disorganized and the cells frequently overlapped forming bilayers, and in multifocal areas were separated from the basement membrane.



Figures 1. Uninfected fetal bovine tracheal (FBT) tissue fixed prior to culturing. Uncultured control.

Fig. 1-a. Note: continuous pseudostratified columnar epithelium, full covering of cilia. 880 x.

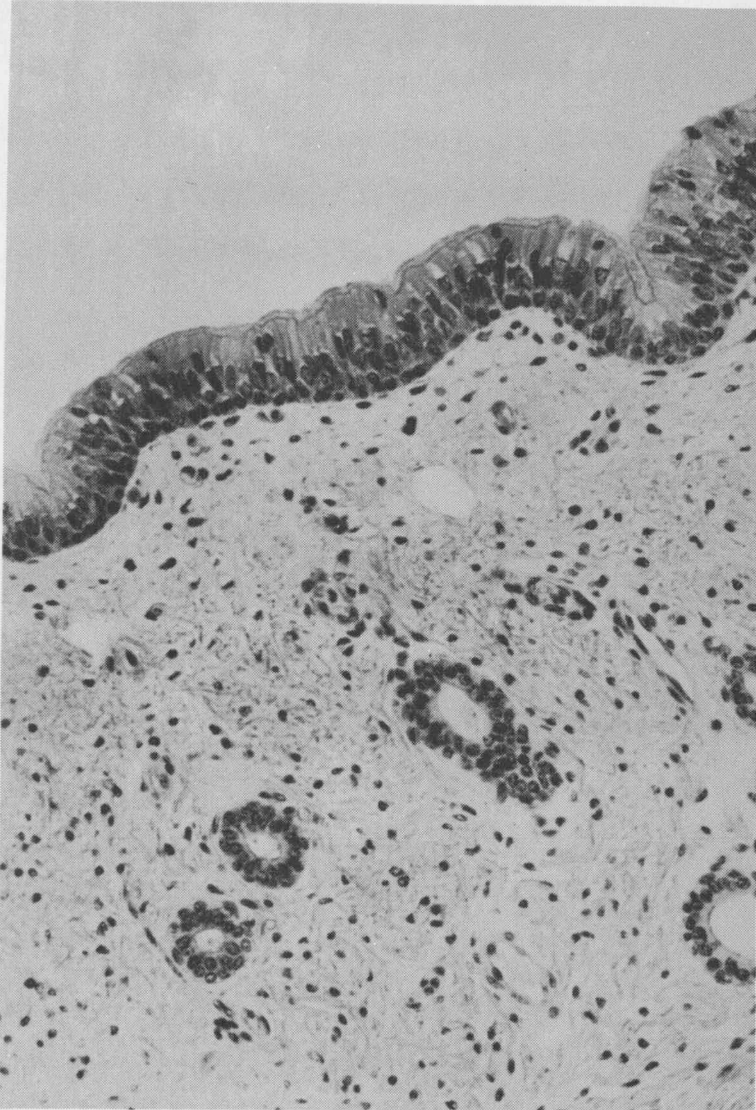
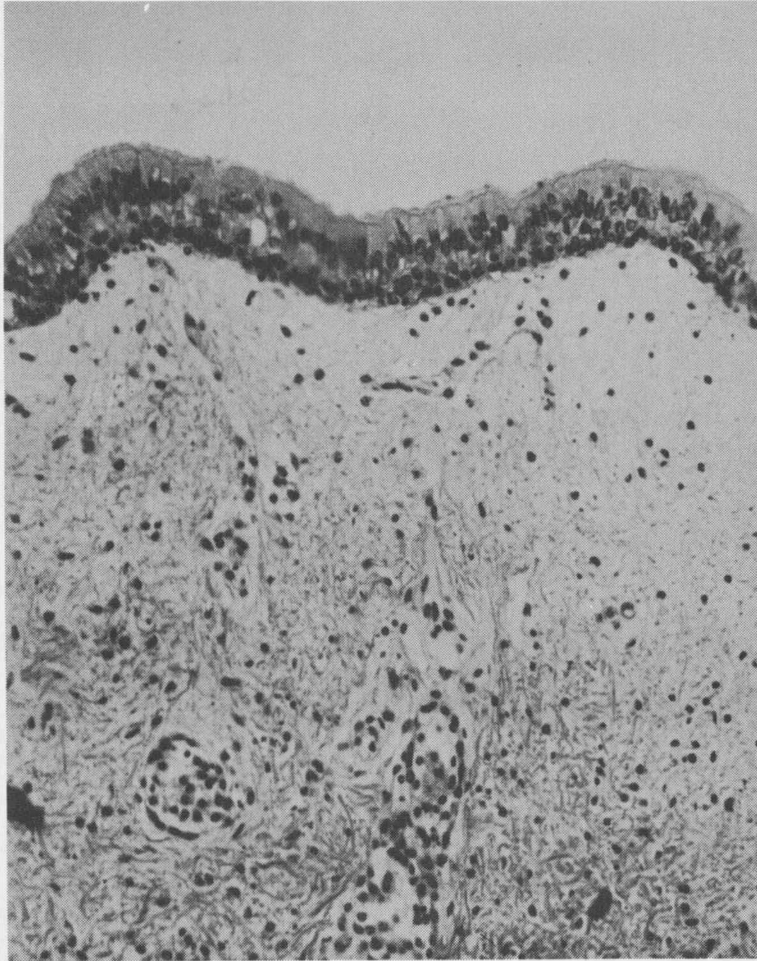


