1974

Isolation, characterization, and pathogenicity studies of a bovine respiratory syncytial virus

Malcolm Herbert Smith

Iowa State University

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Isolation, characterization, and pathogenicity studies of a bovine respiratory syncytial virus

by

Malcolm Herbert Smith

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Veterinary Microbiology and Preventive Medicine
Major: Veterinary Microbiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa

1974
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INTRODUCTION

Bovine respiratory disease has become recognized as a complex and includes a variety of clinical syndromes. As more becomes known about the bovine respiratory disease complex, it soon becomes apparent that each facet of this syndrome must be defined and understood as a separate entity. Little hope can be held out to those who demand solutions until each factor is understood as a contributing part to the sum total of this perplexing and complex disease.

It was in this context that the search for new viral agents involved in this complex disease resulted in the isolation of a bovine respiratory syncytial virus, not as a total answer or a solution to the problem, but as one more agent involved in the disease.

The purpose of this study was to describe the isolation, characterization, and identification of this virus. It also involved the experimental infection of animals in order to study the pathogenesis of the agent and its involvement in bovine respiratory disease.
LITERATURE REVIEW

Research in the area of bovine respiratory syncytial (RS) viruses must first acknowledge previous work done with the human respiratory syncytial (RS) viruses, since research with human RS viruses has laid the foundation and given direction to past and continuing work with bovine RS viruses.

It seems fitting, therefore, to review aspects of research with human RS viruses which provide us with a background of knowledge relative to their isolation history, cytological characteristics in infected cells, pathogenicity studies in experimental animals as well as in humans, and epidemiological data, including recent developments involving vaccine production and testing trials; the more recent work being especially important since it focuses our awareness on the complexity and the unknown factors in the immunological process, both in animals and in humans.

In 1956 Morris, Blount and Savage reported the isolation of an agent they suspected of causing an epizootic of respiratory disease in a colony of chimpanzees (53). The clinical signs of coryza produced by the virus led the investigators to term their isolate the chimpanzee coryza agent (CCA). The epizootic was not severe, involving some 20 animals; however, further studies with the virus have been far reaching, opening broad areas in human respiratory disease research.

The initial inoculation of throat swab material from affected chimpanzees into roller tube cultures of Chang liver cells resulted in
cytopathic effects (CPE) in cells after four days' incubation, characterized by rounding, granulation, and sloughing of cells from the tube wall.

Experimental infections in other species indicated that only chimpanzees were affected by this virus. Intracerebral and intraperitoneal inoculation into day-old mice, weanling hamsters, young rabbits, and guinea pigs, as well as inoculation of the chorioallantoic membrane, amniotic, allantoic, and yolk sacs of 7-10 day chick embryos did not result in infection or apparent virus replication.

A serological survey indicated that the CCA was not antigenically related to many other viruses being studied at the time.

In the course of this study, a laboratory worker who had been in contact with the CCA and with experimentally infected chimpanzees, developed a moderate respiratory infection and, although the CCA was not recovered, there was serological evidence that this virus probably was involved in the illness. This finding resulted in a serological survey of humans with a variety of illnesses. There appeared to be a rather broad antibody response in the human population which stimulated work directed at isolating the same or a similar agent from humans with respiratory disease.

In 1957 Chanock, Roizman and Meyers isolated two viruses indistinguishable from the CCA from children with severe lower respiratory disease (13). These isolates were named the Snyder and Long strains after the patients from whom they were isolated. These viruses were
isolated in the course of a search for new cytopathogenic agents from infants with lower respiratory disease. Several cell types were used for virus isolation attempts, including the KB strain of human epidermoid carcinoma, the Chang strain of human liver epithelium, cells from human amnion, and monkey kidney epithelial cells. Both virus strains showed cytopathogenic effects in the KB and human liver cells, but not in the other cell types. Subpassage from KB cells to human amnion did result in infection, but incubation times were prolonged. The noticeable effect was the production of syncytia in both cell types. Further studies of the isolates indicated their approximate size at 90-130 nm. They also proved to be ether sensitive, would not agglutinate chicken or human "O" erythrocytes, and would not infect embryonated eggs.

Serological studies with other syncytium-producing viruses and with other agents involved in respiratory disease, such as an agent of primary atypical pneumonia and of the psittacosis - lymphogranuloma-venereum (LGV) group failed to indicate any antigenic relationship.

The cytopathogenic effects of the two isolates were indistinguishable from those produced by the CCA. A close antigenic relationship was also shown between the CCA and the new isolates by complement fixation and cross-neutralization tests. Since these agents appeared to be antigenically identical and because of the characteristic syncytium production, the isolates were grouped together as respiratory syncytial viruses (13).
The epidemiologic study that followed the isolation of the Long and Snyder strains established that they were widespread in the human population (10).

In a short time other reports supplied ample evidence that the virus was a leading cause of severe respiratory disease in infants and young children (12, 40, 56). This evidence was obtained not only from serological studies but also by virus isolation. The efficiency in recovering the virus increased greatly when it was recognized that it was extremely difficult to isolate from frozen samples due to its lability.

The recognition of the importance of the virus in human disease stimulated studies of the properties of the virus itself (4, 40, 45). Jordan examined the growth characteristics of the virus in various cell types, different mediums, and in mediums with varying defined components (39). He observed that the capacity of the virus to produce characteristic syncytia was dependent on medium composition as well as on the cell type used. These findings, as well as the capacity of other unrelated viruses to produce syncytia, raised the question as to the appropriateness of the name designation of the virus group. This apparently is still an open question causing continuing confusion in the literature.

Bennett and Hamre also studied the growth and serological characteristics of another RS virus (Randall) strain (4). They also noted variations in cell sensitivity to the virus. They confirmed
earlier reports of the instability of the virus under storage conditions (3). Their studies of absorption and growth cycles indicated that absorption was slow but quite efficient; 90 percent of the virus being absorbed after ten hours. They found that the maximum virus titer was reached 36 hours following infection. No increase in virus titer after 36 hours' incubation, even though CPE was not complete, was interpreted as being an equilibrium point between virus production and inactivation since the virus is rapidly inactivated at 37C. Immunofluorescent examination of infected cells revealed evidence of intracellular viral antigen nine hours after infection and about ten hours before CPE could be observed. Cross-neutralization tests with the other two strains of RS virus confirmed the antigenic similarity reported earlier (3).

Immunofluorescence studies by Kisch, Johnson and Chanock (45) confirmed observations of the replicative cycle observed by others (4, 68). In their more detailed observations, they noted specific fluorescence only in the cytoplasm throughout the replicative cycle (45). They also found no evidence of hemagglutination or growth in embryonated eggs. Their studies noted a tendency for perinuclear fluorescence at all stages of infection, but intranuclear fluorescence was never observed. Intracytoplasmic inclusions were noted with May-Grunwald-Giemsa and immunofluorescent staining but not with acridine orange. Mitotic nuclei were never observed in syncytia with any staining method.
As studies of the virus progressed, the need for a sensitive, reproducible method for detecting minute amounts of antibody, for critical examination of acute and convalescent serum samples, and for quantitating serologic differences among various RS virus strains, resulted in plaque assay methods which provided the sensitivity and accuracy desired (44, 66). The method of Taylor-Robinson and Doggett did not utilize a plaque overlay as did the method of Kisch and Johnson; however, the sensitivity and reproducibility appeared to be the same with either method. In both systems, whether stained or unstained, a "plaque" was defined as a syncytium composed of a number of fused cells.

The instability of the virus was by then well known. Hambling reported on a study of the stability of the virus with variable temperatures and serum concentrations (26). It was found that loss of virus infectivity occurred at any temperature, but the lowest practical temperature and avoidance of refreezing resulted in the highest infectivity for the longest period. An approximate 50 percent decrease in infectivity could be expected in a virus sample stored at -65°C after three months. In contrast, at 55°C no infectivity could be expected after 20 minutes. The addition of serum beyond 5 percent did not increase virus survival time.

The precise classification or grouping of viruses continues to be a perplexing problem. Although biological properties serve as a guide to classification and identification, it has become evident that
more exact and definable criteria must be examined. In this respect, the ultrastructure of the virus particle itself appears to present the most constant features necessary for precise classification.

The respiratory syncytial virus has been considered a member of the myxovirus group by morphological and physico-chemical studies (6, 7, 8). It has further been proposed that the structure of the nucleocapsid of myxoviruses be used to separate myxoviruses into subgroups (70).

Since the RS virus is grouped with myxoviruses and yet possesses special characteristics, such as a lack of any capability to cause hemadsorption to infected cells or to hemagglutinate erythrocytes in infectious tissue culture fluid, presently there have been efforts to examine the ultrastructure of the virus in order to resolve its designation (7, 38, 54).

Early studies of the ultrastructure of the virus produced both agreeing and conflicting results (1, 6, 21, 38). Most investigators agree that the morphogenesis of RS virus resembles that of other myxoviruses. Viral particles range in diameter from 80 to 500 nm with spherical, filamentous and pleomorphic forms. Projections have been observed on mature viruses measuring 15 nm in length. Furthermore, it has been noted that the structure of a newly isolated RS virus differs little from one passaged in cell cultures (38).

The maturation of the virus occurs at the cytoplasmic membrane by budding (54). Elongated forms of virus particles were often seen
to bud from the cytoplasmic membrane with a uniform diameter of 100-130 nm and of variable length (54).

Since the diameter of the nucleocapsid seems to be a critical factor in classifying the RS virus, measurements have been made from many preparations with a conflicting range of 12-18 nm diameter having been reported (6, 7, 21, 38). It is felt that the extreme fragility of the virus plus different methods of fixation are the major causes for this discrepancy. It appears that with meticulous efforts taken in preparation of specimens for examination, a quite constant average diameter is 13 nm (54). This diameter is both above that for influenza viruses (9 nm) and below that for paramyxoviruses (17-18 nm) (54).