Fig. 1-b. Note: continuity of epithelium, glandular structures in lamina propria. 880 x.



Figures 2. Uninfected FBT tissue after two days in culture immediately prior to viral inoculation.

Fig. 2-a. Note: hypocellularity, degeneration of glandular structures and stain variation in lamina propria. 880 x.

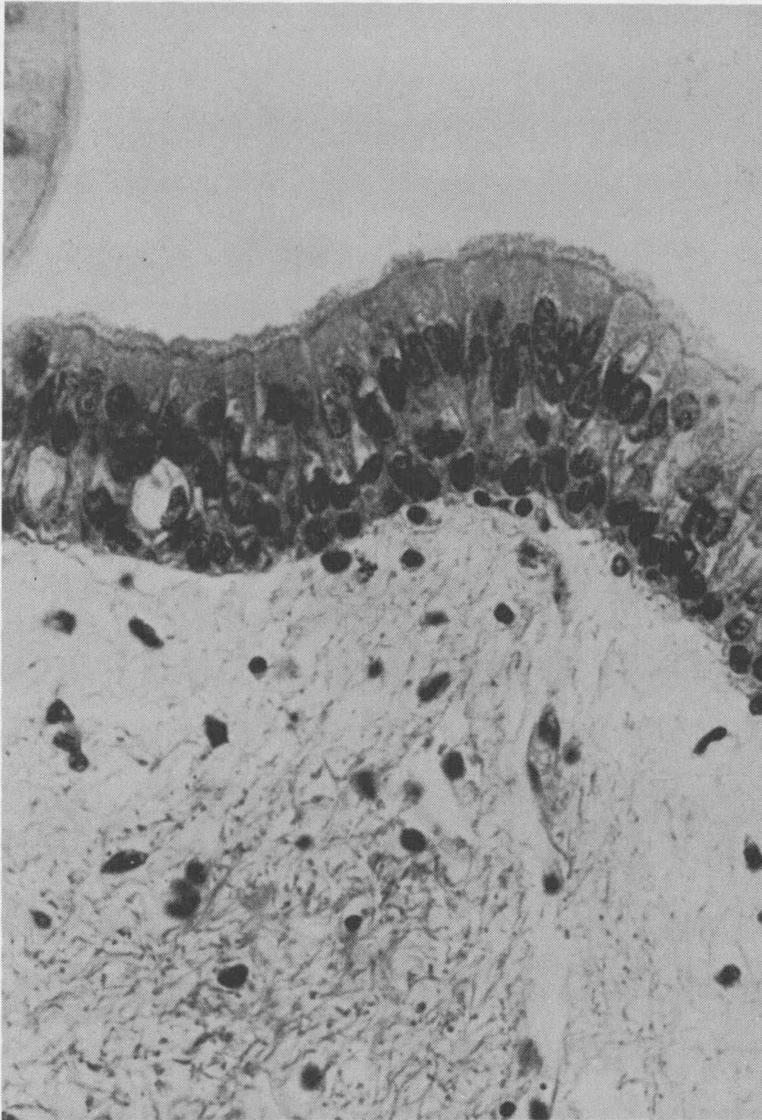
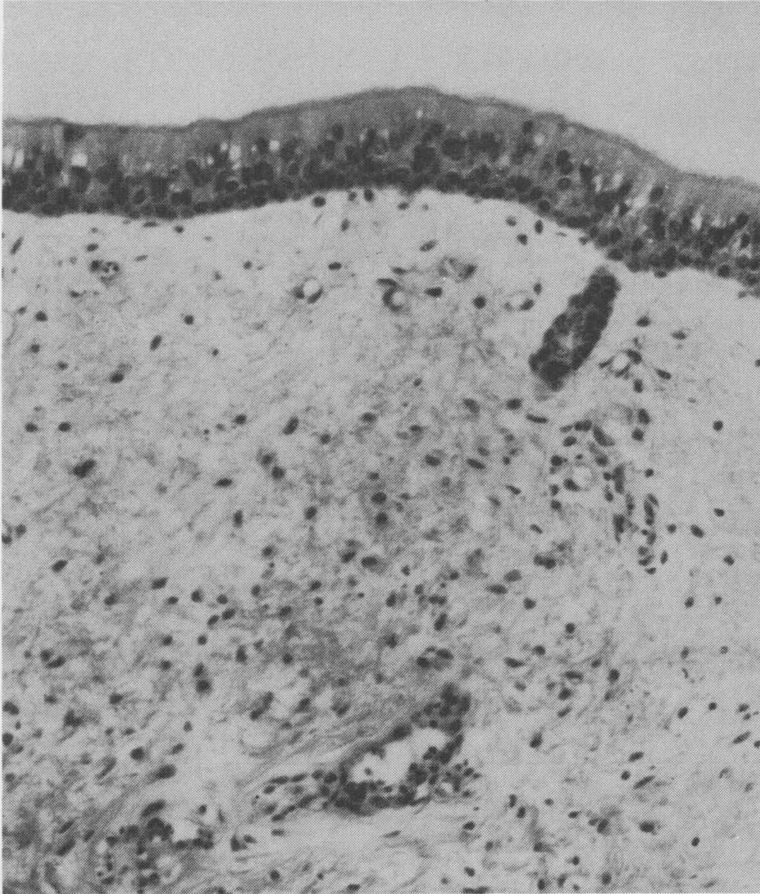