The diameter of the nucleocapsid plus the biological features of RS virus is the basis for some to feel that this virus should be grouped with the pneumonia virus of mice which shares similar characteristics (17, 54).

The internal structure of the inclusions noted in the cytoplasm of infected cells appears to be denser than the inclusions found with paramyxoviruses (54).

In order to study the pathogenesis of the virus and to evaluate any contemplated preventive vaccine, a susceptible experimental animal was needed. Coates and Chanock experimented with several species of animals, including ferrets, rabbits, mink, guinea pigs, marmosets, rats, mice and chinchillas (15). All these animals could be infected as indicated by an antibody response, but clinical illness
could not be produced in any of them. The ferret did, however, show rather extensive histologic changes in the turbinate epithelium. The virus could also be recovered from nasal secretions for seven days after infection. The virus was serially passaged in ferrets 27 times with no increase in virulence. In later studies with temperature-sensitive mutants of the virus, the Syrian hamster was apparently judged to be the most suitable for experimental studies since it supports replication of the virus throughout the respiratory tract and the temperature differential between the nasal turbinate and lungs is similar to that in man (74).

The study of RS virus infection was further examined by infecting human volunteers to observe clinical and serological responses (36, 37, 48, 51). It was noted that many subjects had serum neutralizing titers to RS virus; however, this did not apparently alter the response to the infection of the virus. Natural re-infection with RS virus in adults has subsequently been shown to occur in spite of serum antibody (36).

The possibility of antigenic variation among various RS virus isolates has been examined extensively to determine if antigenic differences did indeed exist or if the virus was capable of antigenic drift (14, 16, 75). Knowledge of antigenic variations was especially important in the light of contemplated vaccine development. Initial studies by Coates, et al, indicated an antigenic difference between two RS virus strains when ferret antiserum was used, but there was no
difference found by using antiserum from infants recovered from RS virus infection (16). A similar finding was noted by Wulff, et al (75). In 1966 a comprehensive study by Coates, et al, of ten strains of RS viruses indicated that antigenic differences do exist but they are slight and re-infection in children with an antigenically indistinguishable virus does occur (14). There was no indication of antigenic drift in the strains isolated over an eight-year period.

Progress in development of a protective vaccine continued. Epidemiological studies by this time provided ample evidence that RS virus was the greatest single cause of severe respiratory illness in infants and young children (12, 56). In 1969 two reports of vaccine trials with young children were published (42, 43). In both trials, an inactivated virus vaccine was used. Serological studies clearly indicated that the vaccine was antigenic. Subsequent outbreaks of RS virus infection occurred in both groups of children, with unexpected results. The children inoculated with the RS virus vaccine who became infected during the outbreak experienced 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 entirely unexpected. There is still no clear explanation for the results obtained; however, it is suspected that serum antibody against RS virus is not only nonprotective, but in some way contributes to the pathogenesis of the observed illnesses (11).
The hypothesis that the severity of RS virus infection probably has an immunologic basis has provoked considerable study in this area. As yet there are no answers but several observations are of interest. A study of the age-illness relationship indicates that the most severe form of RS virus illness involves children at an age when maternally acquired antibody is at its highest level. It is also evident that at this time local antibody, which is not maternally derived, is at its lowest level (11).

The importance of local antibody in protection against RS virus infection has been studied and there is evidence that though local antibody may not influence susceptibility to infection, it has a marked effect on virus replication (11, 43).

In view of this recent evidence, the direction of efforts to induce protection has been altered. A live attenuated vaccine capable of replicating in the upper respiratory tract is now thought to be capable of providing protection without causing the more severe lower respiratory tract infection. Wright, et al. reported on results in hamsters using a temperature-sensitive mutant of RS virus capable of replicating in nasal turbinates but not in the lungs (74). Evidence has been produced using this mutant virus to infect adult subjects, that it is capable of inducing protection against a challenge with wild-type RS virus (73). However, it was found in this study that the rise in nasal and serum antibody titers was not sufficient to explain the protection observed. It is possible that immunological phenomena
other than local antibody or other non-immunological factors may be responsible for the protection observed. This area of RS virus research is very active at the present time (11).

As stated earlier, the purpose in reviewing human RS virus research is to place the investigation into bovine RS virus in proper perspective. By using human RS virus research as a guideline, it should be possible to pursue the study of bovine RS virus more efficiently and also to examine areas that potentially could be profitable to both the human and bovine research efforts.

An introduction to bovine respiratory syncytial viruses came in 1963 when English investigators in human RS virus research realized that the calf serum they were using in their tissue culture medium contained factors that inhibited syncytium production in human RS virus infected cells (19). Subsequent studies fairly well established that these inhibiting factors were specific antibodies and, therefore, the same or a closely related virus must be present in cattle (19). This apparently stimulated a search for a respiratory syncytial virus in cattle.

In 1970 Paccaud and Jacquier published a report of an outbreak of bovine respiratory disease in Swiss cattle (55). The outbreak began in 1967 with several farms being involved. They collected specimens from two farms where respiratory disease was present. It is interesting that only animals less than seven years of age showed signs of clinical illness even though the older animals were in close
contact with those with disease. It was also noted that cows rather than calves experienced a more severe clinical illness.

There were two viruses isolated from the two outbreaks examined. It soon became apparent that the isolates were identical and, furthermore, that they manifested many of the biological characteristics seen in human RS virus isolates. On the second passage in primary bovine embryonic kidney (ECK) cells syncytia began to appear after twelve days of incubation at 36°C. The first indication of CPE was that of granular, ballooned cells, progressing to syncytium formation with additional incubation. With additional passages CPE was noted in 5-6 days. It was further noted that maintenance medium supplemented with 2-5 percent calf serum and/or 0.1 mg proflavine sulfate per ml enhanced the formation of syncytia (30, 55).

Several cell types proved insensitive to the bovine isolate, including human embryonic fibroblastic cells, KB, HeLa, BHK 21, secondary rhesus kidney cells and primary embryonic sheep kidney cells. The infected monolayers of sensitive cells would not hemadsorb human, calf, guinea pig, or chick erythrocytes. The same negative results were obtained when hemagglutination tests were made with infectious tissue culture fluids.

Infected cell monolayers were stained with Seller's May-Grunwald-Giemsa, hematoxylin and eosin, and acridine orange stains. Cytoplasmic inclusions were prominent in all stained preparations except with acridine orange, where optically empty spaces appeared in
the otherwise flame-red cytoplasm. This latter observation was an
indication that the inclusions did not contain nucleoprotein.

The physical and chemical properties of the bovine isolate
closely resembled those found with human isolates. It was found to
be very heat labile, sensitive to low pH, and to lipid solvents. It
was observed that 99 percent of residual virus infectivity was lost
when filtered through an 0.45 u filter. This unexpected finding was
thought to be due to the formation of virus aggregates which were
retained by the filter.

Serological studies were conducted with acute and convalescent
serum samples of infected cattle. There were sero-conversions in both
affected herds, including the animals from which the viruses were
isolated. This was concluded as evidence of the bovine origin of the
isolate and that the virus was involved in the respiratory outbreak.
Cross-neutralization tests with the bovine isolate and a human RS virus
using guinea pig antiserum were also conducted. The results of these
tests 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 isolate. No production of serum
antibody titers could be found in horses. One of the bovine isolates
was neutralized by over 50 percent of the human serums tested, further
indicating the close relationship of the two viruses.
From these studies and observations, the investigators con­cluded that their isolates were bovine respiratory syncytial viruses.

Later in 1970 Inaba, et al. described a respiratory epizootic that swept through Japan during 1968 and 1969 (33). Their report was brief but it clearly indicated that the virus isolated from this outbreak was a bovine respiratory syncytial virus and its close antigenic relationship to the human RS virus was confirmed.

Also, in 1970, a report from Belgium indicated that a bovine RS virus had been isolated from cattle in that country (72). A subsequent report of a serological survey of cattle in Belgium indicated that nearly 40 percent of the animals tested had an antibody titer to the bovine RS virus (71).

In 1971 investigators in England reported the isolation of a bovine RS virus from cattle in England (35). Studies on the physico-chemical or serological characteristics of the virus were not elaborated upon.

A recent serological study with various animal species was conducted to detect serum antibodies to RS virus in Canada (5). Although this was a limited survey, there was an indication of antibody titers not only in cattle, but also in sheep. This report did not indicate whether a human or bovine RS virus was used as an antigen in the complement fixation tests; however, it is assumed that it was a human RS virus isolate. It was concluded from this study that since a high percentage of sheep did have RS antibody, it would well be worth
considering this animal as an experimental model for the study of human RS virus infection.

In 1973 the Japanese investigators reported results of ultrastructure studies with the bovine RS virus isolated in that country (34). Negative stained preparations clearly indicated the pleomorphism of the virus with spherical, elongated, and distorted forms. Dimensions of particles varied from 80-450 nm, with an average of 200 nm. The internal structure measured 11-15 nm in diameter, which is consistent with findings with the human RS virus. In ultrathin sections, budding particles also appeared pleomorphic, with spherical and filamentous forms observed at the plasmalemma. These investigators concluded from their studies and observations that the bovine RS virus should be included in a separate group of metamyxoviruses as has been suggested by others (7, 8, 17, 38, 54).

In 1969 Malmquist, et al. reported on the isolation and characterization of a virus causing syncytia formation in bovine spleen cells (50). The new isolate was designated as a bovine syncytial virus because of the consistent formation of syncytia in infected cells. This virus has been shown to be morphologically and antigenically distinct from the bovine RS virus¹. This is an instance where confusion in the literature will remain as long as RS viruses retain their present designation.

¹Dr. M. Van Der Haaten, N.A.D.L., Ames, la. - personal communication.
In concluding this review, it is apparent that there is much to be accomplished in the area of bovine RS virus research, particularly relative to the epidemiology and pathogenesis of the disease. It is also apparent that continued research with the bovine RS virus can be helpful in both animal and human disease.
MATERIALS AND METHODS

Cell Cultures

Two embryonic bovine kidney cell lines were used; Madin-Darby bovine kidney\(^1\) (MDBK) and a bovine kidney cell line developed in Georgia (GBK) by Solarzono\(^2\). In addition, low passage cell types were obtained from a neonatal calf. These included cells from the lung (IBL), trachea (IBTr), thyroid (IBThyr), aorta (IBA), and spleen (IBS).

The tissues for low passage cell types were taken aseptically and placed in Dulbecco's modification of Eagle's minimum essential medium\(^3\) (MEM) with antibiotics in the concentrations as mentioned later. The tissues were finely minced with a scalpel and the fragments were placed in 250 ml plastic tissue culture flasks with MEM, 10 percent fetal calf serum\(^4\) (FCS), and antibiotics. After incubation for 1-2 weeks at 37°C in a 5 percent carbon dioxide atmosphere, the cells had grown out from the tissue fragments. The medium was removed and the cell surface was carefully washed with Rinaldini enzyme solution (R-saline), a calcium and magnesium free Tyrodes-like solution (62). The cell surface was then flooded with 0.1 percent trypsin\(^4\) and 0.02

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\(^1\)American Type Culture Collection

\(^2\)R. F. Solarzono, University of Missouri, Columbia, Mo.

\(^3\)Grand Island Biological Co., Grand Island, N. Y.

\(^4\)Difco Laboratories, Detroit, Mich.
percent versene\textsuperscript{1} in R-saline (RTV) for 3-5 minutes. The RTV was then removed and the cells were incubated at 37°C until they detached from the flask surface. The cells were harvested in 5 ml MEM and dispersed by vigorous pipetting 3-4 times. The cells were then passed to new flasks using MEM with 10 percent FCS and antibiotics. By the second passage antibiotics were usually not necessary. Most of the cell types could be passed 8-10 times before noticeable slowing of growth was noted.

When cells were needed for virus studies, they were removed from the flask as described and suspended in MEM with 2 percent FCS at a multiplicity necessary to form a monolayer when attached. Virus inoculations were usually done with the cells in suspension (60).

Virus isolation attempts were made in plastic tissue culture tubes\textsuperscript{2}. Plastic tissue culture plates with 24 wells\textsuperscript{3} were used for virus titrations and serum neutralization tests. In both instances 1 ml of cell suspension was used to provide monolayers.

The cells used in this study were grown and maintained in 250 ml plastic tissue culture flasks\textsuperscript{2}. Buffers used were 0.076 percent sodium bicarbonate and a combination of organic buffers\textsuperscript{4}: N, N'-bis (2-hydroxyethyl)-2-aminoethane sulfonic acid (BES), N-2-hydroxyethyl-piperazine-N'2-ethanesulfonic acid (HEPES), and N-2-hydroxyethyl-

\textsuperscript{1}J. T. Baker Chemical Co., Phillipsburg, N. J.

\textsuperscript{2}Falcon Plastics, Oxnard, Calif.

\textsuperscript{3}Linbro Chemical Co., New Haven, Conn.

\textsuperscript{4}Nutritional Biochemicals Corp., Cleveland, Ohio
piperazine propane sulfonic acid (HEPPS) in final concentrations of 10, 15, and 10 nm respectively (20).

Antibiotics used were potassium penicillin G\textsuperscript{1} (100 I.U./ml), streptomycin sulfate\textsuperscript{1} (100 \text{ug/ml}), and amphotericin B\textsuperscript{2} (10 \text{ug/ml}). Antibiotics were used only in initial low cell passage preparation and in virus isolation attempts from nasal secretion material. All cells were propagated at 37°C in a 5 percent carbon dioxide atmosphere.

All tissue culture and virus isolation procedures were conducted in a positive air flow hood\textsuperscript{3}.

Organ Cultures

A trachea from a neonatal calf was obtained and incubated in MEM with antibiotics for one hour before further processing. Tracheal rings were separated with a scalpel and excessive tissue trimmed away. The rings were then cut into 8-10 mm\textsuperscript{2} sections and placed in six-chambered plastic tissue culture trays\textsuperscript{4}. The surface of the trays was scratched to facilitate attachment. Then MEM with 10 percent FCS and antibiotics was added to a level that just left the tracheal epithelium exposed. For virus studies the medium was removed and MEM with 2 percent FCS and antibiotics was substituted and 0.1 ml of a

\textsuperscript{1}Eli Lilly & Co., Indianapolis, Ind.
\textsuperscript{2}Fungizone, E. R. Squibb & Sons, New York, N. Y.
\textsuperscript{3}Plasticage Corp., Jackson, Mich.
\textsuperscript{4}Linbro Chemical Co., New Haven, Conn.
virus suspension was dropped directly on the epithelial surface. Ciliary activity was observed daily using an upright microscope with indirect lighting. The explants could be maintained for over six months with bi-weekly changes of medium.

Virus Isolation

On January 1, 1971, this laboratory (the Veterinary Medical Research Institute) was contacted relative to an outbreak of acute respiratory disease in a herd of cattle in central Iowa. The clinical history of the herd was scanty, but apparently the outbreak was in a group of 300-400 lb. calves of mixed breeds and origins which had been purchased some 2-3 weeks previously from a local sale barn. There was no known previous vaccination or treatment history. On January 1, 1971, the majority of the calves were obviously ill with a mild cough, anorexia, and pyrexia. There were two calves showing signs of incoordination, which in acute respiratory disease is often an indication of an extremely high temperature. Nasal secretions were taken from five calves with a sterile cotton swab and expressed into 2 ml MEM with 2 percent FCS and antibiotics. Serum samples were taken at this time and again four weeks later. The calves sampled were given identifying numbers: FSI-1, FSI-2, FSI-3, FSI-4, and FSI-5. Hereafter, in this discussion, these numbers may indicate various things, including nasal secretion samples, serum samples, or virus isolates, but they all will reflect the identity of the animal of origin and the context in which the item is discussed should identify it.
precisely. Eventually the designation of FS1-1 will be synonymous with "the bovine respiratory syncytial virus," and "the bovine isolate FS1-1".

The nasal secretion material from the five calves was held at 4C for 1-2 hours and then frozen at -70C until virus isolation attempts were made.

Virus isolation attempts were made by thawing the nasal secretion suspension and inoculating 0.1 ml into duplicate tubes containing the cell types mentioned previously. One tube of each type was placed in a stationary rack and incubated at 33C and 37C respectively. The tubes were examined daily with an inverted microscope for 10-12 days or until cell degeneration was noted. Samples were subpassaged five times if no cytopathogenic effect (CPE) was noted. If any suggestion of CPE developed, the samples were subpassaged eight times. Subpassage was accomplished by inoculating 0.1 ml of medium, cells, and cell debris into new tubes prepared as before. If CPE was noted, the cells were dislodged and with the supernatant medium were frozen at -70C. Before isolation attempts were discontinued, or when CPE developed, a hemadsorption test was made. The method described by Hotchin, et al. was used for this test (31). The medium was removed from the monolayer, the monolayer was washed three times with warm R-saline, 1 ml of 1 percent guinea pig erythrocytes in Alsever's solution was added and the tube was incubated for one hour at room

1Unitron Instrument Co., Newton Highlands, Mass.
temperature. The solution was then removed and the cell layer was washed once and then examined under a microscope for evidence of hemadsorption.

Virus Stocks

The bovine isolate, FS1-1, and a human respiratory syncytial virus (RS)\textsuperscript{1} were grown in GBK cells until 75 percent of the cell sheet showed signs of CPE. The virus was harvested, with cells, following one freeze-thaw cycle. Aliquots of each virus were then frozen and stored at -70°C.

Virus Titration

The bovine respiratory syncytial virus was titrated by inoculating 0.1 ml of half log. dilutions of virus suspension into 1 ml GBK cell suspensions as previously described. All titrations were performed in quadruplicate. Titors were determined after five days' incubation at 37°C in a 5 percent carbon dioxide atmosphere as 50 percent infective doses (TCID\textsubscript{50}) calculated by the method of Reed and Muench (59). The human respiratory syncytial virus was titered in the same manner.

Serum

Paired serum samples were taken from the animals from which the bovine RS virus was isolated, one at the time of illness and a second four weeks later.

\textsuperscript{1}Long strain. American Type Culture Collection (KB6L2)
Serum samples from animals used in experimental infections were taken at the time of infection and at intervals thereafter.

Hyperimmune serum against parainfluenza-3 virus produced in a goat was made available by this laboratory. Hyperimmune serum against the bovine syncytial virus described by Malmquist, et al. (50) was provided by Dr. M. Van Der Maaten, N.A.D.L., Ames, Iowa.

Hyperimmune serums against the bovine respiratory syncytial virus and the human respiratory syncytial virus were produced in rabbits by injecting 2 ml of virus suspension intraperitoneally at weekly intervals for six weeks. The suspension injected contained approximately 4,000 plaque forming units (PFU) of virus. Serum was harvested two weeks following the last injection.

All serums were heat inactivated at 56°C for 30 minutes. In addition, serums from experimentally infected animals and from rabbits were absorbed with an equal volume of GBK cell suspension. This mixture was incubated for three hours at room temperature, then centrifuged at low speed and the supernatant was stored at 4°C until used.