Fig. 2-b. Note: depth and fold of epithelium, cilia. 2,200 x.



Figures 3. FBT 24 hrs. post RSV inoculation.
Uninoculated cultures.

Fig. 3-a. Note: healthy eptihelium, mild cytoplasmic vacuolization, nuclear pyknosis, stages of degeneration of glandular structures. 880 x.

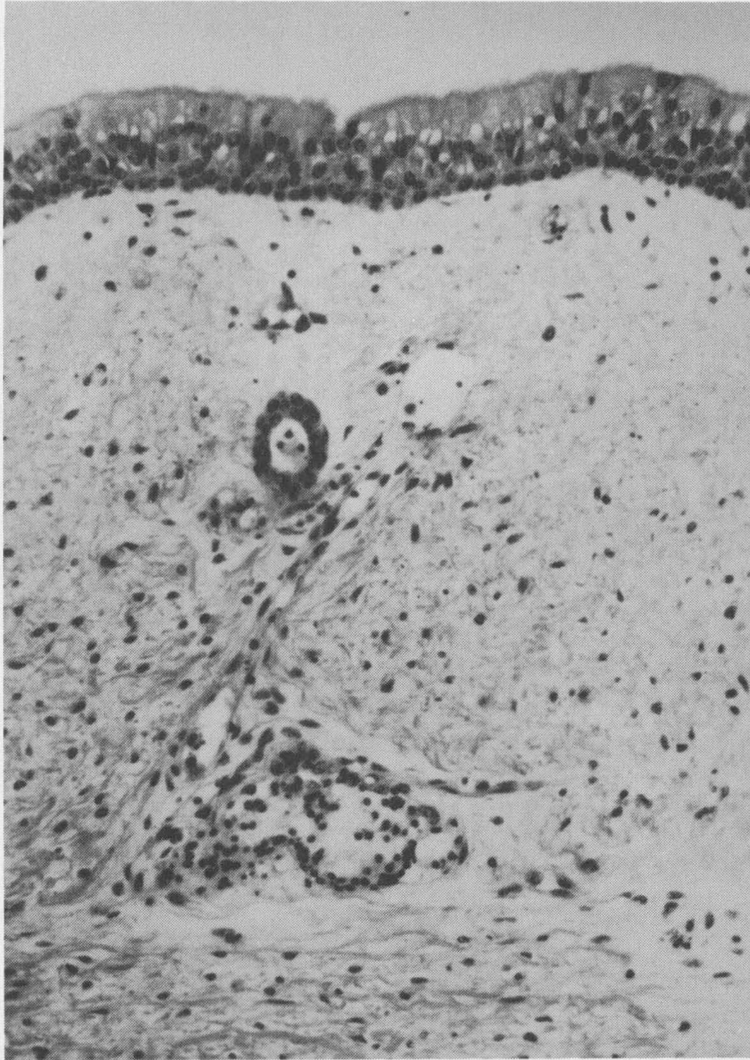


Fig. 3-b. Note: pyknotic nuclei in epithelial cells, stages of degeneration in glandular structures. 880 x.