Serum Neutralization

Two-fold dilutions of serums were incubated with equal volumes of virus suspension containing 50-150 PFU for three hours at room temperature. After this incubation period, .25 ml of the serum-virus mixture was inoculated into duplicate wells of a 24-well plastic
tissue culture plate containing a monolayer of GBK cells from which growth medium had been removed. Absorption was allowed to proceed for three hours at room temperature. Excess fluid was then removed and 1 ml of equal volumes of 2X MEM and 1 percent agarose was added. An incubation period of five days at 37°C in a 5 percent carbon dioxide atmosphere followed. The agar was then removed and the cell surface was washed with warm R-saline. The monolayer was then fixed and stained simultaneously using a combination of one volume methanol, one volume 1 percent basic fuchsin in methanol, and three volumes 1 percent methylene blue in methanol (58). After a ten minute fixation and staining period, the mixture was removed and the monolayer was washed with tap water. The monolayer was then examined with an inverted microscope and plaques were counted. A plaque was defined as a syncytium or an empty space from which a syncytium had detached in the process of fixing and staining. The antibody titer was determined as the reciprocal of that serum dilution which reduced the plaque number by 50 percent. Appropriate controls were processed in a similar manner.

Determination of Essential Lipid Content

The method described by Feldman and Wang (22) was used to determine the sensitivity of the virus to lipid solvents. Twenty volumes of virus suspension containing approximately 1000 pfu/ml

1Marine Colloids, Inc., Rockland, Maine
was mixed vigorously with one volume of reagent grade chloroform for five minutes. The mixture was then centrifuged at low speed for five minutes and the supernatant was titrated to determine residual infectivity. The virus was determined to contain essential lipid if no infectivity was noted. Appropriate controls were processed similarly.

**Determination of pH Sensitivity**

The method described by Ketler, et al. (47) was used in this test. One volume of virus suspension was incubated with ten volumes of MEM adjusted to pH 3 for 30 minutes at room temperature. Residual infectivity was then titrated. A rhinovirus and an enterovirus were used as virus controls.

**Determination of Nucleic Acid Type**

The indirect method of nucleic acid determination was used in this test (28). Monolayers of GBK cells in 24-well plastic tissue culture plates were washed two times with warm R-saline. Half-log dilutions of the test virus and the virus controls were made in MCM without FCS. Two plates with cell monolayers were then inoculated with .1 ml of each virus dilution--four replicates per dilution. The virus was allowed to absorb for one hour at room temperature and the excess fluids were then removed. To one plate 1 ml MEM without FCS and to the second 1 ml of MEM containing $10^{-4}$ M 5-bromodeoxyuridine

1Sigma Chemical Co., St. Louis, Mo.
(BUDR) was added. After a 5-day incubation period at 37°C in a 5 percent CO₂ atmosphere, the monolayers were examined for signs of CPE and the virus titer was calculated as described. Parainfluenza-3 (PI-3) and infectious bovine rhinotracheitis (IBR) viruses were used as controls. A virus was determined to be insensitive to the thymidine analog, thus containing RNA, if there was not at least a 2 log drop in the virus titer in the virus dilutions to which BUDR was added.

Determination of Virus Size

A modification of the method described by Hsuing was used to approximate the size of the virus (32). Nalgene filters of .45 u and .20 u average pore diameter were used. An aliquot of MEM (10-15 ml) was drawn through the filter and discarded. This was to insure thorough wetting of the filter. Following this, a sample of 50 ml of virus suspension was drawn through the filter and the residual virus infectivity in the filtrate was titrated as described.

Cytopathogenic Effect and Staining Characteristics

The cytopathogenic effect (CPE) was observed using standard methods with hematoxylin and eosin, May-Grunwald-Giemsa, and acridine orange stains (57, 69). Unstained preparations were observed in infected monolayers of various cell types prepared in 24-chambered plastic tissue culture trays as described. For stained preparations

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1 Indicator virus strain obtained from Veterinary Biologics Division, U.S.D.A., Ames, Iowa
2 Nalgene Labware Division, Rochester, N. Y.
round coverslips were prepared by dipping in 95 percent ethanol and flaming. They were then placed in plastic tissue culture dishes. Then .5 ml of a cell suspension in MEM with 2 percent FCS was carefully placed on the coverslip. The cell suspension was prepared as described earlier. The cells were then infected in suspension using .05 ml virus inoculum. The cells were allowed to attach for 2-3 hours following which 2 ml MEM with 2 percent FCS was added to flood the coverslip and wash away unattached cells. At predetermined time intervals the coverslips were removed, washed with R-saline, and fixed in a manner appropriate for each staining procedure. Carnoy's fixative was used for acridine orange staining, methanol for May-Grunwald-Giemsa, and 10 percent buffered formalin for hematoxylin and eosin. After staining and mounting, the cells were examined and photographed through a binocular Leitz Ortholux microscope. Photographs were taken with Panatomic-X black and white, and high speed Ektachrome color films.

Determination of Mycoplasma Contamination

Throughout this study samples of all pertinent items were cultured to determine if mycoplasma contamination was present. The method of Frey, et al. (23) was used. Petri plates with M-96 medium

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1Falcon Plastics, Oxnard, Calif.
2Ernst Leitz, Wetzlar, Germany
3Eastman Kodak, Rochester, N. Y.
were streaked with sample material, incubated in a microaerophilic environment at 37°C for 24 hours, examined under a microscope, then incubated an additional 4-6 days at 37°C if no colonies were noted.

Experimental Infectivity Studies in Hamsters

Based on human RS virus studies, it was considered that much of the work on pathogenesis of the bovine RS isolate could be carried out easily and inexpensively in hamsters if it could be shown that the bovine isolate would replicate in the respiratory tissues of that species. In two trials weanling hamsters were inoculated with infectious virus suspensions. In the first trial six hamsters were anesthetized with ether and .2 ml of virus suspension (100 TCID₅₀/μl) was instilled into each nostril. In the second trial six hamsters were placed in a large beaker, the beaker was covered, and a 4 ml quantity of virus suspension was sprayed into the beaker using a gas-powered atomizer (64). The inoculum in the second trial contained approximately 4000 TCID₅₀/ml of the virus. In both trials the lungs, trachea, and nasal turbinates of one hamster were harvested on alternate days following inoculation. The tissues were pooled, ground in a tissue grinder, and made up to a 10 percent suspension in MEM with antibiotics. A .1 ml aliquot of the tissue suspension was inoculated into GBK cells as described earlier. Samples from each hamster were subpassaged 3 times at weekly intervals in an attempt to recover the virus.

¹A.R.S., Sprague-Dawley, Madison, Wisc.
Experimental Infectivity Studies in Calves

Five calves were used in this study. Three Hereford calves were obtained at birth from a herd maintained by the Veterinary Medical Research Institute (V.M.R.I.). Two Guernsey calves were purchased from a local dairy. Each calf was scrubbed with a .01 percent benzylkonium chloride solution and then placed in isolation units previously fumigated with formaldehyde. Each calf was given 5 ml of a combination of procaine penicillin G (200,000 IU/ml) and dihydrostreptomycin (.25 g/ml) intramuscularly daily for ten days. Serum was obtained from a colostrum-deprived calf used in another study. At birth each calf was given 100 ml of this serum subcutaneously and 100 ml orally. Powdered nonfat milk was reconstituted with equal volumes of Darrows solution and 5 percent glucose and fed to each calf four times daily for ten days. If diarrhea developed, the powdered milk was omitted and only the Darrows solution and 5 percent glucose mixture was fed for three days. After ten days, a commercial milk replacer was gradually substituted. Commercial pelleted feed and good quality hay were made available at two weeks, and by two months the calves were weaned.

1Roccal, Winthrop Laboratories, New York, N.Y.
2Combiotic, Chas. Pfizer & Co., New York, N.Y.
3Darrows solution: 4.0 gm KCl, 6.0 gm NaCl, 15.5 ml 60 percent sodium lactate, q.s. to 1,500 ml with water
4Calf Krunch - 6101-27, Allied Mills, Chicago, Ill.
When the youngest calf was 8 weeks old, the five calves were infected intranasally with the bovine RS virus using a gas-powered atomizer as described by Sinclair and Tamoglia (64). The inoculum was 4 ml of virus suspension containing approximately 4000 PFU of virus. The calves were observed three times daily for clinical signs of illness. Rectal temperatures and respiration rates were taken daily. Blood samples were taken daily from one day before infection until seven days after infection. They were examined for total white blood cell count, differential white blood cell count, hematocrit, plasma fibrinogen, and total serum protein. Serum samples were taken before infection and one week, one month, and two months after infection.

Nasal secretions were obtained twice daily from one day before infection to ten days after infection. The nasal secretion samples were handled and virus isolation attempts were made using the same procedures as described earlier.

Serological Survey

Serum samples from 32 cattle were examined for the presence of serum neutralizing antibodies to the bovine RS isolate FSI-1. The animals tested were divided into three categories.

The first category consisted of 37 animals with respiratory disease. Paired serum samples were available from these animals, one taken at the acute phase of respiratory disease and one taken 3-4 weeks later.
The second category consisted of 37 animals with no obvious sign of respiratory disease. One serum sample was tested from these animals.

The third category consisted of calves from an Iowa State University herd. These calves, hereafter referred to as Albia calves, were from a herd of mixed-breed and cross-bred cows. Calves from this herd had a history of having an outbreak of respiratory disease each year within two weeks after being moved from one farm to another, a distance of about 40 miles, and mixed with other calves in a feedlot. This part of the survey was designed to follow these calves by serological testing from before being moved until after they experienced an outbreak of acute respiratory disease. The first serum sample was taken from 84 calves on October 18, 1972, which was two weeks before the anticipated move. Nasal secretion material was also taken at this time and the samples were pooled, five to a pool, and virus isolation attempts were made as described earlier. The calves did not show signs of respiratory disease within the time predicted. A second serum sample was taken from 25 of the calves on January 12, 1973. On January 25, 1973, the calves had an outbreak of acute respiratory disease. At this time five animals with signs of respiratory disease were examined. Nasal secretions and two blood samples were taken, the second sample being taken with anticoagulant in order to harvest white blood cells from the buffy coat. The nasal secretion material was inoculated into four cell types as described earlier. The
cell types used were MDBK, GBK, and low-passage lung and spleen cells. In addition, .05 ml of the nasal secretion material was inoculated onto bovine tracheal epithelium of organ cultures as described. The buffy coat was harvested and dispersed in 1 ml MEM. Then .1 ml of this cell suspension was inoculated into cell cultures for virus isolation in the same manner as nasal secretion material. All isolation procedures were done in duplicate tubes and a tube from each sample was incubated in stationary racks at 37°C and 33°C respectively. A final serum sample was taken on March 2, 1973, from 20 calves in this group.
RESULTS

Isolation and Culture Characteristics

Frozen nasal secretion samples obtained from five animals (FS1-1--FS1-5) with acute respiratory disease were rapidly thawed at 37°C and inoculated into several cell types as described. These cells included MDBK, GBK cells and low passage lung, trachea, synovial, aorta, and spleen cells obtained from a neonatal calf as described in Materials and Methods.

One sample, FS1-4, showed obvious CPE by the fifth day of incubation in all cell types. This isolate exhibited hemadsorption capability and was subsequently identified as PI-3 by serum neutralization.

The remaining samples were subpassaged five times at 12-14 day intervals before any obvious changes were noted. On the fifth passage, FS1-1 exhibited slight CPE in GBK and lung cells at 12 days of incubation. The GBK cells usually showed obvious signs of degeneration at 12-14 days of incubation and therefore were not used for initial isolation. The low passage lung cells could be maintained for up to 60 days with no signs of degeneration; therefore, these cells were utilized in initial isolation attempts.

The three samples remaining showed no signs of CPE after the eighth passage and isolation attempts were terminated.

The two isolates, FS1-1 and FS1-4 exhibited identical cytopathic characteristics in lung cells. The first changes noted were small
areas where 2-3 cells showed extreme granularity with shrinkage of cytoplasm resulting in eventual opacity. These cells detached in about 24 hours and floated in the medium. In early passage the medium in the tubes showed a rapid shift to an acid pH. After 10-12 passages this rapid shift was no longer noted. Subsequent to this type of CPE it was noted that syncytium formation occurred with 10-15 cells coalescing to form a very granular, vacuolated mass. In these early passages the syncytia developed a deep yellow to brown color. This also disappeared by the tenth passage. Because of the early acidity noted, it was suspected that mycoplasma infection might be involved but repeated attempts to isolate mycoplasma were fruitless. The only difference noted between the two isolates was that FSl-4 showed an extreme hemadsorbing capability whereas FSl-1 would not hemadsorb. After 4-5 passages FSl-4 ceased to exhibit the granularity noted earlier and produced CPE characteristic of many Pi-3 isolates, with many scattered, rounded, refractory cells and small syncytia. At this time the FSl-4 isolate was frozen and efforts were directed toward characterizing and identifying FSl-1.

The syncytia produced by FSl-1 increased in size with subsequent passage until cell masses containing 150 or more nuclei were noted. It was difficult to increase the virus titer, although several methods were used; such as roller tubes, incubating at various temperatures, and harvesting the virus at various times of incubation. However, by about the eighth passage CPE was noted by 5-7 days and the titer was
increased to $10^{3.3-3.5} \text{TCID}_{50}/\text{ml}$. At this time it was noted that the
GBK cells were more permissive to virus growth, producing larger
syncytia with shorter incubation periods until by the fifteenth passage
CPE was noted at 2 days with maximum cell involvement by 5-6 days.
The GBK cells were then used for subsequent studies since the lung
cells exhibited much slower growth after 6-8 subpassages. The virus
titer could not be increased above $10^{3.5} \text{TCID}_{50}/\text{ml}$, and it was even
difficult to maintain the titer at this level.

All other cell types could be infected with FS1-1, but
virus titers remained at low levels and the CPE was never very extensive.
In addition, infection in these cells, including MDBK cells, did not
result in syncytium formation. In the low passage cells, small
areas of granularity developed which did not enlarge. These areas
could have easily been mistaken for nonspecific changes. The MDBK
cells exhibited focal areas of cell degeneration, eventually spreading
to include most of the monolayer. The cells would shrink, become
granular and detach. Later, when low passage bovine thyroid cells
were used, production of syncytia resulted much like that in GBK cells.

Tracheal organ cultures were also infected with FS1-1 in an
attempt to increase the virus titer. This failed to produce a virus
titer above that mentioned earlier although the virus could be
recovered from the organ cultures as long as 40 days after infection.
Many of the organ cultures showed a marked decrease in ciliary activity
at 5-6 days after infection. However, the activity appeared to return to normal in 24-48 hours.

Various concentrations of fetal calf serum were used in the maintenance medium in an attempt to enhance CPE and increase the virus titer. There was no enhancement of CPE nor increase in virus titer when concentrations of FCS greater than 2 percent were employed.

Cytological Characteristics

The May-Grunwald-Giemsa (MG-G) staining method proved to be the most useful in monitoring changes in cells as CPE progressed. GBK cells were grown and infected on round coverslips as described and at various times of incubation a coverslip was removed and stained.

The density of the cell monolayer and the multiplicity of infection produced slight differences in the CPE noted. A dense monolayer infected with 100-500 TCID\textsubscript{50} produced smaller syncytia and more focal areas of granularity.

The MG-G stained preparations revealed two types of CPE. The focal areas of granular cells appeared very dense with shrunken cells and degenerating nuclei. The syncytia observed were composed of a large cytoplasmic mass with the nuclei tending to aggregate toward the center. The syncytia seen early in incubation appeared to be composed of viable cells and it was only in late incubation stages that the cytoplasm and nuclei appeared to degenerate. Many of the syncytia contained quite dense inclusions surrounded by a clear halo. As CPE progressed, the cytoplasm appeared to shrink, with cytoplasmic
strands extending from the syncytia. Eventually the syncytia would exhibit faintly staining cytoplasm with a lacy appearance and pyknotic nuclei.

The hematoxylin-eosin stained preparations showed the same changes noted in the MG-G stained monolayers except that the inclusions were eosinophilic.

Acridine orange stained monolayers revealed syncytium formation somewhat earlier than with the two previous procedures. The focal areas containing granular cells were densely stained with the nuclei showing evidence of karyorhexis. The cytoplasm in syncytia did not stain as intensely as that in uninfected cells; however, the nuclei appeared quite normal. There were spaces in the cytoplasm devoid of fluorescence, which could indicate the location of inclusions as noted by others (4, 54).

Physical and Chemical Characteristics

Table 1 summarizes the characteristics of the bovine RS virus isolate compared with control viruses.

The test for lipid solvent sensitivity indicates that the FS1-l isolate does contain essential lipid. The effectiveness of the test is indicated by a rise in titer of the enterovirus used as a control.

The results of the acid sensitivity test indicate that the new isolate is sensitive to a low pH.

The sensitivity of FS1-l to BUDR indicates that it contains RNA. The results of this test were often inconsistent and the test had to
be run many times before any conclusion could be reached. This test, as well as the test for acid sensitivity, will be discussed in more detail later.

Determination of the approximate size of the virus by using membrane filtration is certainly not a sensitive one. However, it does give an approximation and the difficulties and inconsistencies of the test will be discussed in the next section.

The Antigenic Relationship Between the Bovine RS Virus and a Human RS Virus

The results of this study are summarized in Table 2. The Long strain was used as the human prototype virus and antiserum to both the bovine and human isolates was prepared in rabbits as described. The antigenic relationship between the bovine isolate and the human respiratory syncytial virus was established.

The Antigenic Relationship of FSI-1 with other Syncytium-Producing Viruses

This study was conducted, first, because of the close resemblance of FSI-1 to FSI-4 in cytopathic characteristics and, second, because of the confusion many have of the bovine respiratory syncytial virus and the bovine syncytial virus mentioned earlier. The results of neutralization tests with a prototype PI-3 virus, FSI-4, various field PI-3 isolates using both rabbit and bovine antisera clearly
indicated a distinct lack of antigenic relationship. Neutralization tests with the FSl-1 isolate and the bovine syncytial virus antiserum proved in several tests that there was no serological relationship between the two.

Examination for Mycoplasma Contamination

As indicated earlier, mediums used, cells, and virus samples were periodically checked for the presence of mycoplasma. It is unusual, in this laboratory, to consistently find no mycoplasma contamination; however, even in the initial field isolates, no mycoplasma could be recovered. This is not proof, of course, that there was no mycoplasma present; it was just not isolated. In addition, bacterial or fungal contamination was present only sporadically, usually traced to careless laboratory techniques. The absence of antibiotics in all mediums except for initial isolation provided an efficient monitor for indicating failures in technique.

Experimental Infectivity in Hamsters

The results of experimental infectivity efforts in hamsters were uniformly negative. In spite of three subpassages, there was never any indication of virus recovery. Also, there was never any indication of clinical illness in the hamsters. Neither infectivity nor pathogenesis studies in hamsters was pursued.

_Bovine syncytial virus antiserum provided by Dr. M. Van Der Maaten, N.A.D.L., Ames, Iowa_
Experimental Infectivity In Calves

The five experimental calves used in this study were inoculated intranasally with the FSl-l isolate using an atomizer described by Sinclair and Tamoglia (64). The virus had been passaged 18 times when the calves were inoculated.

There were no clinical signs of illness until the fourth day after infection when calves 3 and 5 developed a temperature of 104.0°F. On this day these two calves were obviously ill with increased respiratory rates, anorexia, serous nasal discharge, dry muzzle, and malaise. The following day their temperatures had returned to near normal and, though they still showed signs of illness, they could have been missed without close observation. Calf 4 developed a slight temperature rise (102.4°F) five days after infection. At this time it was anorectic but otherwise quite normal. The remaining calves (1 and 2) did not develop increased temperatures nor did they exhibit any signs of illness.

Virus was recovered from only one calf (calf 4) on the sixth day after infection.

The clinical and serological results of the experimental infection are summarized in Table 3.

The hematological results are summarized in Table 4. The blood picture reveals two items of interest. All five calves showed an average decrease in the white blood cell count of 28 percent at some point from 3-6 days after infection. In addition, the three calves
that had clinical signs of illness had an average 69 percent relative increase in immature white blood cells, whereas the two calves that had no signs of illness had a 55 percent relative decrease in the same cell type.

All five calves developed hyperemic nasal mucosae. The significance of this finding is uncertain because of the twice daily nasal swabbing.
Fig. 1. Uninfected bovine embryonic kidney (GBK) cells after 36 hr. incubation. May-Grunwald-Giemsa stain. X250.

Fig. 2. Cells (GBK) infected with bovine isolate F51-1 after 3 da. incubation. Early syncytia - dark staining cells (left-center) would appear granular unstained. X130.
Fig. 3. Cells (GBK) infected with FS1-1 after 5 da. incubation. Note large and small syncytia. Cells in lower center exhibit spindling. X130.

Fig. 4. Cells (GBK) infected with FS1-1 after 5 da. incubation. Numerous inclusions are present. X250.
Fig. 5. Cells (GBK) infected with FSI-1 after 5 da. incubation. Sparse cell population illustrates cell recruitment for syncytia formation. X250.

Fig. 6. Cells (GBK) infected with FSI-1 - 5 da. incubation. Note large syncytium with cytoplasmic strands. X400.
Fig. 7. Uninfected low passage bovine thyroid (IBThyr) cells after 36 hr. incubation. X250.

Fig. 8. Cells (IBThyr) infected with FSI-1 after 5 da. incubation. Large, dark staining cells are ballooned cells. Large syncytia with numerous inclusions present. X250.
Fig. 9. Cells (IBThyr) infected with FS1-1 after 3 da. incubation. Note cytoplasmic vacuoles and inclusions. X250.

Fig. 10. Enlargement of Fig. 9. X400.
Fig. 11. Cells (GBK) infected with FSI-1 after 30 hr. incubation. Most cells show no evidence of infection but early syncytium formation can be seen in the upper right and in the center of the photomicrograph. Acridine orange stain. X250.

Fig. 12. Cells (GBK) infected with FSI-1 after 3 da. incubation. The syncytium in the center would appear as granular CPE in unstained preparations. Acridine orange. X130.
Fig. 13. Cells (GBK) infected with FS1-1 after 5 da. incubation with a large syncytium. Acridine orange. X250.

Fig. 14. Cells (GBK) infected with FS1-1 after 7 da. incubation. The empty spaces in the cytoplasm are apparently inclusions. Acridine orange. X250.
Table 1. The Comparative Physical and Chemical Characteristics of Bovine Isolate F51-1

<table>
<thead>
<tr>
<th>Criteria</th>
<th>F51-1</th>
<th>RS virus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PI-3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Enterovirus&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Rhinovirus&lt;sup&gt;c&lt;/sup&gt;</th>
<th>1BR&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>untreated</td>
<td>3.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.0</td>
<td>4.3</td>
<td>6.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>sensitivity</td>
<td>treated</td>
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<td>0.0</td>
<td>0.0</td>
<td>6.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>Acid</td>
<td>untreated</td>
<td>3.5</td>
<td>4.0</td>
<td>n.d.</td>
<td>6.0</td>
<td>3.3</td>
</tr>
<tr>
<td>sensitivity</td>
<td>treated</td>
<td>1.2</td>
<td>1.8</td>
<td>n.d.</td>
<td>6.0</td>
<td>0.0</td>
</tr>
<tr>
<td>BUDR&lt;sup&gt;g&lt;/sup&gt;</td>
<td>untreated</td>
<td>3.5</td>
<td>4.0</td>
<td>4.3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>sensitivity</td>
<td>treated</td>
<td>2.5</td>
<td>2.5</td>
<td>4.0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Particle size&lt;sup&gt;h&lt;/sup&gt;</td>
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<td>4.0</td>
<td>n.d.</td>
<td>n.d.</td>
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</tr>
<tr>
<td></td>
<td>0.45 u</td>
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<td></td>
<td>0.20 u</td>
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<td>0.0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

<sup>a</sup> Respiratory syncytial virus (Long-KB6L2) American Type Culture Collection

<sup>b</sup> Parainfluenza-3, reference virus, obtained from Veterinary Biologics Division (V.B.D.) U.S.D.A., Ames, Iowa

<sup>c</sup> Reference enterovirus (LCR-4) and rhinovirus (FS1-43) obtained from Veterinary Medical Research Institute (V.M.R.I.), Ames, Iowa

<sup>d</sup> Infectious bovine rhinotracheitis virus, reference strain (Cooper) obtained from V.B.D., Ames, Iowa

<sup>e</sup> Infectious virus titer expressed as 10<sup>-6</sup> TCID<sub>50</sub>/ml

<sup>f</sup> n.d. = not done

<sup>g</sup> 5-bromo-deoxyuridine

<sup>h</sup> Nalgene Labware Division, Rochester, N. Y.
Table 2. Antigenic Relationship Between Bovine Isolate FSI-1 and a Human Respiratory Syncytial Virus (Long Strain), as Shown by Neutralization Tests

<table>
<thead>
<tr>
<th>Rabbit Antiserum</th>
<th>Virus</th>
<th>Long Strain</th>
<th>FSI-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit #1 (Long)</td>
<td></td>
<td>2048&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64</td>
</tr>
<tr>
<td>Rabbit #2 (Long)</td>
<td></td>
<td>2048</td>
<td>32</td>
</tr>
<tr>
<td>Rabbit #1 (FSI-1)</td>
<td></td>
<td>64</td>
<td>512</td>
</tr>
<tr>
<td>Rabbit #2 (FSI-1)</td>
<td></td>
<td>64</td>
<td>256</td>
</tr>
</tbody>
</table>

<sup>a</sup>Neutralizing titer expressed as the reciprocal of that serum dilution reducing the plaque number by 50 percent.
Table 3. Summary of Results of Experimental Infection

<table>
<thead>
<tr>
<th>Calf No.</th>
<th>Max. temp.</th>
<th>Virus recovery</th>
<th>Clinical signs</th>
<th>Pre-infection</th>
<th>Post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pre-infection</td>
<td>Post-infection</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>60 days</td>
</tr>
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Serum antibody titer expressed as the reciprocal of that serum dilution reducing the plaque number by 50 percent.
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<tr>
<th>Calf No. 1</th>
<th>Time relative to infection (days)</th>
<th>PCV</th>
<th>WBC</th>
<th>Band. Seg.</th>
<th>Seg. pro.</th>
<th>Fibrinogen</th>
<th>Temp.</th>
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<th>Temp.</th>
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<td>72</td>
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Table 4. (continued)

Summary of Hematological Studies in Experimental Calves

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<th>Time relative to infection (days)</th>
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<th>Seg. pro- fibrinogen</th>
<th>Temp.</th>
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</table>
Table 5. A Serological Survey of Iowa Cattle for Serum Neutralizing Antibodies Against Bovine isolate FSI-1

<table>
<thead>
<tr>
<th></th>
<th>Sample #1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sample #2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sero-conversions&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pos.</td>
<td>neg.</td>
<td>pos.</td>
</tr>
<tr>
<td>Animals with respiratory disease</td>
<td>37&lt;sup&gt;d&lt;/sup&gt;(51)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>35(49)</td>
<td>50(70)</td>
</tr>
<tr>
<td>Animals without respiratory disease</td>
<td>37(98)</td>
<td>1(2)</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Acute phase sample from animals with respiratory disease

<sup>b</sup>Convalescent phase sample from animals with respiratory disease

<sup>c</sup>A seroconversion was indicated by at least a 4-fold rise in antibody titer

<sup>d</sup>Antibody titer expressed as the reciprocal of the serum dilution which reduced the number of plaques by 50 percent. A positive antibody titer was considered to be 16 or greater.

<sup>e</sup>Percent of group tested
Table 6. The Presence of Serum Neutralizing Antibody Against Bovine Isolate FSi-1 in a Herd that had Experienced Annual Outbreaks of Respiratory Disease (Albia Calves)

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</tr>
</thead>
<tbody>
<tr>
<td>Date serum sample taken</td>
<td></td>
<td></td>
<td></td>
<td>Serocon-</td>
</tr>
<tr>
<td>Date serum sample taken</td>
<td></td>
<td></td>
<td></td>
<td>pos.(^a)</td>
</tr>
<tr>
<td>Number of animals tested</td>
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<td>20</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

\(^a\)Antibody titer expressed as the reciprocal of the serum dilution which reduced the number of plaques by 50 percent. A positive antibody titer was considered to be 16 or greater.


\(^c\)A seroconversion was indicated by at least a 4-fold rise in antibody titer.

Table 7. A Summary of Observations of Calves from which FSi-1 was Isolated

<table>
<thead>
<tr>
<th>Calf Number</th>
<th>Temperature (°C)</th>
<th>Clinical signs of illness</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSi-1</td>
<td>40.6 (105.0°F)</td>
<td>Nasal discharge, Anorexia, Mild cough</td>
</tr>
<tr>
<td>FSi-2</td>
<td>40.4 (104.8°F)</td>
<td>&quot;</td>
</tr>
<tr>
<td>FSi-3</td>
<td>39.8 (103.6°F)</td>
<td>&quot;</td>
</tr>
<tr>
<td>FSi-4</td>
<td>40.6 (105.0°F)</td>
<td>&quot;</td>
</tr>
<tr>
<td>FSi-5</td>
<td>40.6 (105.0°F)</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
Table 8. Serological Study of Calves from which FSI-1 was Isolated

<table>
<thead>
<tr>
<th>Calf Number</th>
<th>FSI-1 Acute</th>
<th>Convalescent</th>
<th>PI-3&lt;sup&gt;a&lt;/sup&gt; Acute</th>
<th>Convalescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSI-1</td>
<td>16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64</td>
<td>64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>128</td>
</tr>
<tr>
<td>FSI-2</td>
<td>neg</td>
<td>32</td>
<td>128</td>
<td>64</td>
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<tr>
<td>FSI-3</td>
<td>32</td>
<td>128</td>
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<td>512</td>
</tr>
<tr>
<td>FSI-4</td>
<td>64</td>
<td>128</td>
<td>256</td>
<td>128</td>
</tr>
<tr>
<td>FSI-5</td>
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<td>-</td>
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</tbody>
</table>

<sup>a</sup>PI-3 indicator virus strain obtained from Veterinary Biologics Division, U.S.D.A., Ames, Iowa

<sup>b</sup>Sample taken at time of acute illness - antibody titer expressed as reciprocal of serum dilution reducing plaque number by 50 percent

<sup>c</sup>Antibody titer expressed as reciprocal of serum dilution that neutralized 100 TCID<sub>50</sub> virus.
DISCUSSION

The isolation of the bovine isolate FS1-1 was a result of a continuing effort at this laboratory (V.M.R.I.) to isolate and identify new agents involved in bovine respiratory disease. In the course of the search for new agents, several considerations relative to it have been brought into focus.

One of the most important considerations is the selection of animals to be sampled. The selection of animals very early in a respiratory disease outbreak is most important if one expects to recover viral agents. From the clinical history that was available, it was apparent that the herd from which FS1-1 was isolated was in the early stages of illness.

A second important consideration is the method of handling the nasal secretion sample once it is obtained. Slow freezing and thawing is not conducive to successful virus isolation attempts. In this instance the nasal secretion samples were kept at about 4°C for 1/2-1 hour after which they were rapidly frozen and stored at -70°C. The 1/2-1 hour time interval was the time necessary to obtain the samples and return to the laboratory.

One consistent feature we have found with most new viral isolates is the extended initial incubation period. This is probably due to the low initial titer of the virus in the sample, although it could also be due to the time necessary for the virus to adapt to the cell type used for isolation or possibly to IgA or nonspecific
inhibitors in nasal secretions that are diluted with passage.

This leads to another important consideration in virus isolation attempts. The selection of the cell type or types and growth conditions that provide optimal sensitivity for viral replication is a necessary qualification. In this regard, it has been our experience that a young, actively growing cell is more important than the cell type. It was for this reason that isolation attempts, as well as most characterization tests, were carried out by inoculating cells in suspension (67). The procedure of inoculating cells in suspension gives the virus, in a short time span, optimal conditions for production of progeny virus. Cells in suspension present maximal surface area for virus absorption and, as the cells settle, attach, spread out, and divide, the virus is presented with optimal conditions for attachment, penetration, and replication. It is also important that the cell type have a longevity under incubation conditions sufficient to allow observation of CPE before spontaneous degeneration supervenes. It was observed with FSl-1, as well as with other new isolates, that most cell types of superior longevity would allow replication of the virus even though there might not be much obvious CPE. As an example, the low passage lung cell (IBL) could be maintained for more than 60 days with infrequent changes of the maintenance medium and in early passages the FSI-1 isolate would show only focal areas of CPE and never involve the entire monolayer, although virus could be recovered from supernatant
fluids for extended periods. We have come to the conclusion that 2-3 cell types would give the investigator optimum efficiency in isolating new viruses. This is not only because of adequate sensitivity but it also allows the time necessary to thoroughly examine samples daily for minute changes that so often are the only signal that a cytopathic agent is present.

Observations in the early isolation work with samples from the FSI-1 - FSI-5 group of calves were often confusing and difficult to interpret. The initial CPE manifested by FSI-1 and FSI-4 were so alike that, except for the hemadsorption test, it was difficult to conclude that the two isolates were not identical. In this regard the hemadsorption test has proven most useful and reliable in characterization studies. Also, the early acid production was difficult to explain. In spite of intensive efforts it was impossible to isolate any mycoplasma from either FSI-1 or FSI-4.

The use of tracheal organ cultures in an attempt to enhance the virus titer was a disappointment. This, however, may not be a result of insensitivity of the tracheal epithelium to the virus but is more likely a result of the lability of the virus at incubation temperatures. It was also of interest that the cilia on the tracheal epithelium showed a marked decrease in activity 5-6 days after inoculation of the virus. These studies should be repeated and if this observation is consistent, it could bear strongly on the study of the pathogenesis of disease caused by the virus. It is also interesting that the tracheal
epithelium continued to produce virus for at least 40 days. Therefore, even though the organ cultures did not fulfill our expectations in increasing virus titer, they did provide an opportunity for observations worthy of further study.

Efforts to increase the virus titer in other ways were uniformly unsuccessful. Various incubation temperatures, roller cultures, harvesting virus at various time intervals, and various concentrations of FCS were methods employed in an attempt to increase the virus titer. Another method used was to trypsinize the monolayer from a roller bottle at the time when about 25 percent of the cells showed indications of CPE, suspending the cells in the medium, and allowing them to reseed. Since most of the virus was shown to be strongly cell associated it was felt that the opportunity for cell-cell and thus cell-virus contact would be maximized in this fashion. This attempt to increase the virus titer also failed. There will be a continuing effort to increase virus yield in order to obtain a cell-free virus suspension necessary for critical cross-neutralization and comparative serological studies. There are several possible reasons why the virus titer cannot be increased, but perhaps the most logical one would take into account the lability of the virus at incubation temperatures which, accompanied by a high proportion of incomplete virus particles, would place an absolute limitation on virus yields, regardless of other circumstances. It should be noted that the human RS virus (Long strain), once adapted to GBK cells, would produce a
10-fold higher yield than the bovine isolate with no difficulty.

Some of the changes that occur in a monolayer of cells infected with the bovine isolate FS1-l are illustrated in the accompanying photomicrographs. It was impossible to depict some of the early changes except in unstained monolayers and photographs of reproducible quality showing these changes have not been obtained. However, the important and obvious changes are illustrated in May-Grunwald-Giemsa stained preparations in Fig. 1-10, and with acridine orange stained GBK cells in Fig. 11-14.

As noted earlier, the density of the cell monolayer has a significant effect on some of the changes seen. In a contiguous monolayer of infected cells a granular CPE with ballooned cells and cells with contracted cytoplasm and pyknotic nuclei was a prominent feature. Syncytia in these areas tend to be smaller except in later stages of infection. In areas where cells are not so numerous there are larger syncytia with a definite tendency for cells to be "recruited" to form syncytia. Recruitment of cells is illustrated in Fig. 5. Also, in less densely populated areas of infected cells, spindling of cells and the presence of long cytoplasmic processes become more obvious.

As CPE progresses, the nuclei in the larger syncytia tend to aggregate toward the center of the syncytial mass. This is illustrated in Fig. 3. It is only in late stages of CPE that pyknotic nuclei are noted in syncytia.
The observation of cytoplasmic inclusions has been of major interest and importance to many investigators (4, 33, 39, 55). However, in our preparations it is difficult to become convinced that inclusions noted are of viral origin. These inclusions are seen in Fig. 4 and 8. Some observers indicate that these inclusions do not contain nucleoprotein since they show up as empty spaces with acridine orange stained preparations as noted in Fig. 14 (55). It is not difficult to find what most would consider typical inclusions; however, there are gradations from these "typical" inclusions to unquestionable artifacts or cell debris. These observations have been noted both in human RS virus and bovine RS virus infected cells. Studies using fluorescent antibody (FA) techniques indicate that focal areas of intense cytoplasmic fluorescence appear at the same time that inclusions are noted in similar preparations with stains such as May-Grunwald-Giemsa (45). Therefore, the inclusions noted are probably authentic. They could represent viral protein free of any nucleic acids.

A method to determine serum antibody levels in animals to FSI-1 was developed only after extensive efforts. The conclusion was reached that the cell association of FSI-1 precluded any serum neutralization test other than using a plaque reduction technique similar to that described by Kisch and Johnson (44). This method produced the most consistent results; however, it appears that unless
a virus stock can be produced of adequate titer and devoid of cell debris, serum neutralization tests will often be erratic.

The characteristics of FSI-1 summarized in Table 1 place it in the paramyxovirus group. Other unique features, including a lack of hemadsorbing or hemagglutinating capability, tend to keep respiratory syncytial viruses in a category of their own.

The chloroform test for the presence of essential lipid is rapid and efficient. There is good correlation between the ether test and the chloroform test (22). From the results of this test, the bovine isolate clearly possesses essential lipid.

It is also obvious that FSI-1 is sensitive to low pH. This test is not always as clear-cut as described in the literature. Control viruses must be chosen with care since some viruses classified as being sensitive to low pH are not, or at best give variable results. It appears that the test for sensitivity to low pH is primarily suited to separate rhinoviruses from enteroviruses.

The nucleic acid type of FSI-1 was determined by using BUDR, a thymidine analog which is incorporated into the deoxyribonucleic acid (DNA) of the cell and of any DNA containing virus, thus blocking viral replication. A virus containing ribonucleic acid (RNA) would not be affected since thymidine is not essential to its replication. The bovine isolate, FSI-1, was decreased substantially in titer following the use of BUDR, but after numerous tests, it was determined that the reduction in titer was not due to a direct
effect of BUDR. Since BUDR interrupts normal cell DNA synthesis, and, knowing the lability and cell association of FS1-1, it was concluded that these factors together were sufficient to account for the decrease in virus titer. It was fortunate that since the control DNA virus (IBR) was completely inhibited a judgment could be made that FS1-1 did, in fact, contain RNA as indicated by less than a 2-log reduction in titer in the presence of BUDR. Earlier attempts to obtain information as to the type of nucleic acid in FS1-1 were made with the acridine orange staining technique. However, this test proved to be completely unreliable even with known control viruses.

A study to determine the approximate size of FS1-1 by ultrafiltration studies was undertaken in the face of anticipated problems. By this time the lability and cell association of FS1-1 were well recognized. However, results obtained as noted in Table 1 are in agreement with observations of others (55).

In order to designate the new isolate, FS1-1, as a bovine respiratory syncytial virus, it must show a close antigenic relationship to the human RS virus and yet be unquestionably of bovine origin (33, 55). Cross-neutralization tests with rabbit antiserum to both the bovine and human isolates as summarized in Table 2 indicate the antigenic similarity between the two viruses. There is also a clear indication that the two isolates are not identical. Proof that FS1-1 is of bovine origin comes from several sources.
Kapikian, et al. have agreed that, in the case of human rhinoviruses, an isolate could be considered of human origin if there was a 4-fold or greater rise in neutralizing antibody to the isolate in the convalescent serum from the patient from whom the isolate was recovered (41). If this arbitrary rule could be applied to the bovine RS virus, the neutralizing antibody in the animal from which FSI-1 was isolated shows a 4-fold rise as indicated in Table 8.

Serological studies, especially as noted in Tables 5 and 6, would give additional evidence as to the bovine origin of FSI-1. The percentage of cattle, with or without respiratory disease, that showed serological evidence of exposure to FSI-1, or to an antigenically similar virus, provides additional proof of its bovine origin. In addition, the seroconversions noted in Tables 5 and 6 provide not only evidence as to the bovine origin of FSI-1, but also give some indication that it could be involved in the pathogenesis of some bovine respiratory disease outbreaks.

The observation that respiratory syncytial viruses are not unique in their syncytium-producing capability would pose the question as to what antigenic relationship the bovine isolate might have to other viruses of bovine origin known to produce syncytia. It was particularly important to determine if any antigenic relationship existed between FSI-1 and FSI-4 since their initial cytopathic characteristics were so similar. Many PI-3 isolates often produce syncytia, especially in early passage. Also, the virus described by
Malmquist, et al. consistently produces syncytia in cell culture (50). The results of neutralization tests with reference strains of PI-3 and with FS1-4 indicated that there was no antigenic relationship existing between FS1-1 and these viruses. Antiserum to the bovine syncytial virus would not neutralize the bovine isolate, FS1-1.

Attempts to infect hamsters with the bovine respiratory syncytial isolate met with failure. Studies of others in hamsters, with temperature-sensitive mutants of the human RS virus, appeared to be quite successful (74). It was therefore disappointing that the bovine isolate did not appear to replicate in any respiratory tissues of the hamsters as determined by re-isolation attempts and serological studies. Additional trials with a higher titered virus may be successful. The hamsters would be an excellent laboratory model if they could be infected with the virus.

There appears to be an over-emphasis by some on the ability of any new agent to produce illness in calves. This is understandable, particularly if prevention of bovine respiratory disease is the investigators' goal. However, it must be kept in mind that any laboratory manipulation of a new virus isolate could alter its pathogenic capability. Therefore, it was not expected that the bovine isolate would produce a fulminating infection in experimentally infected calves. The expectations with FS1-1 were that the virus would infect the calves, replicate, and be shed so that its recovery from nasal secretions could be accomplished.
The experimental calves were all considered to be colostrum deprived. Calves 1 and 2 definitely did not receive colostrum. Calves 3, 4, and 5 were thought to be colostrum deprived since they were taken from their dams shortly after birth. It is apparent that all three calves must have received some colostrum. A later check of the serum antibody level of the dams of calves 3-5 proved that at the time of calving they all possessed a high antibody titer against FS1-1. This was certainly unexpected since at this time the wide-spread occurrence of antibody to the bovine isolate had not been determined. However, the fact that calves 3-5 had serum neutralizing antibody to the bovine RS virus may have been as fortuitous as it was unexpected, since these three calves were the only ones to show any signs of clinical illness. The presence of humoral antibody in humans, especially in infants and children, without local secretory antibody is somehow linked to the more severe manifestations of respiratory disease (43).

The nature and the extent of the response to experimental infection in the five calves are summarized in Tables 3 and 4. There is certainly no indication that the virus precipitated a severe clinical illness. However, there are aspects of the data that are worthy of attention. The temperature response in even the obviously ill calves was not remarkable. This has also been the observation in human illness with RS virus (10, 12, 13). If the basic steps in the pathogenesis of severe clinical illness in humans is due to some form
of allergic reaction as suggested by some, the temperature response would probably not be a major factor (24).

Since the clinical manifestations of illness were not significant enough in any calf to justify killing it for postmortem examination, any speculations relative to histopathological changes will not be considered. Future studies with the bovine isolate in combination with other agents should provide sufficient material for histopathological studies.

Virus infections are classically linked with a leukopenia. This was noted in all five calves subsequent to experimental infection as indicated in Table 4. The leukopenia was not marked but was well below the range expected in young animals (63). There was also a shift to the left in calves 3, 4, and 5, but, again, it was not marked. The leukopenia and left shift would indicate, however, that the calves were experiencing an active infection. The plasma protein and fibrinogen levels were measured and, as Table 4 indicates, the variability would hardly allow any conclusion to be drawn.

The recovery of the virus from only one calf when apparently all five were infected was disturbing. In trials with the human virus, it was not unusual to recover virus from a low percentage of those infected (3, 9, 10). It may be that nasal washings would be more efficient in virus recovery attempts.

In Table 5 a summary of a serological survey to determine the incidence of serum neutralizing antibody to a bovine RS virus produced
evidence of exposure to the virus of totally unexpected proportions. It must be kept in mind, however, that the close antigenic relationship between the human RS virus and the bovine counterpart could influence to a great degree any conclusion that might be reached relative to the importance of bovine RS virus in bovine respiratory disease. It would seem imperative, therefore, that comparative serological studies be continued since the natural history of disease due to a respiratory syncytial virus is important in human as well as in bovine respiratory disease.

The results of serum neutralization tests on the Albia calves as summarized in Table 6 give strong support to the assumption that a bovine RS virus could be involved in respiratory disease, perhaps even as the inciting agent. It was considered that if it were possible to isolate a virus from any group of calves, there should have been an isolation from this one since the time of sampling, the handling of the samples, the numerous cell types employed, and the culturing of buffy coat cells seemed to provide optimum conditions for virus isolation. If a bovine RS virus was involved in the outbreak, it is understandable, with our present knowledge relative to the lability of the bovine isolate, that no virus recovery was made. After the discovery of the dramatically uniform seroconversions in the calves tested, the conclusion was reached that further serological tests in this herd could provide data for an epizootiological study of
considerable significance. A project with these considerations in mind is in progress at the present time.

A summary of clinical findings in calves FS1-1 - FS1-5 in Table 7 provides only a fraction of the information that would be desirable to give a clear picture of the outbreak and its consequences. The only conclusion to be drawn from the information available is that the calves were obviously experiencing an outbreak of respiratory disease.

Table 8, showing the results of a serological study of the calves from which FS1-1 was isolated, presents an interesting yet confusing picture. It would seem evident that the bovine RS isolate was involved in the illness of all of the four calves tested. However, the antibody titers were not as high as those in other calves, including the Albia calves. The serum antibody titers to PI-3, in addition to isolation of PI-3 from FS1-4, would indicate that perhaps this virus was more than just casually involved. There was only one seroconversion to PI-3 (FS1-3); therefore, it must be a matter of conjecture whether or not PI-3 was a significant factor in this outbreak.

A review of the results and observations presented would indicate that the characteristics of the bovine isolate FS1-1 are those of a respiratory syncytial virus and that there is sufficient evidence that it is of bovine origin. The significance of the new isolate as a pathogen must await further study.
SUMMARY

A search for new agents involved in the bovine respiratory disease complex resulted in the isolation of a virus (FS1-1) from a herd of Iowa cattle with acute respiratory disease. The bovine virus was shown to be closely related to a human respiratory syncytial virus (Long strain) antigenically as well as in other biological characteristics such as its preferential syncytium formation in only certain cell types and in the production of intracytoplasmic inclusions.

Physical and chemical characterization studies of the virus established that it contained essential lipid, ribonucleic acid, was sensitive to low pH, and was approximately 150 nm in diameter. These characteristics placed the new isolate in the paramyxovirus group. A clear designation of the virus as a paramyxovirus is clouded by its inability to cause hemadsorption in infected cells. In this respect it also resembles the human RS virus.

The bovine origin of the new isolate was established by cross-neutralization tests using rabbit antiserum against the human and bovine viruses. Additional proof as to the bovine origin of FS1-1 was obtained by showing a rise in serum antibody titer to the virus of the animal from which it was isolated as well as by the seroconversion of other animals in the same herd.

The widespread exposure of cattle to the bovine isolate or to an antigenically similar virus was established by significant antibody titers to the virus in widely separated Iowa herds with and without
respiratory disease. The possibility of the virus being a cause of some bovine respiratory disease outbreaks was indicated by universal seroconversions in a herd with respiratory disease (Albia calves).

The bovine RS virus was shown to be capable of infecting young calves. The calves had an increased temperature, a leukopenia, and 3 of 5 calves exhibited visually recognizable signs of illness. The virus was recovered from one of the experimentally infected calves.

The results of characterization studies, cross-neutralization tests, a serological survey, and experimental infectivity trials provide sufficient evidence that the bovine isolate can be considered a bovine representative of a respiratory syncytial virus.
BIBLIOGRAPHY


